Factor V Leiden mutation and pregnancy

Haemostasis during pregnancy in non-carriers and carriers of factor V Leiden mutation, with special emphasis on placenta-mediated and venous thromboembolic complications and on blood coagulation and fibrinolysis markers for prediction of complications

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Göteborg 2009

With modern powerful statistical computer software, a false statement can be asserted with a high level of accuracy. Furthermore, if a false statement is repeated many times it will be turned into a truth.

Abstract

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Factor V Leiden (FVL) mutation elevates the risk of venous thromboembolism (VTE) in general. During pregnancy, the haemostatic balance is changed in the direction of hypercoagulability, resulting in an increased incidence of VTE.

42 women were followed longitudinally during pregnancy and the puerperium. Classic and modified activated protein C (APC) ratios decreased during pregnancy. However, the modified ratio was above the lower limit for non-carriers, and can be used to detect the FVL mutation during pregnancy. Increased levels of prothrombin fragment 1+2 (F1+2), soluble fibrin (SF) and D-dimer indicated activation of blood coagulation. Fibrinogen, Factor VIII and plasminogen activator inhibitor type 1 and type 2 levels increased. Free protein S and tissue plasminogen activator activity decreased. Protein C levels remained unchanged. Sonoclot analyses indicated hypercoagulability during pregnancy. The same reference Sonoclot curve can be used throughout pregnancy.

5 986 women were genotyped for the FVL mutation; the prevalence of FVL carriership was 8.1%. 500 carriers and 1 058 controls were followed longitudinally and haemostatic markers were analysed. There were no differences regarding placenta-mediated complications or gestational age at delivery. The incidences of neonatal asphyxia, eclampsia, intrauterine fetal death, intrapartum death and unexplained late miscarriage were low. The incidence of blood loss exceeding 1000 ml at delivery was lower in FVL carriers. There were three VTEs among FVL carriers and none among controls. No difference in superficial thrombophlebitis was found. Genotyping for the FVL mutation in healthy pregnant women without heredity for VTE is doubtful, nor can genotyping be justified in women with obstetric complications.

F1+2 and D-dimer increased during pregnancy and levels were higher than eight weeks postpartum. Alterations in SF were minor or absent. Levels of F1+2 and SF in carriers and non-carriers were the same during pregnancy. Carriers had higher levels of D-dimer than non-carriers during both pregnancy and the puerperium. The levels of all markers were in the same ranges in women with and without complications, and were unaffected by zygosity or additional thrombophilia. F1+2, SF or D-dimer cannot serve as predictors of placenta-mediated complications or VTE in FVL carriers.

Keywords

Activated protein C, D-dimer, Factor V Leiden, fibrinolysis, haemostasis, obstetric complications, platelet function, pregnancy, prothrombin fragment 1+2, soluble fibrin, Sonoclot, thrombosis

ISBN 978-91-628-7904-4

http://hdl.handle.net/2077/21078 Göteborg 2009

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Abbreviations

APC	activated protein C
APTT	activated thromboplastin time
AT	antithrombin
BMI	body mass index
CI	confidence interval
DIC	disseminated intravascular coagulation
DVT	deep vein thrombosis
ELISA	enzyme-linked immunosorbent assay
F 1+2	prothrombin fragments 1+2
F	factor; a following the respective factor means activated
FVL	Factor V Leiden
Hb	haemoglobin concentration
HELLP	syndrome of haemolysis, elevated liver enzymes and low platelets
IUFD	intrauterine fetal death
IUGR	intrauterine growth retardation
LA	lupus anticoagulants
LGA	large for gestational age
LMWH	low molecular weight heparin
MP	mild and moderate preeclampsia
OAC	oral anticoagulants
OR	odds ratio
p1	one-sided p-value
p2	two-sided p-value
PAI-l	plasminogen activator inhibitor type 1
PAI-2	plasminogen activator inhibitor type 2
PCR	polymerase chain reaction
PE	pulmonary embolism
r	Pearson's correlation coefficient
r_s	Spearman correlation coefficient
s-CT	spiral computed tomography
SD	standard deviation
SF	soluble fibrin
SGA	small for gestational age
SP	severe preeclampsia
TAFI	thrombin-activated fibrinolytic inhibitor
TF	tissue factor
TFPI	tissue factor pathway inhibitor
t-PA	tissue plasminogen activator
u-PA	urokinase plasminogen activator
VTE	venous thromboembolism
vWF	von Willebrand factor

