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Venous thromboembolism during pregnancy and the impact of thrombophilia in pregnancy complications

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ACADEMIC DISSERTATION

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Abbreviations

aCL	anticardiolipin
APC	activated protein C
aPL	antiphospholipid antibodies
aPS	antiphospholipid syndrome
APTT	activated partial thromboplastin time
ART	assisted reproductive technology
ASA	acetylsalicylic acid
AT	antithrombin
β_2-GPI	β_2-glycoprotein I
DIC	disseminated intravascular coagulation
DVT	deep venous thrombosis
EPCR	endothelial protein C receptor
GPL	unit of anticardiolipin antibody IgG
HIT	heparin induced thrombocytopenia
ICAM	intracellular adhesive molecule-1
IL	interleukin
IUGR	intrauterine growth restriction
LA	lupus anticoagulant
LMWH	low molecular weight heparin
MPL	unit of anticardiolipin antibody IgM
MTHFR	methylene tetrahydrofolate reductase
PAI	plasminogen activator inhibitor
PAR	protease activating receptor
PE	pulmonary embolism
PLG	plasminogen
PROM	preterm rupture of membranes
PTS	post-thrombotic syndrome
RM	recurrent miscarriage
STB	syncytiotrophoblast
TAFI	thrombin activatable fibrinolysis inhibitor
TAT	thrombin-antithrombin complex
TFPI	tissue factor pathway inhibitor
TM	thrombomodulin
t-PA	tissue plasminogen activator
UEDVT	upper extremity deep venous thrombosis
UFH	unfractionated heparin
u-PA	urokinase plasminogen activator
CUS	compression ultrasonography
VCAM	vascular adhesive molecule-1
VTE	venous thromboembolic event
vWF	von Willebrand factor

Abstract

Venous thromboembolism (VTE) are the greatest single cause of maternal mortality in pregnant women in developed countries. Pregnancy is a hypercoagulable state and brings about an enhanced risk of deep venous thrombosis (DVT) in otherwise healthy women. Traditionally, unfractionated heparin (UFH) has been used for treatment of DVT during pregnancy. We showed in our observational study that low molecular weight heparin (LMWH) is as effective and safe as UFH in the treatment of DVT during pregnancy. Although DVT during pregnancy is often massive, increasing the risk of developing long-term consequences, namely post-thrombotic syndrome (PTS), only 11% of all patients had confirmed PTS 3–4 years after DVT. In our studies the prevalence of PTS was not dependent on treatment (UFH vs. LMWH). Low molecular weight heparin is more easily administered, few laboratory controls are required and the hospital stay is shorter, factors that lower the costs of treatment.

Cervical insufficiency is defined as repeated very preterm delivery during the second or early third trimester. Infection is a well-known risk factor of preterm delivery. We found overpresentation of thrombophilic mutations (FV Leiden, prothrombin) among 42 patients with cervical insufficiency compared with controls (OR 6.7, 95% CI 2.7–18.4). Thus, thrombophilia might be a risk factor of cervical insufficiency possibly explained by interaction of coagulation and inflammation processes.

The presence of antiphospholipid (aPL) antibodies increases the risk for recurrent miscarriage (RM). Annexins are proteins which all bind to anionic phospholipids (PLs) preventing clotting on vascular phospholipid surfaces. In this study plasma concentrations of circulating annexin IV and V were investigated in 77 pregnancies at the beginning of pregnancy among women with a history of RM, and in connection to their aPL antibody status. Control group consisted unselected pregnant patients (n=25) without history of adverse pregnancy outcome. Plasma levels of annexin V were significantly higher at the beginning ($\leq 5^{th}$ week) of pregnancy in women with aPL antibodies (lupus anticoagulant, aCL, antiphosphatidylserine, antiprothrombin, and/or anti-β2GPI) compared with those without aPL antibodies (P=0.03). Levels of circulating annexin V were also higher at the 6^{th} (P= 0.01) and 8^{th} week of pregnancy in subjects with aPL antibodies (P=0.01). Results support the hypothesis that aPL could displace annexin from anionic phospholipid surfaces of syncytiotrophoblasts (STBs) and may exert procoagulant activities on the surfaces of STBs

Recurrent miscarriage (RM) has been suggested to be caused by mutations in genes coding for various coagulation factors resulting in thrombophilia. In the last study of my thesis were investigated the prevalence of thrombomodulin (TM) and endothelial protein C receptor polymorphism EPCR among 40 couples and six women suffering RM. This study showed that mutations in the TM or EPCR genes are not a major cause of RM in Finnish patients.

Introduction

Venous thromboembolism(VTE) is the greatest single cause of maternal mortality in pregnant women in developed countries (Greer 1999). Normal pregnancy is associated with several changes in all levels of hemostasis, as increased concentration of procoagulants, decreased levels of natural anticoagulants and diminished fibrinolytic activity render pregnancy a highly hypercoagulable state (Bremme 2003). Venous thromboembolism is rare in healthy pregnant women as natural anticoagulants slow up exessive fibrin formation and finally the fibrinolytic system gets rid of the formed fibrin. However, thrombophilias, either acquired or hereditary, may shift the hemostatic balance towards enhanced coagulation (Greer 2003). Thrombophilias can be found in as many as 50% of patients with VTE during pregnancy (Greer 1999).

Acquired and hereditary thrombophilias have been associated with increased risks of pregnancy complications such as recurrent miscarriage, late fetal loss, preeclampsia, intrauterine growth restriction and placental abruption (Robertson *et al* 2006). One of the major acquired thrombophilias is related to antiphospholipid (aPL) antibodies. An association between these antibodies and pregnancy complications was described as early as in the 1980s (Harris *et al* 1987, Branch *et al* 1989), but the pathophysiology is still unclear. Antiphospholipid antibodies are known to promote coagulation activation via many mechanisms and they lead to thrombotic events in the placenta. The latest pathophysiological concept is related to annexins, natural local anticoagulants. Targeting of the annexin V anticoagulant shield may be a significant mechanism for thrombosis and pregnancy losses related to antiphospholipid antibodies (Rand *et al* 1994, Rand *et al* 1997).

Growing evidence from case-control studies and recent meta-analyses (Rey *et al* 2003, Kujovich 2004) has shown an association between hereditary thrombophilia and recurrent miscarriage. Beside the well known hereditary thrombophilias related to coagulation pathways (F V Leiden, Prothrombin), studies in mice highlight also an important role for the thrombomodulin (TM) and endothelial protein C receptor (EPCR) system in placental development and maintenance of pregnancy (Healy *et al* 1995, Gu *et al* 2002). However, the relevance of these mechanisms as regards pregnancy-associated complications such as recurrent miscarriage (RM) has remained unknown. Thus, it is interesting to discover the prevalence of TM and EPCR polymorphism in humans, especially in women suffering from RM.

Despite intensive research, the etiology of preterm delivery and cervical insufficiency is far from being solved. The interaction between coagulation

and inflammation is well known (Esmon 2003). Thrombin has a key role in hemostatic mechanisms and in a variety of activities that result in augmentation of the inflammatory response as well. Thrombin has the ability to regulate inflammatory processes (Esmon 2003). It could play a more important role in the pathogenesis of cervical insufficiency and preterm delivery than is actually recognized, since it enhances decidual matrix metalloproteases (MMPs). These MMPs are strongly linked to premature rupture of the membranes (Stephenson *et al* 2005). On the other hand, thrombin itself has a uterotonic effect (Elovitz *et al* 2000, O'Sullivan *et al* 2004). These data prompted us to discover if hereditary thrombophilias (with increased thrombin formation) are overpresented in women with cervical insufficiency and preterm delivery

Low molecular weight heparin (LMWH) has been shown to be as safe and effective as unfractionated heparins (UFHs) in the treatment of VTEs in nonpregnant patients. Low molecular weight heparin has several advantages over UFH, as easier administration and more predictable pharmacokinetics lead to less monitoring during treatment. However, at the time when this study was conducted, no comparative studies on short- and long-term outcome with LMWH and UFH had been published. In the future, it will be necessary to discover if we can improve pregnancy outcome with antithrombotic medication in patients with thrombophilia and a history of adverse pregnancy outcome.

Review of the literature

1. Hemostasis during pregnancy

Normal pregnancy is associated with several changes in all aspects of hemostasis. Owing to hormonal changes, increasing concentrations of procoagulants, decreased numbers of anticoagulant factors and diminished fibrinolytic activity (hemostatic mechanism, appendix I) result in pregnancy being a hypercoagulable state in order to prevent maternal hemorrhage after delivery (Bremme 2003, Brenner 2004). Changes in the clotting system are most marked near term and immediately postpartum. However, the hypercoagulable state increases the risk of venous thromboembolism. This hypercoagulabe state returns to normal 4–6 weeks postpartum (Hellgren 2003).

The placenta is an unique organ with dual blood circulations: maternal blood flows in the intervillous space and decidual blood vessels while fetal blood flows inside placental villi. The hemostatic balance is very sensitive in the placenta. There is a continuous low level fibrin production in the placenta reflected in raised levels of plasma D-dimer (Morse 2004, Kline *et al* 2005). Fetal well-being depends critally on the supply and flow properties of the uteroplacental system (Fig. 1) (Bremme 2003).

Fig. 1. Hemostatic mechanisms in circulation and placenta

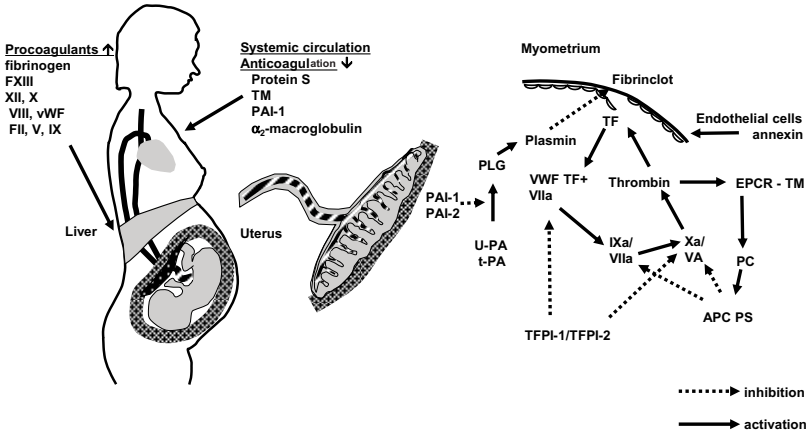


Fig 1. Shows increased production of coagulation factors: fibrinogen, prothrombin, V, VIII, IX, X, XII, XIII and vWF. Systemic changes in anticoagulantory mechanism and local placental hemostatic balance.

During endovascular trophoblast invasion, tissue factor (TF) expression in human endometrial stromal cells prevents postimplantational hemorrhage. An increased TF expression brought about by estradiol (E2) during progestin-induced decidualization has been shown (Lockwood *et al* 2000). Placental cells such as syncytiotrophoblasts are a rich source of TF and they are important for the maintenance of hemostasis in the placenta. Erlich *et al* (1999) studied transgenic mice with low expression of TF. They found that 18% of the mice had fatal postpartum hemorrhage and as many as 40% had fatal mid-gestational hemorrhage.

2. Hereditary thrombophilias

Inherited coagupathies are major causes of thromboembolic disease (table 1). Moreover, the increased risk for maternal complications, hereditary thrombophilias mey predispose for pregnancy complication with different mechanisms.

2.1 Thrombophilias affecting natural anticoagulation

2.1.1 FV Leiden mutation

The most common hereditary thrombophilia is Factor V (FV) Leiden mutation, which is found in approximately 5% (2–15%) of Western populations (Rees *et al* 1995). Normally activated protein C inhibits coagulation cascade by splitting activated factor V. The phenomenon of activated protein C (APC) resistance was first described by Dahlbäck *et al* (1993). The genetic basis, substitution of adenine for guanine at nucleotide 1691 of the factor V gene (G1691A), which causes the arginine at residue 506 of the factor molecule to be replaced by glutamine (Arg506Gln), was described a year later by Bertina *et al* (1994). This mutation slows down the proteolytic degradation of factor Va by activated protein C, leading to increased generation of thrombin (Seligsohn and Lubetsky 2001). Resistance to APC has been found in 24–60% of women with pregnancy-associated VTE (Hellgren *et al* 1995, Hallak *et al* 1997). In the Finnish population the prevalence of FV Leiden mutation has been found to be lower (2.1–2.9%) than in some other Nordic countries (Kontula *et al* 1995, Zoller *et al* 1996, Helio *et al* 1999, Larsen *et al* 1998, Prochazka *et al* 2003).

2.1.2 Deficiencies of Antithrombin, protein C and protein S

Antithrombin deficiency is the most severe thrombophilic condition associated with a 70 to 90 percent lifetime risk of VTE (Girling and de Swiet 1998). In family studies women with antithrombin defieciency, the risk for pregnancy-associated VTE without anticoagulation has mentioned as high as 40% (Zotz et *al* 2003). Antithrombin is synthesized in hepatocytes.

In addition to its thrombin inhibitory properties, it can also inactivate coagulation factors Xa, IXa, VIIa and plasmin, for example (Bombeli *et al* 1997). Antithrombin activity is increased by heparin binding up to 1000-fold (Lockwood 1999). There are several point mutations which can cause mostly dominantly inherited antithrombin deficiencies (Rao *et al* 1997). Two different types of antithrombin deficiency have been described: 1) low functional and immunoreactive antithrombin, and 2) Low functional but normal immunoreactive antithrombin. The prevalence of antithrombin deficiency is low, 1/600–1/5000 (Tait *et al* 1994)

Protein C and its cofactor protein S are produced in the liver and they are vitamin K-dependent enzymes. Activated protein C is an important part of the inhibitory pathway of the coagulation mechanism. Deficiency of protein C is mainly of two types: 1) both immunoreactive and functionally active protein C are reduced, and 2) immunoreactive levels are normal but activity is reduced (Lockwood 1999). There is also a wide variety of genes and mutations associated with protein C. The prevalence of protein C deficiency is low (0.2–0.5%), but type II deficiency is relatively common in Finland, accounting for approximately one half of all protein C defects and, interestingly, virtually all cases with type II deficiency have been found to carry one single mutation, W380G (Levo *et al* 2000). The prevalence of protein S deficiency is approximately the same as that of protein C and both show autosomal dominant inheritance. Protein S deficiency is of three types: 1) reduced total and free protein S, 2) normal protein S but reduced APC cofactor activity, and 3) normal total protein S but reduced free protein S levels (Lockwood 1999). The lifetime risk of VTE associated with either protein C or S deficiency is about 50% (Allaart *et al* 1993, Gouault-Heilmann *et al* 1994) and both are associated with adverse pregnancy outcome (Robertson *et al* 2006). Protein S levels are decreased during normal pregnancy, and therefore diagnoses of protein S deficiences should be made outside pregnancy.

2.2 Thrombophilias affecting procoagulants

2.2.1 Prothrombin gene 20210A mutation

Poort *et al* (1996) described a mutation in the 3´ untranslated region of the prothrombin gene. The mutation, the result of a guanine to adenine substitution at position 20210, leads to significantly elevated plasma prothrombin levels. The mutation is present in 1–2% of the healthy population and it increases the risk of VTE 3-fold (Rosendaal *et al* 1998) (table 1). There are no studies concerning the prevalence of prothrombin mutation in Finland. Among patients with their first episode of VTE, prothrombin mutation has been found in 6% (Zotz *et al* 2003).

2.2.2 High level of factor VIII

The significance of a high level of FVIII is unclear, but it is evident that high levels increase the risk of deep venous thrombosis (Kraaijenhagen *et al* 2000). A constantly high level of FVIII (> 150 IU/dL) is considered to be abnormal. Most instances of high levels of FVIII are acquired and/or transient, appearing in cases of infection and estrogen treatment, but there is also a hereditary form (Bank *et al* 2005).

2.2.3 Hyperhomocystinemia

Hyperhomocystinemia is known to cause direct endothelial injury through increased oxidative stress (Rao *et al* 1997), to induce impairment in endothelial synthesis of vasodilatory substances, to increase the expression of procoagulants, and increase platelet aggregation (Rao *et al* 1997). Most mild or moderate forms of hyperhomocystinemia are the result of homozygosity of the 667C-T methylene tetrahydrofolate reductase (MTHFR) mutation, the prevalence of which among Europeans is about 11% (Molloy *et al* 1997). Although hyperhomocystinemia is a risk factor of arteriosclerosis, its role solely in pregnancy complications is not defined (Rey *et al* 2003, Jääskeläinen *et al* 2006)

There are numerous of studies on polymorphism of coagulation factors (e.g. TF, TFPI, fibrinogen, XII, XIII), but the clinical relevance has not been ascertained (Bertina 2001).

Table 1. Relative risk and probability of pregnancy-associated thrombosis in regard to hereditary coagulation factors in unselected women (without familial thrombophilia)

Genetic defect	Relative risk (95% CI)	Probability of pregnancy-associated VTE
FV Leiden mutation heterozygous	5.3 (3.7–7.6)	0.26%
homozygous	25.4 (8.8–66)	1.5%
Prothrombin G20210A mutation	6.1 (3.4–11.2)	0.37%
Protein C deficiency < 50%	13.0 (1.4–123)	0.8%
Antithrombin deficiency< 85%	3.0 (1.1–8.7)	0.19%
< 60%	119	7.2%
Protein S deficiency	Not known	Not known

Adapted from (Zotz et al 2003).

3. Acquired thrombophilias

Hypercoagulable state can be also acquired due to f.ex.infection, medication and change in physiologic status as occurs with pregnancy. Acquired thrombophilias increases risk both venous and arterial side thrombosis. The major causes of acquired thrombophilias is shown in table 2.

Table 2. Pathogenetic factors of acquired thrombophilia (Greaves 2004)

Immobilization
Pregnancy
Cancer
Operation
Hypovolemia
Infection
Thrombocythaemia
Estrogen treatment
Antiphospholipid antibodies
Acquired deficiency of protein C, S or antithrombin

3.1. Activated protein C (APC) resistance

The phenomenon of activated protein C resistance is mainly explained by FV Leiden mutation, as previously described. However, APC resistance has also been associated with certain factors such as antiphospholipid antibodies and cancer (Bokarewa *et al* 1995, Haim *et al* 2001). Hormonal changes during pregnancy, and oral contraceptives, can induce APC resistance, mainly via changes in protein S levels (Cumming *et al* 1995, Castoldi *et al* 2004).

3.2. Essential thrombocythaemia

Essential thrombocythaemia is a chronic myeloproliferative disorder. According to the conventional criterion for thrombocythaemia, the platelet count is above 600×10^9/L (Lengfelder *et al* 1998). Causes of reactive thrombocytosis are inflammation, infection hemorrhage and iron deficiency. Thrombocythemia increases the risk of thrombosis in high-risk patients (elderly, earlier VTE), whereas the risk of thrombosis in low-risk subjects is similar to that observed in the normal healthy population (Ruggeri *et al* 1998).

3.3. Antiphospholipid syndrome

Antiphospholipid syndrome (aPS) is an autoimmune disorder in which patients have antibodies against phospholipid structures in their blood and at least one clinical manifestation such as adverse pregnancy outcome or thromboembolism (primary aPS). Antiphospholipid antibodies (aPL) are

found among 1–5% of young healthy people (Petri 2000). Antiphospholipid antibodies can be present in association with some autoimmune conditions (secondary aPS), especially systemic lupus erythematosus (SLE). Among such patients aPLs have been found in 30% (Love and Santoro 1990).

The clinical manifestations related to these antibodies include both arterial and venous thrombosis, spontaneous pregnancy losses and often thrombocytopenia. The results of a recent meta-analysis showed significant associations between aPLs and both early and late fetal loss and an increased risk of preeclampsia (Robertson *et al* 2006). The etiology associated with these antibodies is unclear and even their antigenic targets are not fully established. Thus, aPS is classified as an autoimmune disorder. Some bacterial infections such as syphilis and Lyme disease can also induce aPL production (Rand 2003). The first observation of a false-positive serological test for syphilis was made by Moore and Mohr in 1952, and later Harris *et al* (1987) and Hughes (1985) also described this clinical phenomenon. The diagnostic criterias of aPS (Miyakis *et al* 2006) are presented in table 3.

Table 3. Diagnostic criteria of aPS

1. Clinical history of vascular thrombosis or pregnancy morbidity.

2. Laboratory evidence of Lupus Anticoagulant (LA) or at least a medium titer of IgM or IgG anticardiolipin antibodies (aCLs), or specific anti-β_2 glycoprotein I antibodies. The abnormalities should be present twice, at least six weeks apart.

There are seasonal changes in the prevalence of aPL in the normal population, with a higher prevalence in the winter time compared with summer (perhaps related to viral infections, which have been connected to a rise in aCL). However, correlation of the seasonal prevalence of aPL and VTE has not been established (Luong *et al* 2001). Although antiphospholipid syndrome has been classified as an acquired thrombophilia, familial clustering of raised of aPL antibodies exists (Hellan *et al.* 1998) and HLA linkage has been shown (Sanchez *et al* 2004).

The pathogenesis of aPS is not clear but antigenic targets and cofactors are known. Anti- β_2-glycoprotein I is a highly glycosylated single-chain protein that may have a role in recognition of anionic phospholipids by aPLs (Schultz 1997). The physiological function of anti-β_2-glycoprotein I is not fully understood and its role as an independent risk factor of thrombosis is unclear (Rand 2002, de Groot and Derksen 2005). Other cofactors associated with aPLs such as prothrombin, FV, proteins C and S, high and low molecular weight kininogen and annexins have been described (de Groot *et al* 1996). Thromboxane dominance has also been shown to be related to aPL in pregnant women with SLE. This may contribute to adverse pregnancy outcome (Kaaja *et al* 1993b). Moreover, in addition to the established procoagulative properties of aPL, there is evidence that

aPL can directly interfere with decidual endovascular trophoblast invasion (Sebire *et al* 2002).

Accumulated data concerning the pathogenetic mechanisms associated with aPLs is shown in table 4. There are many biological processes in which aPLs seem to play role but it is difficult to determine whether they are clinically relevant or not.

Table 4. Suggested thrombotic mechanisms of aPLs

Interference with a phospholipid- or other polyanionic-dependent antithrombotic mechanism
Disruption of the annexin V shield
Interference with protein C
aPL binding with proteins C and S
inhibition of protein C
acquired protein C resistance
Inhibition of tissue factor pathway inhibitor (TFPI)
Impairment of phospholipid-mediated autoactivation of Factor XII and reduced fibrinolysis
Inhibition of heparin-antithrombin complexes
Promotion of tissue factor expression/synthesis on monocytes and endothelial cells
Vascular injury/stimulation of apoptosis
Injury to endothelium
Induction of apoptosis of vascular cells
Release of membrane-bound microparticles
Promotion of cellular adhesion to vascular surfaces
Stimulation of platelet function
Platelet activation: increased thromboxane production
Release of membrane-bound microparticles
Others
Increase in endothelin-1
Cross-reactivity to oxidised LDL
Increase in PAI-1

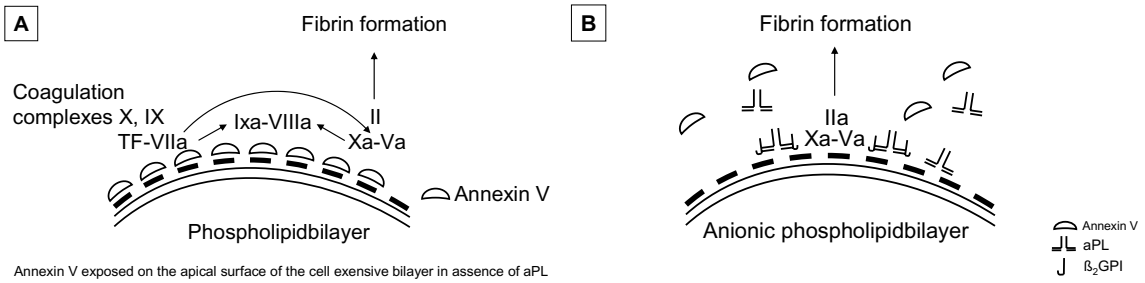
Adapted from J. Rand (2002)

4. The role of annexins IV and V

Annexins are a family of structurally related proteins which all have high affinity to negatively charged anionic phospholipids (PLs) in the blood vessels, acting in a calcium ion-dependent manner. The best-known annexin, annexin V, has anticoagulant properties and the capacity to displace coagulation factors from anionic phospholipid surfaces (Rand *et al* 1997).

In the placenta, annexin V is localized on the apical surfaces of the syncytiotrophoblasts (STBs) and it is necessary for the maintenance of placental development and its integrity (Wang *et al* 1999). Wang *et al* (1999) infused polyclonal anti-annexin V antibodies into pregnant mice, causing placental infarction and pregnancy wastage. The physiological function of annexin V is still not fully understood. However, it is known that it has anticoagulant properties and it is speculated to have a role in placental apoptosis (Bonet *et al* 1992, Krikun *et al* 1994). Anti-annexin V antibodies have been detected in subjects with an elevated incidence of intrauterine fetal loss, preeclampsia and arterial and venous thromboses (Matsuda *et al* 1994a, Kaburaki *et al* 1997, Matsubayashi *et al* 2001). Di Simone *et al* (2001) speculated that anti-annexin V antibodies could affect embryo implantation and worsen pregnancy outcome by way of syncytiotrophoblast apoptosis and inhibition of trophoblastic gonadotropin secretion (Matsuda *et al* 1994b, Kaburaki *et al* 1997, Wang *et al* 1999). Antiphospholipid antibodies are known to promote coagulation activation via many mechanisms (table 4). One of the proposed mechanisms could be displacement of annexin V, with anticoagulant properties, from the surfaces of STBs by antiphospholipid antibodies (Rand *et al* 1994, Rand *et al* 1997), which may lead to activation of coagulation complexes.

Fig. 2. Pathophysiological mechanisms of annexin on syncytiotrophoblasts surfaces (Adapted J. Rand, Thromb Res 2004 with permission from Elsevier)



Compared with annexin V, much less is known about annexin IV. Annexin IV expression has been found in the basal layer of STBs in the placenta (Masuda et al 2004). Annexin IV seems to enter the maternal blood circulation just after delivery and it has been speculated to have a preventative role in DIC (Masuda et al 2004).

5. Venous thromboembolic disease

Venous thromboembolism (VTE) represent the greatest single cause of death in pregnant women in developed countries (Gates 2000). Deep venous thrombosis and pulmonary embolism are two different manifestations of one disease (Ginsberg 1996). Pulmonary embolism (PE) is estimated to cause 50 maternal deaths in pregnancy every year in the United Kingdom (Greer 1999). The incidence of VTEs has been estimated to be 1/1000–1/2000 pregnancies, which is 5–10 times higher than the incidence in nonpregnant women (1/10 000) (Greer 1997). In Finland, at least 2–7 deliveries with associated pulmonary embolism are confirmed every year. Venous thromboembolism, overall, are the main cause of maternal mortality (2/100 000 live births) (Gissler M, Finnish Birth Register, personal communication). The etiology of deep venous thrombosis during pregnancy is multifactorial and common risk factors are shown in table 5.

Table 5. Major risk factors of DVT during pregnancy

Age above 35 years
Obesity
Immobilization
Operative delivery, especially cesarean section
Thrombophilias
Infection

All factors of Virchow´s triad (hypercoagulability, venous stasis and vascular damage) occur during pregnancy. As a result of anatomic factors during pregnancy, blood flow velocity is reduced in the femoral veins by approximately 50% from 25 weeks of gestation to the end of pregnancy (Macklon *et al* 1997) and it normalizes to the nonpregnant level around six weeks after delivery (Lindhagen *et al* 1986). Left-side over-presentation has also been described in pregnancy-associated deep venous thrombosis. This is possible because during pregnancy, the left iliac vein is compressed by the left iliac artery and ovarian arteries (Cockett *et al* 1967). Endothelial damage during operative delivery may be a trigger for a cascade that leads to a highly increased risk of venous thromboembolism. The highest risk periods for VTE are during the late third trimester and immediately postpartum, but almost 30% of cases of VTE have been reported in the first trimester (Ginsberg *et al* 1992, Toglia and Weg 1996, Gherman *et al* 1999).