Definitions

Blood loss:
Routinely estimated and registered by the midwife at delivery
Blood pressure:
Measured in the sitting subject's left arm at heart level after a 20-minute rest
Chronic hypertension:
≥ 90 mm Hg diastolic blood pressure before 20 weeks of gestation or ongoing antihypertensive medication
Double heterozygosity:
Heterozygosity for both Factor V Leiden (FVL) mutation and the prothrombin G20210A gene mutation
Birth weight deviation:
100* mean value of ((weight – mean weight for the same gestational age and gender)/(mean weight for the same gestational age and gender)). Gender-specific intrauterine growth curves based on ultrasonically estimated fetal weights were used for comparison (Maršál et al 1996)
Gestational hypertension:
≥ 90 mm Hg diastolic blood pressure on two occasions at minimum 6 hours interval after gestational week 20
HELLP syndrome:
Classified according to Martin et al, i.e. epigastric pain, haemolysis, elevated serum alanine aminotransferase and thrombocytopenia with or without other signs of preeclampsia (Martin et al 1990)
LGA:
Birth weight exceeding two standard deviations (SD) (Maršál et al 1996)
Mild preeclampsia:
≥ 90 mm Hg diastolic blood pressure on two occasions
proteinuria (≥ 1+ reading on dipstick) on two occasions at minimum 6 hours interval and negative urine culture
Neonatal asphyxia:
Apgar score <7 at 5 and 10 minutes
Severe preeclampsia:
As mild preeclampsia but with one of the following additional findings:
≥ 120 mm Hg diastolic blood pressure once or ≥ 110 mm Hg diastolic blood pressure on two occasions at minimum 6 hours interval
proteinuria ≥ 3g per 24 hours
symptoms/signs of organ failure such as renal insufficiency, pulmonary oedema, eclampsia, visual disturbances, intrauterine growth retardation (IUGR) or platelet count < 100 x 10^9/L
SGA:
Birth weight below two SD (Maršál et al 1996)
Superficial thrombophlebitis:
Palpable, tender vessel accompanied by a surrounding area of localized inflammation

Placental abruption:
Abdominal pain, uterine tenderness, frequent small contractions and elevated uterine tone, together with vaginal bleeding or a retroplacental clot, i.e. a condition leading to immediate delivery

Placenta-mediated complications:
Generic term for preeclampsia, IUGR and placental abruption

VTE:
Venous thromboembolism including deep venous thrombosis (DVT) and pulmonary emboli (PE)