Deep venous thromboses related to pregnancy are often massive and therefore the risk of developing venous insufficiency or post-thrombotic syndrome in the long term may be higher than in the nonpregnant state (Holmström *et al* 1999). Although antepartum VTE complications are more common, it has been suggested that puerperal VTE events are

underestimated because these maternal complications are often treated in non-obstetric hospitals.

Upper extremity deep venous thrombosis (UEDVT) is uncommon and represents only 11% of all diagnosed DVTs (Joffe *et al* 2004). Most often, UEDVT is associated with mechanical or anatomic compression or obstruction or severe thrombophilic disorders such as antithrombin deficiency or antiphospholipid syndrome (Prandoni *et al* 1997, Joffe *et al* 2004). Growing evidence, mainly based on case reports, has shown that the risk of UEDVT has increased in connection with the use of assisted reproductive technology (ART), especially if ART cycles have been complicated by ovarian hyperstimulation syndrome (OHSS) (Chan and Ginsberg 2006, Nelson and Greer 2006). Ovarian induction and maturation with gonadotropin-releasing analogs cause marked procoagulant changes in the hemostatic and fibrinolytic systems (Aune *et al* 1991). In severe OHSS (1–2% of cases) is often characterized by ascites, hypoalbuminemia and reduced intravascular volume, which are additional risk factors for thrombosis (Kaaja *et al* 1989, Aune *et al* 1991). Although ART increases the risk of thrombosis, screening for thrombophilias is not cost-effective (Fabregues *et al* 2004), but the risk of thrombosis should be evaluated individually.

Diagnosis of a VTE can be difficult because many of the classic symptoms such as dyspnea, tachypnea, leg swelling and tachycardia have also been associated with normal pregnancy (Garcia-Rio *et al* 1996). Clinical diagnosis of DVT and PE is unreliable. In nonpregnant patients, DVT has been confirmed by objective methods in only about 30% of suspected cases (Ginsberg 1996). In a large retrospective study (Refuerzo *et al* 2003) conducted in pregnant patients with suspected PE, it was shown that symptoms did not differ in patients with confirmed PE compared with those without PE. Pulmonary embolism develops in approximately 15–24% of patients with untreated deep vein thrombosis (Rutherford and Phelan 1986, Gherman *et al* 1999, Winer-Muram *et al* 2002).

In nonpregnant subjects, D-dimer, a specific degradation product of fibrin, has been used as a diagnostic tool of DVT and PE (Bounameaux *et al* 1994). D-dimer has a good negative predictive value as regards excluding DVT and PE outside pregnancy (Bounameaux *et al* 1994). During pregnancy, its specificity is low, which limits its use as a diagnostic tool (Proietti *et al* 1991, Nolan *et al* 1993, Francalanci *et al* 1995a, Francalanci *et al* 1995b). Contrast venography remains the "gold standard" test for the diagnosis of lower extremity DVT (Rabinov and Paulin 1972, Hull *et al* 1983). However, venography is invasive and exposes the patient to radiation, so it is not optimal for pregnant subjects. Thus, diagnosis of deep venous thrombosis in symptomatic subjects is based on the non-invasive compression ultrasound (CUS) test (Heijboer *et al* 1993). In cases of symptomatic patients with suspected calf DVT, normal CUS should be repeated after 2–3 days. If iliac DVT is suspected, pulsed Doppler ultrasonography can help in diagnosing DVT.

When PE is suspected, a radiological test should be performed, because the harmful effects of radiation are minimal compared with the consequences of a missed diagnosis of PE (Ginsberg *et al* 1989a). Pulmonary spiral computer tomography (CT) scans of the lungs has been increasingly used in the diagnosis of PE.

5.1 Treatment of venous thromboembolism during pregnancy

Non-pregnant patients with deep venous thrombosis are usually treated in the acute phase with low molecular weight heparin (LMWH) given subcutaneously (Holmström *et al* 1999). Low molecular weight heparin has been shown to be as safe and effective as unfractionated heparin in the treatment of VTE (Holmström *et al* 1999, Dolovich *et al* 2000). However, in many countries UHF is still used for treatment of DVT during pregnancy because of long experience of its effectiveness, and monitoring (Ginsberg *et al* 1989b, Ginsberg *et al* 1989c, Ensom and Stephenson 2004). The anticoagulation effect of UFH can be neutralized quickly with protamine (Hirsh and Raschke 2004).

Fig. 3. Effects of heparins in coagulation cascade

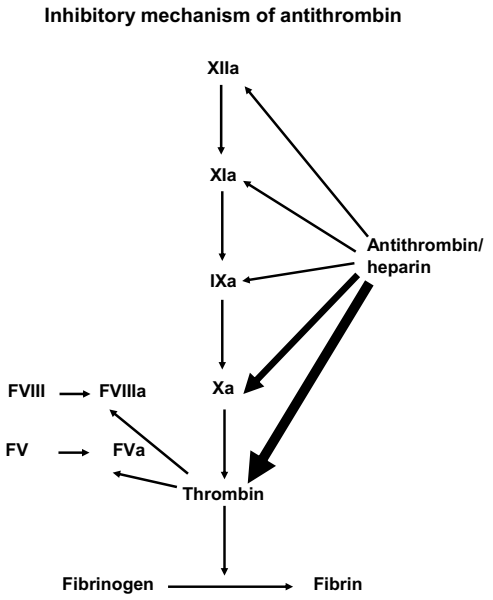


Table 6. Properties of unfractionated heparin and LMWH, Modified from Hirsh et al (2001)

Unfractionated heparin	Low molecular weight heparin
-molecular weight 3000–30 000 (mean 15 000) approximately 45 monosaccharide chains	-molecular weight 1000–10 000 (mean 5000)
-anticoagulant profile and clearance depends on chain length of molecule, higher cleared more rapidly	-longer clearance through renal route
-binds to platelets (PF4), inhibits aggregation	-lower binding to proteins and cells
-increases vessel permeability	-the risk of HIT and osteoporosis is much lower than with unfractionated heparin
-suppresses osteoblast formation, activates osteoclasts	-more stable pharmacokinetics, self-administration
	-favorable IIa to FXa ratio

On the basis of earlier results, low molecular weight heparin (LMWH) (Forestier *et al* 1984, Forestier *et al* 1992), like unfractionated heparin (UFH) (Flessa *et al* 1965), does not cross the placenta and is at present considered to be the drug of choice for the prophylaxis of VTEs during pregnancy (Greer and De Swiet 1993, Toglia and Weg 1996, Pettilä *et al* 1999). Plasma levels of UFH vary depending on the degree of binding to proteins in plasma and on the endothelium (Glimelius *et al* 1978) and UFH requires more monitoring and dose adjustments. Thus LMWH has several advantages over unfractionated heparin such as longer half-life (Weitz 1997), and more stable and predictable pharmacokinetics (Greer 1999), which makes possible subcutaneous once or twice daily self-administration, with minimal laboratory monitoring. Long-term use of UFH is associated with significant maternal side effects during pregnancy such as increased risks of osteoporosis and symptomatic vertebral fractures (2–3%), heparin-induced thrombocytopenia (HIT), and allergy (Nelson-Piercy 1997). Low molecular weight heparin has been shown to be safer than UFH as regards HIT (Warkentin *et al* 1995). Several investigators have also shown no significant effect on bone mineral density when prophylactic doses of LMWH are used (Shefras and Farquharson 1996, Sanson *et al* 1999, Pettilä *et al* 2002).

Up to now, there have been no randomized studies in which UFH and LMWH have been compared in the treatment of DVT during pregnancy. Experience of the use of LMWH for prophylaxis has also encouraged its use in the treatment of DVT. In Finland there are two LMWHs (enoxaparine and dalteparin) available for VTE prophylaxis and treatment. They have both been used during pregnancy.

5.2 Long-term outcome of venous thromboembolism during pregnancy

In addition to a lack of controlled randomized prospective trials concerning the management of VTE during pregnancy, there are few data on the long-term outcome of pregnancy-related DVT. Post-thrombotic syndrome (PTS) is chronic complication of DVT. The reported incidence of PTS varies from 20% to 100% owing to initially different definitions of the syndrome (Gjores 1956, O'Donnell *et al* 1977) and lack of diagnostic criteria (Kahn and Ginsberg 2002). Clinical symptoms of PTS are pain, swelling, pruritus, eczematous skin change, development of secondary varicose veins and even ulceration of the leg (Immelman and Jeffery 1984). There are no gold standards for the diagnosis of PTS but it should be based on the presence of typical symptoms. Confirming venous reflux by means of ultrasonography may help diagnosis (Kahn and Ginsberg 2002). A clear correlation between size, degree of occlusion and location of the initial thrombus has not been documented (Browse *et al* 1980, Prandoni *et al* 1996), but there is evidence for a higher rate of PTS after proximal compared with distal DVT (Lindner *et al* 1986, Holmström *et al* 1999, Mohr *et al* 2000). According to Janssen *et al* (1997), only 10–30% of patients are symptom-free after iliac DVT. The results have been disputed in another study (Philbrick and Becker 1988), but recurrent DVT has been confirmed to be a risk factor of PTS in many studies (Prandoni *et al* 1996, McColl *et al* 2000). There are also studies that suggest that high BMI could be an independent risk factor of PTS (Biguzzi *et al* 1998, Ageno *et al* 2003).

Deep venous thrombosis during pregnancy is often massive and proximal, and the risk of PTS could be expected to be higher than after DVT outside pregnancy (Greer and De Swiet 1993). There are few studies on this issue. McColl *et al* (2000) described a 79% incidence of post-thrombotic syndrome in women who suffered from DVT during pregnancy. As post-thrombotic symptoms in young women may cause impairment in the quality of life of otherwise healthy people, it is very important to evaluate the impact of initial treatment of DVT with subcutaneous LMWH compared with the standard therapy with intravenous UFH.

6. Thrombophilias and pregnancy complications

There is growing evidence that women with thrombophilia are at an increased risk of several severe obstetric complications in addition to VTEs. These include recurrent miscarriage (RM), preeclampsia, intrauterine growth restriction (IUGR), unexplained intrauterine fetal death (stillbirth) and placental abruption (Kupferminc 2003, Rey *et al* 2003, Dudding and Attia 2004). Thrombotic factors could operate at the level of the placenta after gestational week 8, when the placental circulation maintains pregnancy. However, aPLs may have pathogenetic mechanisms other than those related to the thrombotic process, such as apoptosis and the ability to interfere with trophoblast differentiation (Bonet *et al* 1992, Sebire *et al* 2002, Quenby *et al* 2005). Maternal thrombophilia together with natural prothrombotic changes during pregnancy may shift the hemostatic balance towards thrombotic changes in placental capillaries, leading to inadequate fetomaternal circulation and decreased placental perfusion (Khong *et al* 1987, Roberts *et al* 1989, Shanklin and Sibai 1989, Salafia *et al* 1995, Redman *et al* 1999). Retrospective case-control studies suggest a strong causal relationship between thrombophilias and recurrent miscarriages (Robertson *et al* 2006), although conflicting data still exist (Infante-Rivard *et al* 2005). There are also histological studies of the placenta that show a relationship between pregnancy complications, placental pathology and maternal thrombophilia (Arias *et al* 1998, Many *et al* 2001), although there is also conflicting data which failed to show any differences in obstetric complications in regard to specific histologic findings in women with and without thrombophilia (Mousa and Alfirevic 2000). One major problem is the heterogeneity and small sizes of study populations. Moreover, inclusion criteria and even outcomes vary, resulting in certain limitations as regards the conclusions. It is clear that the etiology of these severe obstetric complications is multifactorial, and it seems evident that thrombophilia has an additional role in the pathogenesis of these complications, as recently shown in a Finnish study (Järvenpää *et al* 2006).

Although thrombophilias may be harmful during pregnancy, they also have beneficial effects. It has been shown that FV Leiden carriers have less blood loss during delivery (Lindqvist *et al* 1998) and even less menstrual bleeding compared with non-carriers (Lindqvist *et al* 2001). There are some studies concerning the association between thrombophilic mutations and fecundity. Of interest is the recent study by van Dunne *et al* (2006) showing that fecundity is increased in male but not in female FV Leiden mutation carriers. There are speculations concerning a link between the FV Leiden gene and a fertility gene that may potentially affect sperm count and motility. In one study the implantation rate was reported to be higher if either the mother or the child carried the FV Leiden mutation (Gopel *et al* 2001), but contradictory results indicating an increased number of failures after IVF treatment of thrombophilic women have also been shown (Azem *et al* 2004, Qublan *et al* 2006).

6.1 Recurrent miscarriage and fetal loss

Definitions of recurrent miscarriage (RM) vary but generally it has been characterized as at least three consecutive miscarriages in the first or second trimester of pregnancy (upper limit 22 weeks of gestation, WHO) (Tulppala and Ylikorkala 1999). Late fetal loss (stillbirth) has been defined as intrauterine fetal death beyond 22 weeks of gestation.

Every tenth pregnancy ends in miscarriage and one tenth of these miscarriages are recurrent (≥ three consecutive miscarriages) meaning that 1% of all pregnancies end in recurrent miscarriage (Tulppala *et al* 1993, Li *et al* 2002). Some clinicians define recurrent miscarriage as two or more consecutive miscarriages, which increases the number of cases from 1% to 5% (Hogge *et al* 2003). The risk of miscarriage increases with maternal age (Nybo Andersen *et al* 2000). Etiological factors of RM are shown in table 7.

Table 7. Possible etiological factors of recurrent miscarriage

Acquired and inherited thrombophilias
Genetic abnormalities
Uterine structural abnormalities
Infection
Endocrine abnormalities (luteal insufficiency? PCO?, insulin resistance?, thyroid dysfunction?)
Immune dysfunction (unfavorable cytokine shift Th1→ Th2, autoantibodies?)
Endometrial responsiveness?

Fetal chromosomal defects have been suggested to be the most common reason for sporadic miscarriage, accounting for as much as 50% of all miscarriages (Stephenson *et al* 2002). On the other hand, the frequency of a normal embryonic karyotype increases with the number of miscarriages, this indicating a more important role of maternal factors in pregnancy failure (Ogasawara *et al* 2000, Sullivan *et al* 2004). Both retrospective and prospective studies show that the risk of unsuccesful pregnancy outcome in the following pregnancy increases with the number miscarriages, being 40–45% after three miscarriages (Regan *et al* 1989, Lee and Silver 2000, Nybo Andersen *et al* 2000)

Antiphospholipid syndrome is a well-recognized cause of RM and has been reported in 7–42% of women with RM (Greaves *et al* 2000). In addition to aPL antibodies, there is also a heterogeneous groups of hemostasis-related autoantibodies (e.g. anti-prothrombin, anti-β_2 glycoprotein-1 antibody, anti-phosphatidylserine, phosphatidylethanolamine, and anti-annexin V) which can locally promote hypercoagulation, and interfere with trophoblast invasion and growth (Shoenfeld and Blank 2004).

On the basis of the results of small retrospective studies, and a review, it has been suggested that also essential thrombocythaemia could be a risk factor of miscarriage (Elliott and Tefferi 2003, Niittyvuopio *et al* 2004).

Growing evidence from case-control studies and recent meta-analyses has shown an association between recurrent miscarriage and some, but not all, thrombophilias (table 8). The association seems to be even stronger with late fetal losses (2nd or 3rd trimester) (Rey *et al* 2003, Kujovich 2004, Robertson *et al* 2004).

Table 8. Thrombophilia-associated fetal losses

Thrombophilia	Recurrent miscarriage	Late fetal loss
FV Leiden mutation	+	++
Prothrombin G20210A mutation	++	++
Protein C deficiency	?	?
Protein S deficiency	?	++
Antithrombin deficiency	?	?
MTFHR homozygosity	+/-	+/-
Anticardiolipin antibodies	++	++
Lupus anticoagulant	++	++

Although meta-analyses have partly failed to show an association between protein C and S deficiency, antithrombin deficiency and fetal losses, these deficiencies of the anticoagulative mechanism are capable of causing a severe thrombophilic state and they markedly increase the risk of a VTE (Robertson *et al* 2004). Thus, we can assume that they may have a role in the etiology of RM and fetal loss. On the other hand, in some cases RM could be related to low grade thrombophilias known not to be associated with a VTE (e.g. low positive antiphospholipid antibody levels) (Rai *et al* 1997, Pattison *et al* 2000, Farquharson *et al* 2002). This highlights the special role of the placenta as a most sensitive organ in which a thrombotic event may manifest itself.

6.2 Preeclampsia

Preeclampsia affects approximately 5% of singleton pregnancies. Its etiology is still poorly understood. It is characterized by an abnormal vascular response to placentation in that there is increased systemic vascular resistance (high blood pressure), enhanced platelet aggregation, activation of the coagulation system, and endothelial cell dysfunction (edema, proteinuria) (Sibai 2005). The risk factors of preeclampsia are described in table 9.

Table 9. Risk factors of preeclampsia (couple-related risks) (Sibai *et al* 2005)

Primipaternity
Pregnancies after donor insemination, oocyte donation, embryo donation
Protective effect of partner change in the case of previous preeclamptic pregnancy
Maternal or pregnancy-related risk factors
Extremes of maternal age
Multifetal gestation
Preeclampsia in a previous pregnancy
Chronic hypertension or renal disease
Rheumatic disease
Maternal low birth weight
Obesity and insulin resistance
Pregestational diabetes mellitus
Maternal infections
Pre-existing thrombophilia
Maternal susceptibility genes
Family history of preeclampsia
Smoking (reduced risk)
Hydropic degeneration of the placenta

Gestational hypertension (GH) without proteinuria can represent a mixture of preeclampsia and a heterogeneous group of preexisting hypertensive disorders affecting up to 20% of pregnancies (Morrison *et al* 2002). The first report of an association between early onset (before 34 weeks of gestation) or severe preeclampsia and aPL was described by Branch *et al* (1989). Later, Dekker *et al* (1995) reported an association between an inherited thrombophilic mutation and preeclampsia. Most studies later showed an association between thrombophilia and early onset or severe (< 34th week of gestation or proteinuria > 5 g/day) preeclampsia but not mild or term preeclampsia (Morrison *et al* 2002, Sibai *et al* 2005, Sibai 2005, Robertson *et al* 2006). Recent meta-analyses (Robertson *et al* 2006) have indicated that preeclampsia is significantly associated with FV Leiden and prothrombin mutations, anticardiolipin antibodies, MTFHR homozygosity and hyperhomocystinemia, whereas protein S, protein C, and antithrombin deficiency are not significant risk factors. In a recent large case-control study, Mello *et al* (2005b) showed not only an association between thrombophilia and severe preeclampsia but also a tendency towards increased risks of maternal complications such as early onset of disease (< 28 weeks of gestation), placental abruption, disseminated intravascular coagulation (DIC) and acute renal failure.

6.3 Intrauterine growth restriction

Same vasculopathic findings related to preeclampsia may also be seen in IUGR. However, the association between thrombophilic disorders and IUGR is weaker than in preeclampsia and the evidence is not indisputable (Infante-Rivard *et al* 2002, Verspyck *et al* 2004). The etiology of IUGR is multifactorial, but thrombophilia may have an additional role. A diagnosis of IUGR might be erroneous if only birth weight for gestational age is used. Individual neonates in the low centile groups might not be affected by IUGR but most neonates are constitutionally small (Mamelle *et al* 2001, Mamelle *et al* 2006). In such cases of small-for-gestational age (SGA) infants later prognosis is normal. However, thrombophilia seems to increase the risk of IUGR, although the only significant association has been found with anticardiolipin antibodies (Robertson *et al* 2006).

6.4 Placental abruption

The incidence of placental abruption in Finland is 0.42% of pregnancies (Tikkanen *et al* 2006). General risk factors are maternal and paternal smoking, use of alcohol, placenta previa, preeclampsia, and chorioamnionitis (Tikkanen *et al* 2006). Placental abruption has also been reported to be more prevalent in thrombophilic pregnancies (Kupferminc *et al* 1999) and in women with a family history of venous thromboembolism (Prochazka *et al* 2003). Some Finnish studies have shown no association between FV Leiden mutation or MTFHR polymorphism and placental abruption (Jääskeläinen *et al* 2004, Jääskeläinen *et al* 2006). However, a recent meta-analysis showed that FV Leiden mutation and prothrombin mutation were associated with an increased risk of placental abruption (Robertson *et al* 2006).

Current evidence shows that there are similar vasculopathic findings in preeclampsia, IUGR, fetal loss and placental abruption, and thrombophilia seems to play a role in the etiology of these complications. Although the etiology is multifactorial, the association between thrombophilia and placental pregnancy complications seems to be particularly strong, especially in early-onset and severe forms of complications (Lockwood 2002). Thrombophilias have also been associated with a severe form of preeclampsia, HELLP (hemolysis-elevated liver enzymes-low platelet) (Bozzo *et al* 2001).

6.5 Prevention of thrombophilia-associated pregnancy complications

Most of the data on interventional studies to prevent thrombophilia-associated pregnancy complications concerns RM. However, we must be cautious when interpreting this data. The study populations are heterogeneous and small and the types of thrombophilia are divergent. There is a lack of randomized trials, making comparisons inconclusive. Some small studies (Kutteh 1996, Rai *et al* 1997) showed that unfractionated heparin and acetosalisylic acid

(ASA) can improve pregnancy outcome in patients with RM and aPLs compared with ASA alone. It seems that patients with aPLs and RM benefit from antithrombotic therapy (Empson *et al* 2005). However, Farquharson *et al* (2002) found no beneficial effect of LMWH/ASA compared with aspirin alone in aPL-positive women with RM.

There are some non-randomized observational studies (Brenner *et al* 2000, Carp *et al* 2003) in which improvement of pregnancy outcome with LMWH prophylaxis has been shown. Gris *et al* (2004) showed in their comparative trial that LMWH treatment is superior to ASA in patients with a history of single miscarriage and thrombophilia. Recently, Dolitzky *et al* (2006) compared enoxaparin and ASA in the prophylaxis of RM of unknown etiology and showed no significant differences between these treatments. Successful pregnancy outcomes were 94% (LMWH) vs. 81% (ASA). The results in both groups were better than the spontaneous success rates mentioned in the literature overall. Because intervention was started at 6–12 weeks of pregnancy, after a viable fetus was confirmed, the study population was already selected and biased.

Table 10. Results of antithrombotic interventional studies in patients with a history of recurrent miscarriage (RM), with or without thrombophilia

	Study patients	Intervention	Start of treatment	Controls	Results
Dolitzky *et al* 2006	*n*=104 RM (≥3), unknown etiology, excluded thrombophilia	54 enoxaparin vs 50 ASA 100mg	6–12 weeks of gestation	54/50	82% vs 84% NS
Tzafettas *et al* 2005	RM (≥3) 24 thrombophilic hered/acquired and 27 non-thrombophilic	ASA 80mg and LMWH fraxiparine	confirmed viable pregnancy	no	83% vs 85% NS
Noble *et al* 2005	*n*=50, RM (≥3) aPL positive GPL≥20, MPL≥20 phosphatidylserine ab, LAC	LMWH enoxaparin ASA 81mg vs UFH ASA 81mg	positive pregnancy test	25/25	84% vs 80% NS
Gris *et al* 2004	*n*=160 single fetal loss and thrombophilia	80 LMWH enoxaparin vs 80 ASA 100mg	8 weeks gestation	no	86% vs 29% S
Carp *et al* 2003	*n*=85, RM (≥3) thrombophilic hered/acquired	37 enoxaparin 40mg vs 48 no treatment	confirmed pregnancy	48 no treatment	70% vs 44% S
Farquharson *et al* 2002	*n*=95, RM (≥3) aPL positive GPL≥9, MPL≥5 LAC	51 LMWH 5000 IU + ASA 75mg vs 47 ASA 75mg	before 12 weeks of gestation	no	78% vs 72% NS
Brenner *et al* 2000	RM (≥3) 50 thrombophilic hered/aqcuired	LMWH enoxaparin + low dose ASA for aPL	confirmed viable pregnancy	no	75%
Pattison *et al* 2000	RM (≥3), *n*=20 aPL positive GPL≥5, MPL≥5 LAC	ASA 75 mg vs placebo	confirmed pregnancy	20/20	85% vs 80% NS
Rai *et al* 1997	RM (≥3), *n*=90 aPL positive GPL≥5, MPL≥3 LAC	UFH 5000 IU x 2 + ASA 75mg *n*=45 vs ASA 75mg *n*=45	confirmed fetal heart beats	45/45	71% vs 42% S
Kutteh 1996	RM (≥3) *n*=50 aCL ≥27 GPL ≥23 MPL	UFH 5000 IU x 2 ASA 81mg, *n*=25 vs ASA 81mg, *n*=25	confirmed pregnancy test	25/25	80% vs 44% S

NS = Nonsignificant result, S = Significant result

There are also other treatment options such as low-dose corticosteroids for patients with aPLs. However, in one study, such a treatment regimen did not improve pregnancy outcome and even increased the risk of preterm birth (Laskin *et al* 1997). Women with RM testing positive for severe aPS, have been shown to benefit from intravenous immunoglobulin (IVIG) in some small series (Kaaja *et al* 1993a, Vaquero *et al* 2001, Carp *et al* 2005) but no reduction in pregnancy loss was found in a larger analysis (Empson *et al* 2005) or in an unselected RM population (Scott 2003).

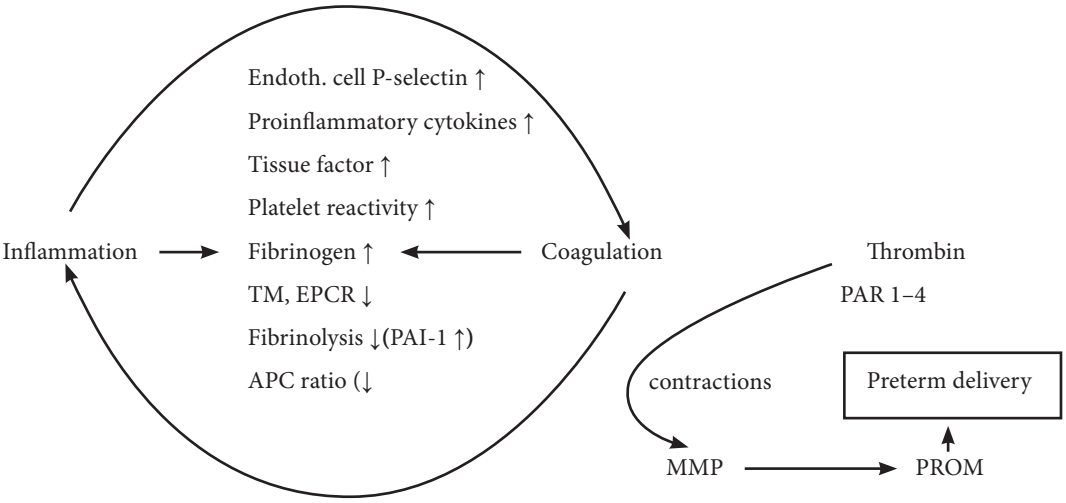
The results of large randomized trials have been published showing no beneficial effect of low-dose ASA in the prevention of recurrence of preeclampsia (Sibai *et al* 1993, Bar *et al* 1997, Caritis *et al* 1998), while there is a lack of data concerning the use of LMWH in this setting. There are only a few (uncontrolled) studies on the treatment or prophylaxis of pregnancy complications such as preeclampsia or IUGR in thrombophilic patients (Riyazi *et al.* 1998, Kupferminc *et al* 2001). Recently, Sergio *et al* (2006) showed that LMWH plus ASA improves pregnancy outcome compared with ASA alone in patients with a history of severe preeclampsia. In the same setting, another study showed no difference in pregnancy outcome (Bar *et al* 2001). We have reported an extreme case of very early-onset preeclampsia in a women with FV mutation and reactio lutealis of the ovaries whose preeclampsia resolved after LMWH treatment and she delivered at term (Saisto *et al* 2004). In one study conducted among women with a previous history of preeclampsia without thrombophilic factors, and homozygous for the angiotensin-converting enzyme (ACE) D allele, LMWH administration reduced the recurrence of adverse clinical outcomes (Mello *et al* 2005a). In one study showed beneficial effect of antithrombin concentrate in acute and severe preeclampsia (Maki *et al* 2000).