Background

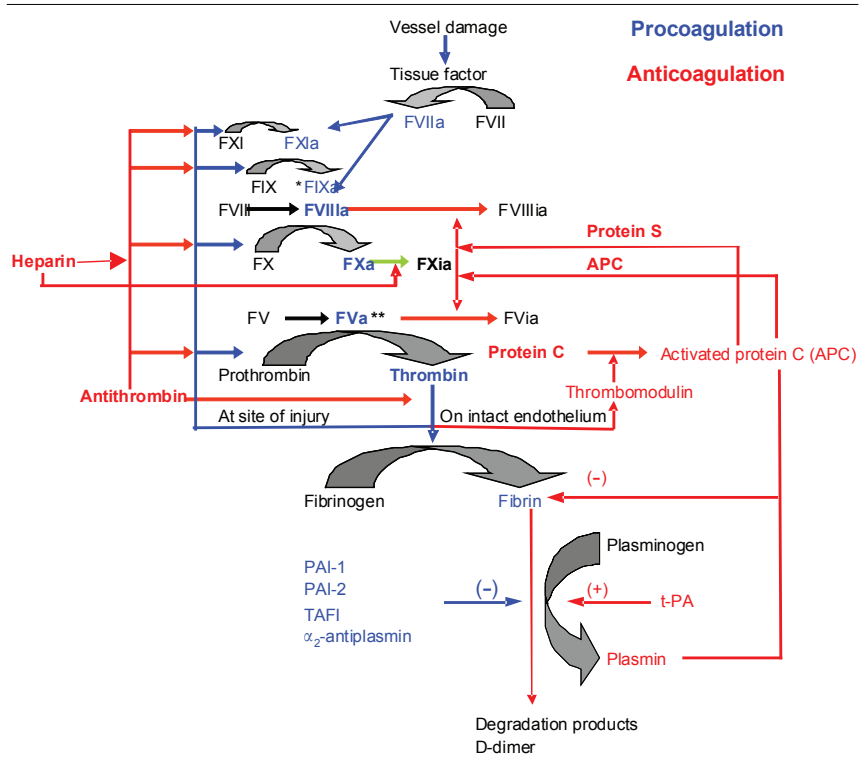
General aspects of haemostasis

The haemostatic system (Figure 1) is designed to maintain blood in a fluid state under physiological conditions, but is primed to react explosively to vascular injury in order to stem blood loss by sealing the defect in the vessel wall. Normal vascular endothelium maintains blood fluidity by inhibiting blood coagulation and platelet aggregation and promoting fibrinolysis. After the vessel is injured, blood is exposed to components from the deeper layer of the vessel wall, including adhesive proteins like collagen, fibronectin, laminin, vitronectin, and von Willebrand factor (vWF), that promote platelet adhesion, and tissue factor, that triggers blood coagulation.

Primary haemostasis is a series of events including vasoconstriction and platelet activation. Vasoconstriction diverts blood from the site of the injury and protects it from exposure to subendothelial structures that activate blood coagulation. Platelets adhere to the injured vessel wall, a process mediated by glucoprotein Ib which binds to vWF on the endothelial cells. In addition, activation of platelets leads to a change in shape, leading to exposure of new receptors on the platelet membrane which makes the platelets able to form aggregates, mediated by the bridging of glucoprotein IIb IIIa to glucoprotein IIb IIIa on another platelet by fibrinogen. This primary platelet plug is very fragile and must, in order to avoid detachment and subsequent resumed bleeding, be stabilised and more firmly anchored to the vessel wall; this is achieved via fibrin formation by the coagulation pathway also called secondary haemostasis.

Secondary haemostasis is a chain reaction involving proteolytic enzymes, the coagulation factors (F), present in circulating blood as inert proenzymes. Before participating in the coagulation pathway, they are activated by proteolytic cleavage after binding to specific protein cofactors on phospholipid surfaces. FVII, present in the circulation in its active form in trace amounts, is one exception. Tissue factor (TF), a membrane protein in adventitial fibroblasts, binds to FVIIa, one of the soluble clotting factors, with high affinity and this complex initiates the blood clotting cascade. The complex between TF and FVIIa activates FX into FXa, which in turn, together with its cofactor FVa, activates prothrombin into thrombin (FIIa) (the initial phase of the blood coagulation or TF pathway). This complex also activates FIX. Thrombin binds to phospholipid surfaces of the platelets, if protein Z is present, and acts on many substrates, resulting in increased formation of thrombin, i.e. the "amplification phase of coagulation" or the "thrombin burst". Thrombin activates FV, FVIII, FXI and FXIII as well as platelets and stimulates platelet secretion. FVIIIa activates FXI which in turn activates FIX. FVIIIa is also a co-factor of FIXa and together they form the "tenase" complex, which activates FX; the cycle continues and more and more thrombin is formed. Thrombin's primary role is the conversion of fibrinogen to fibrin, the main component of a haemostatic plug. The conversion of prothrombin to thrombin and the activation of FX take place on the platelet membrane surface. The final step is the cross-linking of fibrin by FXIIIa. The firm platelet-fibrin clot subsequently retracts, a process that is also platelet-dependent. Thrombin occupies a central role in the process of haemostatic plug formation, influencing its shape, rate of formation and size.

If no systems regulated this process, all circulating blood would coagulate in a couple of minutes. Antithrombin (AT) is the most important physiological *inhibitor* of thrombin and other activated serine proteinases of the coagulation system in circulating blood, TF-FVIIa complex, FIXa, FXa and FXIIa. The created thrombin is modulated by reacting with thrombomodulin on intact endothelium cells close to the injury. This restructured thrombin activates protein C, another important inhibitor of blood coagulation, a reaction augmented by endothelial cell protein C receptor. APC's task is to terminate surplus thrombin generation by inactivating FVa and FVIIIa. Free protein S and FV act as cofactors to APC in its inactivation of FVIIIa. In this reaction, the FV molecule is cleaved by APC at both Arg^{306} and Arg^{506}. The cleavage at Arg^{506} is important for the ability of FV to serve as a cofactor. Protein S, in its free form, also acts as a cofactor for APC in the inactivation of FVa. In this inactivation reaction, cleavage at Arg^{506} is fast but results only in partial inactivation while cleavage at Arg^{306} is slow but results in substantial inactivation.
The active forms of most serine proteases in the clotting system, except FVIIa, have extremely short plasma half-lives (measured in seconds) because plasma contains high concentrations of protease inhibitors. Activated FXa and the TF-FVIIa complex are inhibited by tissue factor pathway inhibitor (TFPI). FXa is inhibited by Z protease inhibitor, a reaction enhanced by protein Z.
Simultaneously with the onset of fibrin formation, the clot starts to dissolve, a process called *fibrinolysis*. Endothelial cells liberate tissue plasmin activator (t-PA) after stimulation by thrombin, firmly bound to fibrin. Fibrin serves as a cofactor enabling efficient activation of plasminogen to plasmin by t-PA. Plasminogen also binds to fibrin; the substrate fibrin is thus the site of both the activator and the proenzyme. Plasmin cleaves both fibrinogen and fibrin to produce degradation products which inhibit thrombin action and fibrin polymerisation, serving as natural anticoagulants. It also exerts positive feedback by cleavage of plasminogen, rendering it more susceptible to activation. Fibrinolysis is subject to multiple regulatory mechanisms. The main inhibitors of fibrinolysis are plasminogen activator inhibitor (PAI)-1, α_2-antiplasmin, lipoprotein (a) and thrombin-activated fibrinolytic inhibitor (TAFI). FXIIIa-induced cross-linking of the fibrin matrix renders it much more resistant to plasmin action. Many other elements, both in circulating blood and membrane-bound in the endothelial cells, enhance or impair haemostasis. All components in the haemostatic system must be intact and balanced in order to avoid VTE events or abnormal bleeding. Furthermore, platelets are fundamental components of haemostasis and their action is affected by both activators and inhibitors.



* Tenase complex, ** Prothrombinase complex, a: activated, ia: inactivated

Figure 1. Coagulation, anticoagulation and fibrinolysis.

Sonoclot analysis

The Sonoclot® analyser presents a global analysis of a patient's tendency to bleed or clot in less than 15 minutes. The method assesses all the different parts of haemostasis with the exception of endothelial action. Routine coagulation workup is more time-consuming and only provides information about the different steps in haemostasis. Some haemostatic tests, such as platelet function tests and fibrinolysis analyses, are not available as rapid tests and bleeding time can be difficult to analyse. Activated thromboplastin time (APTT) and prothrombin time measure the interval from the first steps in the coagulation cascade until fibrin formation starts. The Sonoclot® analyser, on the other hand, provides information about the velocity of total fibrin formation, fibrin polymerisation and fibrinolysis (Hett et al 1995), and imitates haemostasis in vivo. It was invented in order to meet the demand for rapid haemostatic assessments during coronary artery bypass surgery (Tuman et al 1989). Blood is analysed bedside and the device can easily be moved together with the patient between the antenatal ward, delivery ward, operating theatre and intensive care