There are also evidence that heparins may also have beneficial effects other than anticoagulation, such as binding aPLs (Wagenknecht and McIntyre 1992, Franklin and Kutteh 2003), an anti-inflammatory effect (Manduteanu *et al* 2002, Rops *et al* 2004, Xia *et al* 2004) and complement inhibition (Girardi *et al* 2004). Pathogenetic mechanisms should be further elaborated, but if the role of thrombophilia turns out to be important we could have a good chance to improve pregnancy outcome in such patients through the use of LMWH.

7. Interaction between inflammation and coagulation

Tissue factor, as a trigger of the coagulation cascade, is normally present in the circulation at low levels. Inflammatory mediators such as endotoxin and inflammatory cytokines (TNF-α, IL-1α) increase tissue factor expression in monocytes and macrophages, promoting coagulation. An increase of tissue factor expression caused by inflammation shifts the hemostatic balance towards coagulation (Esmon 2003). In addition to its procoagulative effect, inflammation also downregulates natural anticoagulants and inhibits fibrinolytic activity (Esmon 2003). The important natural anticoagulant pathway, the protein C pathway, is downregulated by inflammation. Thrombomodulin and endothelial protein C receptor (EPCR) are inhibited by inflammatory cytokines such as TNF-alpha (Conway and Rosenberg 1988, Fukudome and Esmon 1994) (Fig. 4).

Fig. 4. Interaction between inflammation and coagulation



Thrombin has a key role in hemostatic mechanisms and in a variety of activities that result in augmentation of the inflammatory response as well. Thrombin has the ability to regulate inflammatory processes itself or via protease-activated receptors (PARs) (Dugina *et al* 2002, Esmon 2003). Activating PAR-1, thrombin induces intracellular adhesive molecule-1 (ICAM-1) expression in endothelial cells. ICAM-1 has an important role in the development of the inflammatory response through stimulation of leukocyte adhesion. Thrombin also induces expression of P- and E-selectins, vascular adhesive molecule-1 (VCAM-1), IL-8, IL-6 and chemokines (Dery *et al* 1998, Kaplanski *et al* 1998). Anticoagulatory factors such as antithrombin and protein C have regulatory and even protective capabilities as regards inflammation (Bajzar *et al* 1996, Joyce *et al* 2001, Souter *et al* 2001). Several

factors affecting inflammation and coagulation have structural homologies, for instance tissue factor and cytokine receptors (Morrissey *et al* 1987). There is also evidence that thrombin has a uterotonic effect (Elovitz *et al* 2000, O'Sullivan *et al* 2004). Thrombin also enhances the expression of decidual matrix metalloproteinases (MMPs), this being strongly linked to premature rupture of the membranes (Rosen *et al* 2002, Stephenson *et al* 2005). Premature rupture of the membranes and premature delivery are associated with excess generation of thrombin (Rosen *et al* 2001, Chaiworapongsa *et al* 2002).

7.1 Preterm delivery

The causes of prematurity are multifactorial but uterine infection plays an important role in the etiology of premature delivery (Slattery and Morrison 2002). Any systemic maternal infection during the preterm period can trigger the onset of preterm delivery (Slattery and Morrison 2002). Even periodontal infection has been suggested to be a source of cytokines, increasing the risk of preterm delivery (Offenbacher *et al* 1996, Boggess *et al* 2005). However, infections are most often subclinical, without any signs of maternal infection. Genital tract infections such as bacterial vaginosis (BV) are associated with an increased risk of preterm delivery (Hay *et al* 1994, Goldenberg *et al* 2000). Furthermore, either ascending microbial colonization from the vagina to the uterus, or colonization via the hematogenic route, both causing endotoxin and exotoxin production and activation of inflammatory cytokines (IL-1, IL-8, IL-6 and TNFα) (Lockwood and Kuczynski 1999), leads to induction of prostaglandin synthesis, an increase in the activity of various proteases, contraction and finally preterm rupture of the membranes (PROM) (Goldenberg *et al* 2000).

Although an association between BV and preterm delivery has been shown, the benefit of antibiotic treatment has remained minor (Brocklehurst *et al* 2000, Kekki *et al* 2001). A beneficial effect of prophylactic antibiotic treatment has been shown in cases of PROM (Kenyon *et al* 2001). Despite intensive research, there are still many open questions connected with the pathophysiology and prevention of preterm delivery.

7.2 Cervical insufficiency

Cervical insufficiency is defined as inability of the uterine cervix to retain pregnancy in the absence of contractions or labor. It is clinically characterized by acute, painless dilatation of the cervix, usually in the second trimester, culminating in protrusion and/or premature rupture of the membranes, and premature delivery. The condition was clinically described in the 1950s (Shirodkar 1955) but its etiology has remained unclear. Reported incidences of cervical insufficiency are low, with estimations varying from 1:1800 to 1:182 (Barter *et al* 1958, Harger 1980, Lidegaard 1994). The great variability of incidences in different studies is perhaps the result of different

diagnostic criteria. It has been suspected that various cervical traumas, pregnancy terminations or obstetric lacerations, as well as congenital uterine abnormalities might be risk factors of cervical insufficiency, but evidence is still limited (American College of Obstetricians and Gynecologists 2003). Despite the known risk factors, predicting premature delivery has been very difficult. The main treatment options have been either bed rest or cervical cerclage, the effectiveness of which has not been proven (To *et al* 2004). Cervical insufficiency can be involved in one form of prematurity, in which uterine infection with activation of inflammatory cytokines (IL-1, IL-8 and TNFα) plays an important role (Lockwood and Kuczynski 1999). Interactions between inflammation and thrombosis (Esmon 2003) give us a new viewpoint in regard to premature delivery.

8. Genetic polymorphism of coagulation factors in recurrent miscarriage

There are several studies concerning genetic polymorphism in RM. The most common thrombophilias associated with fetal losses are listed in table 8 . There are also numerous studies concerning identification of polymorphism of coagulation factors (e.g. TF, TFPI, fibrinogen, FXII, FXIII), the clinical relevance of which, even in pregnancy complications, has not been ascertained (Bertina 2001).

8.1 Plasminogen activator inhibitor I (PAI-1) and Coagulation factor XIII

For successful implantation, plasminogen activator inhibitor type 1 (PAI-1) is believed to control maternal tissue during trophoblast invasion. In the coagulation mechanism, coagulation factor XIII finally cross-links fibrin. Homozygosity of PAI-1 4G, and FXIII34 Leu polymorphism have also been associated with RM (Dossenbach-Glaninger *et al* 2003). Impaired fibrinolysis may result in insufficient trophoblast invasion and unbalanced fibrin deposition.

8.2 Thrombomodulin and Endothelial protein C receptor polymorphism

Animal models are one possibility to find out whether genes are essential or not for normal embryonic development. These include two thrombophilia-associated genes, those for thrombomodulin (TM) and for endothelial protein C receptor (EPCR), suspected to be associated with RM. Loss of function of TM causes early post-implantation embryonic lethality before establishment of a functional cardiovascular system in the mouse embryo (Healy *et al* 1995). Embryogenesis is disrupted at two different developmental stages, indicating a crucial role for TM in both. Expression of TM in non-endothelial

placental cells is required for proper function of the early placenta, while the absence of TM from blood vessel endothelium causes excessive activation of the embryonic blood coagulation system (Isermann *et al* 2001).

Deletion of the EPCR gene in mice leads to embryonic lethality before embryonic day 10.5. However, $EPCR^{-/-}$ embryos removed from extra-embryonic membranes and tissues at day E7.5 and cultured *in vitro* developed beyond E10.5, suggesting a role for EPCR in the normal function of the placenta and/or at the maternal-embryonic interface (Gu *et al* 2002). Endothelial protein C receptor is normally detected on giant trophoblast cells, which are in direct contact with the maternal circulation and its clotting factors. If EPCR is not expressed on the giant trophoblast cells, even enhanced expression of EPCR in the embryo cannot rescue the embryo. Conversely, selective EPCR expression on the giant trophoblast cells rescues EPCR-deficient embryos (Li *et al* 2005). Thrombosis is observed surrounding trophoblast giant cells derived from $EPCR^{-/-}$ embryos but not around those derived from $EPCR^{+/+}$ or $EPCR^{+/-}$ cells (Gu *et al* 2002). These observations suggest that extra-embryonic EPCR expression is essential for embryonic viability and plays a critical role in the control of blood coagulation at the feto-maternal interface.

Thrombomodulin and EPCR are glycoprotein receptors that both play key roles in the protein C anticoagulant pathway, the major regulatory mechanism that suppresses coagulation. Thrombomodulin is an endothelial cell surface receptor expressed mainly on the endothelial surfaces of blood vessels and in the placenta. It forms a complex with thrombin, which then converts protein C to activated protein C (Maruyama *et al* 1985, Van de Wouwer *et al* 2004, Dahlbäck and Villoutreix 2005). Endothelial protein C receptor is a type 1 transmembrane receptor, expressed primarily on endothelial cells of large blood vessels and in the placenta and developing cardiovascular system in the fetus. It functions in the protein C pathway by binding protein C and presenting it to the TM-thrombin complex on the endothelium, thereby increasing the rate of protein C activation (Stearns-Kurosawa *et al* 1996, Laszik *et al* 1997, Crawley *et al* 2002).

9. Aims of the study

The aims of the study were to investigate

I. the impact of initial treatment of DVT during pregnancy with LMWH compared with the traditional treatment with UFH on pregnancy and maternal long-term outcome

II. the potential role of hereditary thrombophilias in the pathophysiology of cervical insufficiency by studying the prevalence of hereditary thrombophilias in cervical insufficiency

III. plasma levels of annexins IV and V at the beginning of pregnancy in women with a history of recurrent miscarriage, and the association of these annexin plasma levels with the presence of antiphospholipid antibodies

IV. polymorphism of TM and EPCR in women with a history of RM

10. Material and Methods

Altogether, 153 patients and 818 controls were investigated in connection with this thesis. A description of the studies (I–V) is presented in table 11. The study protocols were approved by the local ethics committee.

Table 11. Patients and methods in Studies I–V.

	Study I	Study II	Study III	Study IV	Study V
Study group	patients with DVT during pregnancy	patients with post-DVT during pregnancy	patients with cervical insufficiency	patients with RM	patients with RM
Design	prospective, observational LMWH vs UFH	prospective, observational LMWH vs UFH	retrospective case-control	prospective comparative	retrospective case-control
Outcome	Maternal outcome of DVT	prevalence of PTS after DVT	prevalence of FV and prothrombin mutation	levels of annexin IV and V associated with the presence of aPL	prevalence of TM and EPCR polymorphism
Number of patients	21 LMWH	25 LMWH 21 from study I	42	68 patients 77 pregnancies 25 controls	86
Controls	10 UFH	10 UFH	617 healthy blood donors	25	191, no history of miscarriage

General hemostatic tests used in studies I–V

In patients using UFH, APTT was measured with ACL Futura and ACL 2000 equipment (Instrumentarium Laboratory, Helsinki, Finland) and the reagent PTT AUTOMATE (Diagnostica Stago, Paris, France).
Anti-Xa measurements (patients with LMWH) were carried out by using a chromogenic substrate assay based on inhibition of bovine factor Xa by heparin-activated antithrombin III (HEPRN method, DuPont aca IN analyser, DuPont Co., Wilmington, DE, USA). APTT (normal range 24–34 sec) was measured by using Platelin LS equipment (Organon Teknika, Boxtel, the Netherlands).

All patients in studies I–V were analyzed for hereditary and acquired thrombophilia. Factor V Leiden was analyzed by the method described by Bertina *et al* (1994), and the G20210A prothrombin mutation by the method described by Poort *et al* (1996). Lupus anticoagulant was studied by using the Russell Viper Venom Test, with pooled normal plasma in confirmatory tests, anti-cardiolipin IgG (normal if <10 GPL) by QUACA Anti-Cardiolipin Elisa (Cheshire Diagnostics Limited, Great Britain), anti-thrombin by Coamatic AT 400 (normal range 84–108% of normal control) (Chromogenix AB, Mölndal, Sweden), protein C (normal range 67–131% of normal control) by Coamatic Protein C (Chromogenix AB, Mölndal, Sweden), APC ratio (normal if >2) by using kits from Chromogenix AB (Mölndal, Sweden), and protein S (normal range 43–126% of normal control) by Liatest Protein S (Diagnostica Stago, Asnieres, France).

Study I

The first 10 consecutive patients received intravenous unfractionated heparin for treatment of acute DVT, and the next 21 patients received low molecular weight heparin (dalteparin). Most patients (29) had DVT in the lower limbs, but two had it in the upper limbs. In all cases, diagnosis was based on compression ultrasonography (CUS, color Doppler). Ultrasonographic examination was repeated 2–6 weeks after starting heparin treatment if symptoms occurred or there was suspicion of recurrence in the affected limb. Treatment length was 7 days in both groups. In the UFH group a bolus of 5000 IU was injected and thereafter the daily dose was 15 000 IU/500 ml infused at 36 ml/h. Treatment was followed by means of repeated plasma APTT measurements (every six hours) and the target value was 50–100 seconds. The next 21 patients were treated subcutaneously with dalteparin, 200 IU/kg/d, divided into two doses. The doses were adjusted by means of anti-Xa measurements; target levels 0.5 and 1.5 IU/ml (before and 3 hours after subcutaneous injection, respectively).

Both groups received either dalteparin (28) or the LMWH enoxaparin (1) for secondary prophylaxis at treatment doses during the following two weeks and thereafter the dose (twice a day) was gradually decreased until delivery. This prophylactic LMWH dose at the end of pregnancy was adjusted

on the basis of plasma anti-Xa measurements (target 3 hours after injection: 0.5–0.7 IU/ml).

The LMWH doses were halved on the day of delivery in both groups. After delivery the patients were treated with warfarin (3–6 months) and LMWH was stopped when INR was 2–3 for at least two days.

Efficacy of treatment was evaluated daily by measuring the circumference of the affected limb at the mid-femoral and crural level and by investigating the occurrence of post-thrombotic symptoms (pain, edema, physical limitations and paresthesia). Re-examination by means of US was performed in cases of worsening of the post-thrombotic symptoms 2–6 weeks after starting treatment. Pulmonary ventilation scans were performed only in cases of clinical suspicion of PE. Platelet levels were followed during both acute treatment and prophylaxis. Episodes of bleeding (hematomas, hematuria, gingival and vaginal bleeding) were recorded.

Study II

Our patients were recruited from a previous open prospective observational study in which LMWH and UFH were compared in the initial treatment of DVT during pregnancy (Ulander *et al* 2002). Thirty-five patients with DVT in the lower limbs were enrolled in the study. Ten consecutive patients received iv UFH for treatment of acute DVT, then the next 25 patients (4 patients additional to those in Study I) received LMWH for the same indication. The diagnosis was based on compression ultrasonographic (color Doppler) examinations.

To evaluate the prevalence of post-thrombotic symptoms, we used a modification of a protocol described by Villalta *et al* (1994). In this protocol, the presence of symptoms (feelings of heaviness, pain, cramps, pruritus and paresthesia) and signs (edema, redness, pain during calf compression, skin hyperpigmentation, new venous ectasia) of PTS was noted. For each item a score of 0 (= no or minimal) to 3 (= severe) was assigned. A score of ≥ 15 indicates severe PTS and a score of 5–14 indicates mild or moderate PTS (**appendix II**).

Duplex Doppler ultrasonographic examination was carried out among 17 patients. Eighteen patients did not want to participate in the US examination, mostly because they were symptomless. Ultrasonography was performed by a single experienced vascular surgeon (A.L). The examination was carried out with color-duplex equipment (Image Point®/Hewlett Packard) using Sonos 5500 in both supine and upright positions. Reflux (> 0.5 sec) was determined in a non-weight-bearing leg in an upright position with the aid of a mechanical distal muscle pump and Veno-Pulse® equipment (Stranden). The examination included scanning of the external iliac, deep and superficial femoral, popliteal and calf veins as well as superficial veins. Valvular incompetence was defined by the presence or absence of reflux after distal compression. The findings were categorized using the international

CEAP (C = Clinical, E = Etiology, A = Anatomy, P = Pathophysiology) classification (Porter and Moneta 1995) (**appendix III**).

Study III

Fifty-eight consecutive patients with a diagnosis of cervical insufficiency (ICD-10: O34.3) treated in Helsinki University Hospital from 1996 to 2003 were first identified. The diagnosis was confirmed from the hospital records. Cases of intrauterine fetal death were excluded, as were twin pregnancies and patients with congenital uterine anomalies. Of the 58 patients, 42 were willing to participate in the study and gave informed consent.

At admission of the patients to the hospital, abnormal cervical culture results, and clinical infection defined by elevated levels of serum C-reactive protein (CRP), or clinical signs of infection were recorded. A possible history of sexually transmitted disease was recorded from hospital files and acute infection was excluded by means of cervical cultures or specific PCR tests.

The control group consisted of 617 healthy Finnish blood donors recruited as first-time donors by the Finnish Red Cross Blood Service. This group included both sexes, as FV Leiden mutation and prothrombin gene G20210A mutation are not linked to gender.

The prevalence of common hereditary thrombophilic mutations (FV Leiden, prothrombin) were examined among patients with a history of cervical insufficiency compared healthy control population.

Study IV

Sixty-eight women with recurrent miscarriage were included in the study. At the time of recruitment, all subjects had positive pregnancy test results before 6 weeks of gestation, which was judged by the last menstrual period. All women conceived spontaneously. The women were examined for the presence of known etiological factors of RM. Hereditary thrombophilia tests were performed as described above. Patients were followed at out-patient clinic and blood samples were collected in the first visit (5^{th} weeks of gestation), 6^{th} and 8^{th} weeks of gestation. Patients aPL status was carefully examined. Annexin IV and V levels were investigated in those visits and compared with the presence of aPL. Moreover, 25 unselected controls without a history of adverse pregnancy outcome were included in the study. These women volunteered to have their annexin levels assessed at the 6th and 8th weeks of gestation.

1. Acquired thrombophilias, defined by the presence of lupus anticoagulant, were assessed by dRVTT and PTT-LA tests. Anticardiolipin antibodies were assayed as described previously in detail (Vaarala *et al* 1993). Concentrations of IgM aCL antibodies were determined in the same way as for IgG aCL antibodies except that alkaline phosphatase-conjugated anti-human IgM was used as the detector antibody. The cut-off level for

positivity was determined as a result exceeding 10 IgG phospholipid (GPL) antibody units for IgG class aCL antibodies, and 20 IgM phospholipid antibodies (MPL) for IgM class aCL antibodies.

2. Antiphosphatidylserine antibodies of IgG class were measured in the same way as aCL antibodies. Microtiter wells were coated with phosphatidylserine (P-6641 / P-8518, Sigma Aldrich, St. Louis, MO), diluted 1:200 in chloroform-methanol (1:3). The wells were left to dry overnight at 4 °C and post-coated with 10% bovine serum in PBS; thereafter the assay was continued as in aCL determinations. The cut-off level for positivity was set at mean + 2 SD (0.184 OD units) of samples from 100 blood donors.

3. A detailed description of the antiprothrombin antibodies has been published previously (Puurunen *et al* 1996). The cut-off limit for positivity was set at the 95th percentile of samples from 200 normal blood donors (0.379 OD units).

4. The assay for anti-β_2 glycoprotein I (anti-β_2GPI) was performed in the similar manner as the ELISA for antiprothrombinwith the exceptions that the concentration of anti-β_2GPI used for coating was 5 µg/ml and the samples were diluted at 1:200. The mean + 3 SD of samples from 98 blood donors (0.143 OD units) was used as the cut-off limit for positivity. All subjects with abnormal values were re-evaluated two months later.

5. Blood samples for assay of annexins IV and V were collected using tubes containing EDTA at the time of recruitment, and at 6 and 8 weeks of gestation. In cases of miscarriage, further blood samples were not taken. Platelet-poor plasma (PPP) was separated by centrifugation twice at 2,540 *g* for 15 min and stored at -70 °C until assayed. Concentrations of annexin IV in plasma were determined by sandwich ELISA using PAB-AX4 to human Anx IV for capture and biotin-conjugated AS17 for detection. The standard curve was obtained using His-rAnx IV. Concentrations of annexin V in plasma were determined by using a human annexin V ELISA kit (Bender MedSystems Products, Vienna, Austria), as previously described.(Masuda *et al* 2004).

Study V

Polymerase chain reaction

DNA was extracted from whole blood collected from patients with RM (n=40 couples and 6 women) and controls (n=191) using Puregene DNA isolation kits (Gentra Systems, Minneapolis, USA). Polymerase chain reactions of TM and EPCR exons (non-coding exons included) were performed in a 25 µl reaction mix containing the following reagents: 50–100 ng of genomic DNA, 1× PCR buffer (Applied Biosystems, Foster City, USA), 2 nmol of each dNTP, 10 pmol forward primer, 10 pmol reverse primer and 0.1 units of AmpliTaq Gold DNA-polymerase (Applied Biosystems, Foster City, USA). Additionally, DMSO (final concentration 5% (v/v)) was added to some of the amplicons. Thermocycling was performed in a PTC-225 DNA Engine Tetrad thermocycler (MJ Research, Waltham, USA). Initial denaturation at 95 °C for 10 min was followed by 35 cycles of denaturation at 95 °C for 30s, annealing for 45 s (temperature depending on the amplicon), and extension at 72 °C for 45 s. Final extension was performed at 72 °C for 10 min. PCR conditions and primer sequences are shown in Table 12. The specificity of the amplification was confirmed by agarose gel electrophoresis before further analysis.

Mutation analysis

Denaturing high performance liquid chromatography (DHPLC) analysis of the samples was carried out using a Transgenomic WAVE® Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, USA) and the associated Navigator software as described by Kaare *et al* (2006). Conditions used for DHPLC analysis are shown in Table 12. Following DHPLC screening, samples showing heterozygous peaks were sequenced in order to determine the nature of the sequence change. Additionally, for each amplicon, 10 samples showing only a homoduplex peak were sequenced to confirm that no variation went undetected. Direct sequencing was performed using BigDye version 3.1 sequencing chemistry and an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, USA).

By means of sequencing, a c.1418C>T variation in TM was detected. This variation was not detected by DHPLC. All samples were genotyped using the restriction enzyme Cac8I (New England Biolabs, Ipswich, USA). Homozygosity for EPCR variations c.655A>G and c.717+16G>C were also detected by RFLP using restriction enzymes AciI and DdeI, respectively. After digestion the restriction pattern was visualized in agarose gel. Homozygosity of EPCR variation c.323-20T>C was genotyped using DHPLC. To detect homozygous variations, 5 µl of the genotyped samples were mixed with 5 µl of a reference sample with no variations, and analyzed on the WAVE System. Homozygosity of TM variation c.1728+23_+40del, and EPCR c.323-9_336dup were genotyped using 3% agarose gels, on which an 18/23 bp

difference in fragment sizes was detected. The nature of variations predicted to change an amino acid were analyzed by the SIFT (Sorting Intolerant From Tolerant) program (http://blocks.fhcrc.org/sift/SIFT.html).

Statistical analysis

Continuous variables were presented as means (+/- SD) ana were analysed by using Student´s *t*-test. In the cases of skewness of data, non-parametric Mann-Whitney test was used to analyse diefferences between studygroups (studies I, II, IV, V). Logistic regression analysis was used in study II to identified risk factors of PTS. In study III was used $\boldsymbol{X}^2$ –test to analyse categorical data. Kruskal-Wallis ANOVA with multiple-comparison Z-Value Test were used to analyse differences between annexin levels in study IV. The statistical analyses were performed by means of NCSS software version 2004 (NCSS Inc., Kaysville, Utah). Values of P of ≤ 0.05 were considered statistically significant.

11. Results

11.1 Outcome of deep venous thrombosis (I, II)

There were no statistically significant differences in baseline maternal or neonatal data between the groups. On the basis of our results, LMWH was as effective and safe as unfractionated heparin in the treatment of DVT during pregnancy. The gestational age of DVT tended to be lower in the LMWH group (21 weeks of gestation) than in the UFH group (27 weeks of gestation), but the difference did not reach statistical significance. The time from the beginning of symptoms to diagnosis (diagnostic delay) tended to be relatively long in both treatment groups (8 days in the UFH group and 7 days in the LMWH group; table 12).

In 23 (74.2%) of the cases DVT was in the left leg and in 24 (77.4%) cases it was in the proximal region of the lower limb. There were no statistically significant differences in thrombophilic data or localization of the DVT between the groups.

Table 12. Thrombophilic data and localization of deep venous thrombosis (DVT) during pregnancy

	UFH (n=10)	LMWH (n=21)	*p*
Weeks of gestation at diagnosis, mean (SD)	27.0 (8.5)	21.0 (9.8)	NS
Personal history of DVT, *n* (%)	1 (10)	4 (19.0)	NS
Family history of DVT, *n* (%)	3 (33.3)	9 (42.9)	NS
Hereditary thrombophilia, *n* (%)	2 (20)	7 (33.3)	NS
Acquired thrombophilia, *n* (%)	0 (0)	1 (4.8)	NS
Localization of DVT, *n* (%)			
Lower limb, proximal	8 (80)	16 (76.2)	NS
Lower limb, distal	2 (20)	3 (14.3)	NS
Upper limb	0 (0)	2 (9.5)	NS
Lower left/lower right limb*	8/2	15/4	NS
Delay in diagnosis, days**	7.9 (9.3)	6.8 (8.1)	NS
Failures of treatment, *n*	0	1 (recurrent DVT)	NS
Dose for LMWH prophylaxis, IU/24 h Mean (SD)	7777 (1954)	7875 (2470)	NS
Symptoms after treatment, *n* (%)	4 (40)	8 (38)	NS
Re-canalized thrombi in US, *n*	1/6	7/11	NS
Hemorrhagic complications during treatment	0	0	NS

*(left vs. right side in all patients, $p<0.05$), NS=not significant
**Time from onset of symptoms to diagnosis and treatment

No PE was detected. One failure of treatment was found in a patient who had proximal DVT. After the acute period of DVT treatment, both groups received equivalent doses of LMWH for thromboprophylaxis. There were no differences in symptoms (pain or edema) in the affected limb after treatment. In 17 patients (54.8%) re-examination of the veins by ultrasonography was performed 4 weeks after treatment and re-canalization was evident in 16.7% and 63.6% of patients in the UFH and LMWH groups respectively, but the difference was not statistically significant (p=0.06, Fisher´s exact probability test).

There were no congenital anomalies or treatment-associated side effects (osteoporosis, thrombocytopenia) during pregnancy. One patient in the LMWH group had hematuria, but this occurred during the prophylactic phase. It disappeared spontaneously after decreasing the dose of LMWH. One infant died after premature delivery at 23 gestational weeks (birth weight of 340 g) as a result of severe preeclampsia. The mother had a positive lupus anticoagulant test result and DVT nine weeks before premature delivery. Epidural anesthesia was given if the dose of LMWH was low/moderate (5000 IU for dalteparin) and given 12 hours before delivery. Epidural anesthesia could be given in 35% of our patients. The decision on mode of delivery was made on the basis of obstetric factors in both groups. Delivery and neonatal data did not differ between the groups and were the same as observed in healthy women at our unit.

There were no differences in long-term outcome after pregnancy-associated DVT, initially treated with either LMWH or UFH. Long-term outcome was evaluated by means of a questionnaire and by duplex Doppler US examination.

The mean doses of heparin for the initial phase of treatment were 25 537 IU/24 hours in the UFH group and 16 000 IU/24 hours in the LMWH group during a one-week period. Both groups received equal doses of LMWH (mean 7500 IU/24 hours) for secondary prophylaxis until the end of pregnancy.

There were no significant differences in demographic data between the treatment groups. In 20 cases (57%) DVT was proximal (femoral, iliac). One patient delivered after the first episode of DVT and one patient had recurrent DVT after the index pregnancy.

Post-thrombotic syndrome scoring was performed according to Villalta *et al* (1994). The analysis was carried out at a mean of 51 ± 26 (SD) months after thrombosis in the UFH group and after 42 ± 17 months in the LMWH group (NS). The mean post-thrombotic score was 5.8 in the UFH group and 4.7 in the LMWH group (NS). Forty-nine percent of all patients had a score of four or less (no signs or symptoms of post-thrombotic syndrome). No patient had ulcers.

Table 13. Long-term outcome of DVT

	UFH (*n*=10)	LMWH (*n*=25)	p
Age, mean (SD)	31.6 (5.1)	32.6 (5.3)	NS
BMI, mean (SD)	23.3 (4.0)	25.2 (4.5)	NS
Multiple DVT	1 [1)]	9 [2)]	
Localization (%)			
Distal	4 (40)	11 (44)	NS
Proximal	6 (60)	14 (56)	NS
Thrombophilia-positive [3)] (%)	2 (20)	6 (24)	NS
Post-thrombotic symptoms (*n*=35)			
Time since DVT (years), mean (SD)	4.3 (2.2)	3.6 (1.4)	NS
Villalta score, mean (SD)	5.8 (4.1)	4.7 (3.5)	NS
Villalta score ≤ 4 (%) (no PTS symptoms)	4 (40)	13 (52)	NS
Villalta score 5–14 (%) (mild or moderate PTS symptoms)	6 (60)	12 (48)	NS
Duplex Doppler US (*n*=17)			
Not performed	5	5	
Normal	1	5	
Superficial venous insufficiency or reflux	1	6	
Deep venous insufficiency or reflux	1	2	
Operated varicose veins	2	2	

[1)] Recurrent DVT after index pregnancy
[2)] All before index pregnancy
[3)] Two patients positive for lupus anticoagulant, one patient with high anticardiolipin antibodies (IgG), six patients with FV Leiden mutation (heterozygote) and one patient with a constantly high level of FVIII (> 150 IU)

Seventeen patients were examined by means of duplex Doppler ultrasonography. Seven patients (1 in the UFH group and 6 in the LMWH group, NS) had superficial venous insufficiency and three patients (1 in the UFH group and 2 in the LMWH group, NS) had deep venous insufficiency. One patient without leg ulceration had severe PTS as defined by symptoms and ultrasonography. Four patients had been operated on for varicose veins.

Table14. Prevalence of post-thrombotic symptoms according to localization of DVT

Localization of DVT	No symptoms (Villalta score ≤ 4)	Mild or moderate PTS (Villalta score 5–14 or superfic. ven. insuff.)	severe PTS (Villalta score ≥ 15 or deep ven. insuff.
Distal DVT	5/15 (33.3%)	8/15 (53.3%)	2/15 (13.3%)
Proximal DVT	13/20 (65%)	6/20 (30%)	1/20 (5%)

The total incidence of post-thrombotic symptoms was 51%. In multivariate logistic regression analysis no prognostic factors were found as regards severe post-thrombotic symptoms (Villalta score ≥ 15 or deep venous insufficiency).

11.2 Prevalence of FV Leiden and prothrombin G20210A mutation in cervical insufficiency

Thrombophilic mutation was found significantly more often among women with a history of cervical insufficiency compared with controls (OR 6.7, 95% CI 2.7–18.4; table 15). The prevalence of cervical insufficiency was 2 per 1000 deliveries in our hospital. The mean age of the study population was 36 years (range 25–46 years) and the mean body mass index (BMI) during the last pregnancy was 22 kg/m^2 (range 18–35 kg/m^2). Nine patients with past *Chlamydia trachomatis* infection were found.

Forty-two patients had had 122 pregnancies. Of the thrombophilic patients, 1/7 (14%) had had a history of dilatation and curettage as a result of legal pregnancy termination and another one (14%) had had cervical laceration. The prevalence of cervical procedures or traumas was greater in non-thrombophilic patients (74%) than in thrombophilic patients (28%).

Table 15 . Thrombophilic mutations (FV and FII) in patients with a history of cervical insufficiency (*n*=42), and general population (*n*=617)

	Patients, n (%)	Controls, n (%)	OR (95% CI)
FV Leiden	4 (9.5)	15 (2.4)	4.2 (1.5–13.6)
Prothrombin G20210A	3 (7.1)	3 (0.5)	15.7 (3.4–70.9)
FV Leiden or Prothrombin G20210A	7 (16.7)	18 (2.9)	6.7 (2.7–18.4)

The patients were also tested for other hereditary thrombophilias and one patient with repeatedly low protein C activity but a normal antigen level was found. None of the patients had antithrombin or protein S deficiency. These findings were not included in the statistical analysis and not analyzed in the controls.

11.3 Annexin IV and V levels in early pregnancy in patients with a history of RM

Sixty-eight women with a history of RM participated in the study. There was a total of 77 pregnancies in the study group. The demographic data between studygroups (aPL positive vs aPL negative) did not differ. Seventy-three percent of the subjects were primary aborters without successful pregnancies. Hereditary or acquired thrombophilic disorders were found in 53% (36/68) of the subjects. Table 16.

Table 16. Demographic data of patients in study IV

	Antiphospholipid antibodies	
	positive	negative
Patients	26	42
BMI	23,5	24
Age	31,6	31,9
Primary aborters	16	33
Secondary aborters	10	9
Number of miscarriages		
3 first trimester	19	23
4 first trimester	3	10
5 first trimester	2	0
on second trimester (>22 th)	5	7
Controls		25
miscarriages		3

Plasma levels of annexin V were significantly higher at the beginning of pregnancy in women with aPL antibodies (lupus anticoagulant, aCL, antiphosphatidylserine, antiprothrombin, and/or anti-β_2-GPI) compared with those without aPL antibodies (p=0.03). Levels of circulating annexin V were also higher at the 6th patients among aPL antibodies (P= 0.01) than aPL negative patients and controls. At 8th weeks of gestation in subjects with aPL antibodies annexin V levels were also higher than aPL negative patients (P=0.01) but the difference between aPL positive and controls did not reach statistical significance

Plasma levels of annexin V were also studied in relation to pregnancy outcome. A tendency towards higher plasma levels of annexin V was observed in those whose pregnancies ended in miscarriage compared with those with successful pregnancy, although the results did not reach statistical

significance (p=0.10). The plasma levels of annexin V were also analyzed in relation to the presence of anti-β2GPI antibodies, but no statistically significant difference was found.

Plasma levels of annexin IV at the first visit in women with aPL antibodies were similar to those at 6 and 8 weeks of gestation. Moreover, there were no significant differences in plasma annexin IV levels between women with and without aPL antibodies. The plasma level of annexin IV was not of prognostic value as regards pregnancy outcome.

11.4 Prevalence of TM and EPCR polymorphism in recurrent miscarriage (RM)

In total, 86 cases (40 couples and 6 women) with a history of unexplained RM and 191 controls were screened by means of DHPLC for mutations in TM and EPCR genes. Two sequence variations in the TM gene and four in the EPCR gene were found (table 17). We did not find significant differences in the prevalence of TM and EPCR polymorphism among couples suffering from RM compared with controls.

All variations were detected in both patients and controls. There were no significant differences in the allele or genotype frequencies between patients and controls for any of the sequence variations. The 40 couples included in this study were also analyzed to determine if the variations existed in both partners. The common variations c.323-20T>C, c.655A>G, and c.717+16G>C in the EPCR gene and c.1418C>T in the TM gene were all detected in both partners of a couple, enabling a homozygous state in the fetus. Additionally, the newly identified 1728+23_+40 deletion was detected in a heterozygous state in both partners of one couple.

Table 17. TM and EPCR sequence variations. Locations of the variations, predicted amino acid changes, and the numbers of patients and controls carrying the rarer allele in a hetero/homozygous state. Numbering of the nucleotides is relative to the adenine in the ATG start codon in the reference sequence.

DNA variation (predicted amino acid change)	Location	Heterozygous patients (homozygous patients) n=86	Heterozygous controls (homozygous controls) n=191
TM variations			
c.1418C>T (Ala455Val)	exon 1	41 (5)	84 (8)
c.1728+23_+40del[1]	3´UTR	5	5
EPCR variations			
c.323-20T>C	intron 2	41 (15)	95 (33)
c.323-9_336dup (TyrProGlnPheLeuSTOP)	exon 3	2	1
c.655A>G (Ser219Gly)	exon 4	22 (1)	49 (2)
c.717+16G>C	3´UTR	41 (15)	93 (34)

[1] Novel variation (not previously reported)

12. Discussion

12.1 Venous thromboembolism

The established treatment for DVT during pregnancy before this study was administration of unfractionated intravenous heparin (Greer and De Swiet 1993, Lowe 1997). It is still widely used in many countries (e.g. the US). There is a lack of randomized studies on the treatment of DVT during pregnancy in regard to comparison of LMWH with unfractionated heparin. At present, doses of LMWH in the treatment of DVT during pregnancy have been settled (1 mg/kg or 100 IU/kg twice daily) for the initial treatment, but the duration of the treatment phase and the doses during the rest of the pregnancy are not yet fixed. Recommendations for duration of the treatment phase and the doses of LMWH towards the end of pregnancy are based only on the results of small observational studies, and expert recommendations (Bates *et al* 2004, Greer and Hunt 2005). In our study the starting doses for DVT treatment were slightly higher than recommended by the manufacturer, with a mean daily dalteparin dose of 16 000 IU per mean body weight of 70 kg. After the initial treatment phase all patients were given treatment doses of LMWH for another two weeks. Thereafter the dose was gradually decreased to a high prophylactic level depending on the anti-Xa results. In our study one recurrence of DVT was observed in the early secondary prophylactic phase and thus we recommend extension of the treatment phase to at least 4–6 weeks, continuing with doses near the treatment dose (80%; twice a day) until delivery. Nowadays, there are two main alternatives for treatment: 1) the dose is adjusted according to total body weight and continued to the end of pregnancy, because pregnancy itself is a hypercoagulable state and the risk of recurrence is higher towards the end of pregnancy, and 2) after the acute phase of treatment the dose of LMWH is reduced because of concerns that therapeutic doses of LMWH may carry a risk of osteoporosis and bleeding (Greer and Hunt 2005). Our clinical practise follows the first alternative. Anti-FXa-based dose adjustments could be made in the steady-state phase of treatment of VTE. Low molecular weight heparin does not cross the placenta; thus fetoplacental weight is not relevant (Greer and Hunt 2005). Moreover, adipose tissue receives a comparatively small blood supply (Greer and Hunt 2005). The need for FXa measurements during prophylactic treatment of LMWH has not been defined. There is no evidence to show that a certain FXa level during pregnancy prevents VTE and many experts recommend

a fixed dose of LMWH throughout pregnancy (Greer and Hunt 2005). However, some authors recommend higher prophylactic doses of LMWH in morbidly obese patients (Scholten *et al* 2002). Whether or not peak or nadir values of FXa should be measured is also unclear.

The efficacy of treatment can be evaluated by assessing post-thrombotic symptoms and the patency of thrombotic veins. Our study showed no differences in the occurrence of post-thrombotic symptoms after either of the heparin treatments during pregnancy. Diagnostic delay (time from symptoms to beginning of treatment) reflects difficulties in diagnosing DVT during pregnancy (Garcia-Rio *et al* 1996). However, a delay of one week (mean in both groups) before diagnosis seemed not play a role in the frequency or severity of post-thrombotic syndromes. In 55% of the patients, compression ultrasonography was repeated. The re-canalization rate of the thrombotic vein tended to be higher in the LMWH group than in the UFH group, but as this evaluation was offered only to patients with symptoms, we cannot give any definitive answer as to whether or not LMWH is better in this sense.

The prevalence of PTS after DVT is unknown, the frequency ranging between 20% and 100% (McColl *et al* 1999) depending on predisposing factors and wide variability of criteria of PTS. There are different clinical scales to diagnose PTS. Recently, Kahn *et al* (2006) reported that the Villalta scale may overestimate mild PTS, whereas the Ginsberg method (Ginsberg *et al* 2000) could be better as regards the most severe cases of PTS. In our study, symptomatic patients were also examined by means of duplex Doppler US, and thus the diagnosis of severe PTS could be confirmed.

Severe PTS (PTS score ≥ 15 or deep venous insufficiency) was observed only in three patients of 27 (11%), whereas the total incidence of mild or moderate post-thrombotic symptoms was 51% in our study (Ulander *et al* 2003). Our results do not support the opinion that pregnant patients with DVT are at an elevated risk of developing severe PTS. Similar results have been reported previously (Rosfors *et al* 2001). In their 16-year follow-up study showed that proximal DVT is a risk factor as regards the development of PTS. On the other hand, there is also evidence that localization of DVT (distal or proximal) is a relatively poor prognostic factor of PTS (Janssen *et al* 1997). However, the prognosis is poorest when thrombosis has largely affected the deep venous vasculature, causing valvular incompetence. Only 10–30% of patients are symptom-free after iliac DVT (AbuRahma *et al* 2001, Janssen *et al* 1997). Since DVT during pregnancy is often massive and proximal (in our study 59%), it has been concluded that these patients are at an extremely high risk of developing PTS. In addition, the prolonged venous stasis caused by the gravid uterus, and lack of physical exercise at the end of pregnancy may worsen the outcome of DVT. Recurrent DVT has been reported to enhance the risk of PTS 6-fold compared with patients without recurrence (Prandoni *et al* 1996), and the risk of recurrent DVT is highest

during the first year after the first episode of DVT (Holmström *et al* 1999). One explanation for the low incidence of severe PTS is that our patients were otherwise healthy young women without malignancies associated with high risks of recurrent DVT and PTS (Holmström *et al* 1999). Another explanation could be effective initial phase treatment of thrombosis, which has also been mentioned as a good prognostic factor in the prevention of PTS (Ziegler *et al* 2001). Uterine compression at the end of pregnancy predisposes women to DVT. During the early postpartum period the risk of recurrence in these healthy active women decreases as a result of uterine decompression, but increases as a result of activation of coagulation. In our study 25% of the patients with DVT had a history of DVT, thus prophylaxis during pregnancy cannot be emphasized enough. In multivariate analysis no significant risk factor of PTS was found.

Our studies have certain weaknesses such as small sample size and non-blinded design. At the time of the study most non-pregnant DVT patients were treated with LMWH, which had been proven to be safer than UFH in prophylactic use (Pettilä *et al* 2002). To perform a randomized prospective study aimed at investigation of pregnancy outcome and/or maternal long-term outcome would have been unrealistic. However, according to our results, LMWH seems to be as good as UFH in the acute phase of treatment of DVT in terms of safety and efficacy. There is also evidence for effectiveness in the treatment of pulmonary embolism (Kaaja and Ulander 2002). There were no bleeding complications or cases of heparin-induced thrombocytopenia during the treatment period in either of the heparin groups. Increasing evidence concerning maternal side effects has shown the superiority of LMWH over UFH, giving LMWH certain advantages, for example in long-term use (Greer and Nelson-Piercy 2005, Warkentin and Greinacher 2004). In Finland this was the first randomized study on LMWH and UFH, although with a small number of patients, and it can be called a landmark study, as after it the era of UFH started to decline. Our results are similar to those in other, larger, review articles (Greer and Nelson-Piercy 2005, Sanson *et al* 1999). The safety profile, administration and costs of treatment with LMWHs are superior compared with UFH, thus LMWH has replaced UFH for treatment and prophylaxis of DVT during pregnancy. However, further prospective randomized studies are needed to establish the definitive dose and duration of LMWH in the treatment of DVT during pregnancy.

12.2 The role of thrombophilias in cervical insufficiency

Our preliminary data suggest that hereditary thrombophilia might be an additional risk factor as regards cervical insufficiency and even premature delivery. Cervical insufficiency has similarities with characteristics of premature delivery, although it has often been handled as a separate medical condition. Differences between these conditions may be artificial and thus we can hypothesize that cervical insufficiency is an extreme mode of premature delivery.

Interactions between infection, inflammation and thrombosis are well known. Inflammatory mechanisms upregulate procoagulant factors, downregulate natural anticoagulants and inhibit fibrinolytic activity. Moreover, inflammatory mediators appear to increase platelet reactivity and production of TF (Esmon 2003). The role of thrombin is pivotal in the coagulation system but it is important in augmentation of the inflammatory response as well. There is also evidence that thrombin itself has an uterotonic effect (Elovitz *et al* 2000, O'Sullivan *et al* 2004). Premature delivery and premature rupture of the membranes are associated with excess generation of thrombin (Rosen *et al* 2001, Chaiworapongsa *et al* 2002). Thrombin enhances the expression of decidual matrix metalloproteinases (MMPs) and this is strongly linked to preterm rupture of the membranes (Rosen *et al* 2002, Stephenson *et al* 2005). Diminished blood flow in the spiral arteries resulting from activation of coagulation factors may cause hypoxia and lead to enhanced effects of inflammatory cytokines. Thus it can be hypothesized that in women with thrombophilia, increased thrombin generation could be an additive factor as regards the induction of cervical insufficiency.

Our preliminary study showed that thrombophilia may be an additional risk factor or premature delivery. However, the role of antithrombotic treatment is not clear. Patients at risk of premature delivery are likely to be immobilized, which is an independent risk factor of DVT. Thus, LMWH treatment should be considered, especially in cases with defined thrombophilia. Patients with defined cervical insufficiency should be tested for thrombophilias.

12.3 The role of new local natural anticoagulants (annexins IV and V) in RM

We showed in our longitudinal study that soluble plasma annexin V levels were higher in subjects with aPL antibodies compared with those without aPL antibodies. The physiological function of annexin V is still not fully understood.

It was recently confirmed that plasma purified from the blood of antiphospholipid-positive subjects can reduce annexin V binding to phospholipids (Rand *et al* 2004). The same study group also reported decreased annexin V levels in patients with a history of aPL syndrome with thrombosis compared with non-aPL thromboembolic patients (Rand *et al* 2004). The authors hypothesized that in aPL syndrome aPLs not only inhibit annexin V binding to phospholipid structures, but also bring about resistance to the anticoagulant activity of annexin V. However, there are conflicting data concerning plasma levels of annexin V in aPL syndrome. A study conducted in non-pregnant patients with systemic lupus erythematosus and secondary aPL syndrome showed significantly higher annexin V levels compared with controls, but no correlation was found between the presence of aPL antibodies and a history of thromboembolic complications (Van Heerde *et al* 2003). We found no statistically significant correlation between annexin V levels and pregnancy outcome, although there was a tendency towards lower plasma annexin V concentrations in early pregnancy in subjects whose pregnancies were successful compared with those whose pregnancies again ended in miscarriage. Rand *et al* (2006), in their retrospective case-control study, showed reduced plasma annexin V levels in women with a history of RM. Their study was performed when the women were in a non-pregnant state, thus not correctly reflecting the situation in early pregnancy. Recently published data showed that annexin V levels were higher in amniotic fluid in pregnant women suffering from IUGR at 18–24 weeks of gestation and the authors speculated that there may be some kind of displacing process of annexin V from the placenta to amniotic fluid (Van Eerden *et al* 2006).

Although annexins IV and V have similar anticoagulant properties, their particular effects during pregnancy seem to be different. Masuda *et al* (2004) showed that plasma levels of annexin IV in pregnancy were unchanged compared with the non-pregnant state and they remain unchanged throughout pregnancy. Interestingly, plasma levels of annexin IV increased rapidly in the maternal circulation after delivery. This phenomenon has been suggested to reflect an anticoagulant role of annexin IV in the prevention of DIC (Masuda *et al* 2004). In our study, annexin IV levels in early pregnancy did not differ as regards the presence or absence of antiphospholipid antibodies, or the outcome of pregnancy. Our results are similar to those presented in the currently published literature.

Final connections between plasma annexin V levels, anticoagulant activity and thromboembolic complications cannot be made. However, we can

speculate that a local source of annexin V on placental anionic phospholipid surfaces is probably important for maintenance of pregnancy and placental development, and its role in the systemic circulation remains unclear. It should be pointed out that in the present study it is possible that the number of subjects was not large enough to achieve statistically significant results as regards pregnancy outcome.

12.4 Polymorphism of TM and EPCR genes

It has been shown that many cases of RM and other pregnancy complications are caused by defects in maternal hemostatic responses, leading to disturbances of the uteroplacental vasculature and, in some cases, subsequent fetal loss. While mutations in TM or EPCR genes may cause thrombophilia in the mother, and thereby constitute a risk factor for spontaneous abortions, homozygous mutations in the fetus may cause miscarriage via other mechanisms (Isermann *et al* 2001). In mice an important role for the TM-protein C-EPCR system in placental development and maintenance of pregnancy is firmly established (Healy *et al* 1995, Gu *et al* 2002), but the relevance of these mechanisms as regards pregnancy-associated complications in humans remains unknown. The data on mouse models, the known sequence homology of murine and human TM and EPCR genes, and the similar type of placentation in both species (Dittman and Majerus 1989, Cross *et al* 1994), however, suggest that TM and EPCR genes are candidates as regards RM.

We studied two thrombophilia-related genes, those for TM and EPCR, for possible mutations in patients with RM. Excluding the common variants, the mutation rate in TM and EPCR genes was low. Our results suggest that clear-cut mutations in the TM or EPCR genes are not a major cause of RM in Finnish women. However, some mutations and variants may play a role, as indicated by the mouse models. In this study we detected two interesting variations, c.323-9_336dup in the EPCR gene and 1728+23_+40del in the TM gene, which may have a role in spontaneous abortions. The c.323-9_336dup variation is a truncating mutation in exon 3 of the EPCR gene, previously identified in a patient with pregnancy loss. The 1728+23_+40del mutation is a newly identified variation in the 3'UTR region of the TM gene. As these mutations were rare in our series, more patients/couples should be studied to determine their exact role in RM.

13. Conclusions

I. Low molecular weight heparin is as good as unfractionated heparin in the acute phase treatment of deep venous thrombosis during pregnancy. The safety profile and ease of administration of LMWHs are superior compared with UFH and thus use of LMWH is recommend during pregnancy.

II. The prevalence of severe post-thrombotic syndrome (PTS) after deep venous thrombosis was low, although DVT during pregnancy is often massive and proximal. There were no correlations between treatments and prevalence of PTS. Low molecular weight heparin seems to be effective and safe in the long term.

III. Hereditary thrombophilia can be an additional risk factor as regards cervical insufficiency and even premature delivery. This could open new treatment modalities (LMWH).

IV. Soluble plasma annexin V levels correlated with the presence of aPLs at the beginning of pregnancy in women with a history of RM. Annexin V, as an anticoagulant protein of phospholipid surfaces, can be displaced by aPLs, leading to enhancement of coagulation. However, possibly because of a small number of patients, we were unable to show if this association could be related to pregnancy outcome.

V. Our results suggest that mutations in the TM or EPCR genes are not a major cause of RM in Finnish women.

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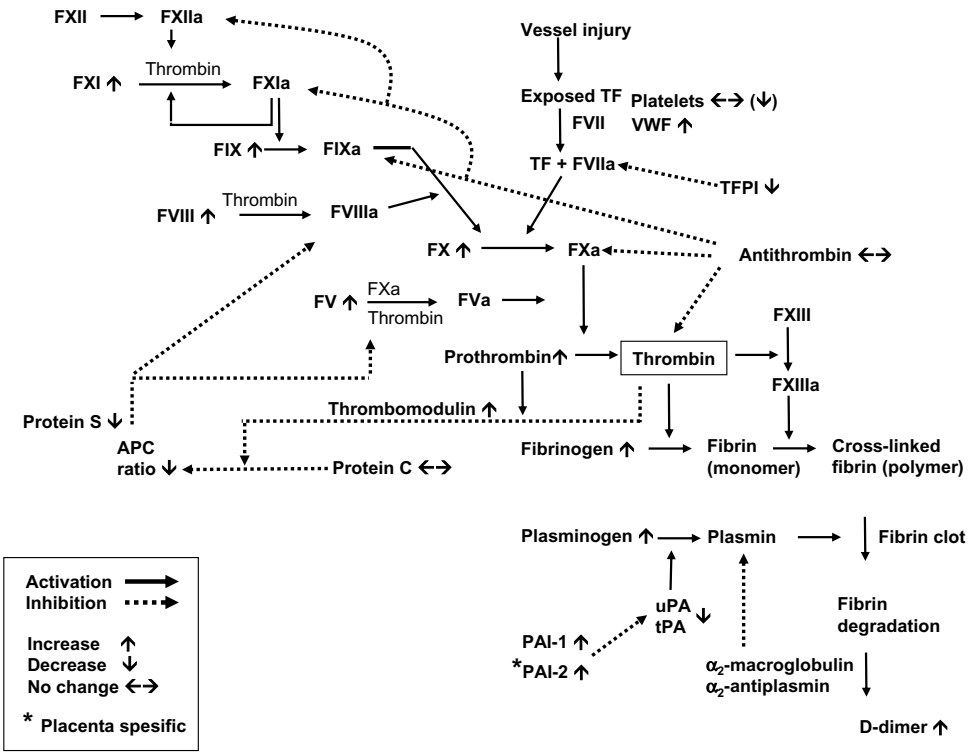
Appendix I: Hemostatic mechanism during pregnancy



Appendix II. Clinical scale of Post Thrombotic Syndrome (PTS) (Villalta *et al* 1994)

0=no symptoms 1=mild 2=moderate 3=severe

Signs
pain
cramps
pruritus
paresthesia

Symptoms
edema
hyperpigmentation
teleangiectases
redness

Total score ≥ 15 means severe PTS, 5–14 means mild or moderate PTS

Appendix III. Classification of lower extremity venous disease (CEAP) (Porter and Moneta 1995)

C. Clinical findings (0–6), A=asymptomatic S=symptomatic

class 0:	No sign of venous disease
1	Teleangiectases, livero reticularis
2	Varicose veins
3	Edema without skin changes
4	Varicose disease-associated skin changes such as hyperpigmentation, eczema
5	Skin induration as above and old healed ulceration
6	Skin induration as above and active ulcer

E. Etiological classification, C=congenital, P=primary, unknown etiology, S=secondary/acquired

E_C / E_P / E_S

A. Anatomy, Superficial / deep / perforator

Superficial veins A S1–5

1	Teleangiectases
	Vena saphena magna
2	above knee
3	below knee
4	Vena saphena parva
5	Superficial, no association with main veins

Deep veins A D6–16

6	Vena cava inferior
	Vena iliaca
7	communis
8	interna
9	externa
10	gonadal, lig. latum
	Vena femoralis
11	communis
12	profunda
13	superficialis
14	Vena poplitea
15	Vena tibiales (ant/post/peron)
16	Muscle veins (gastrocnemius, soleus)

Perforator	A P17, 18
17	Femoral
18	Crural

P. Pathophysiology, PR=reflux, PO=obstruction, PR/O=reflux and obstruction