unit. Coagulation status is assessed in whole blood, allowing in vivo coagulation system interactions with platelets and red blood cells, providing useful information on platelet function.
The following variables are analysed in a Sonoclot curve: sonact time, clot rate, secondary rate, peak amplitude, peak time and downward rate. The sonact time is the time from coagulation activation until fibrin formation starts. The clot rate reflects the rate of fibrin formation. The secondary rate reflects platelet interaction with formed fibrin. The peak time reflects platelet number and function. The peak amplitude depends on platelet number and fibrinogen concentration as well as on the degree to which the cuvette is filled, the degree of rouleau formation and the haematocrit. The downward rate reflects both clot retraction and fibrinolysis.
Sonoclot analysis is considered to be a valuable complement to an ordinary routine coagulation workup (Saleem et al 1983) and has been found to be a better predictor of postoperative haemorrhage than ordinary haemostatic analyses and useful in the process of individualising and optimising the use of blood components and haemostatic drugs (Ekbäck et al 1995, Saleem et al 1983, Stern et al 1989, Tuman et al 1989). The benefits of the Sonoclot® analyser have been documented in connection with liver transplantation (Chapin et al 1989), hip and knee surgery (Ekbäck et al 1995, Schött et al 1995), neonatal care (Blifeld et al 1986, Yang et al 1995), treatment of DIC in sepsis with plasmapheresis (Schött et al 1995) and the determination of hypercoagulability (Amirkhosravi et al 1996, Francis et al 1994, Peck 1979, Perkash et al 1993, Pivalizza et al 1997, Spillert and Lazaro 1987, Sugiura et al 1982). The Sonoclot® analyser might be useful in managing obstetric haemostatic complications. Very few studies have been published concerning its use during pregnancy (Goldstein 1989, Liszka-Hackzell and Schött 2004, Steer 1993, Steer and Krantz 1993). Reference intervals for the Sonoclot variables in pregnant women are as yet unknown.

Haemostasis during pregnancy and postpartum

During pregnancy the haemostatic balance changes in the direction of hypercoagulability, thus decreasing the risk of bleeding complications in connection with delivery, but also raising the risk of thromboembolic complications. Platelet activation increases and the platelet count may decrease moderately during the third trimester as a result of increased platelet consumption in the uteroplacental unit. The concentrations of FV, FVII, FVIII, FIX, FX, FXII, vWF and fibrinogen increase. The inhibition of blood coagulation is decreased by decreased levels of protein C inhibitor and free protein S. However, the increased levels of TFPI inhibit the enhancement phase by inactivating FXa. The levels of AT and protein C remained unchanged. The PAI-1 level is increased, mainly due to secretion from the endothelial cells in the placenta, and the basal activity of t-PA is decreased (Ishii et al 1994), but the mean velocity of clot lysis in the euglobulin clot lysis test does not differ from that in non-pregnant adults, suggesting that the fibrinolytic system remains active and compensates for increased fibrin formation (Smith et al 2003). D-dimer levels thus increase, reaching maximum levels after delivery. Fibrinolytic activity probably occurs primarily in the systemic circulation, while fibrinolysis in the placenta is depressed due

to increased secretion of PAI-1. PAI-2, secreted by the placenta, affects the systemic fibrinolysis process to a lesser extent. It does not inhibit single-chain t-PA and its effect on two-chain t-PA is 100-fold weaker than that of PAI-1. The more efficient inhibition by PAI-2 of urokinase plasminogen activator (u-PA) than of t-PA suggests that its primary role may be in regulating extracellular u-PA, for instance during growth and remodelling of the placenta (Thorsen et al 1998).

Markers of blood coagulation activation and fibrinolysis

Ongoing blood coagulation and fibrinolysis can be measured by different markers of blood coagulation activation, fibrin formation and fibrin degradation. After activation of the blood coagulation system, prothrombin is converted to thrombin by the splitting of the prothrombin fragments F1+2 (F1+2). The resulting enzyme, thrombin, is rapidly inactivated by AT, forming the thrombin-antithrombin complex. Quantification of F1+2 makes it possible to monitor even a minor degree of blood coagulation activation. Only one type of assay for F1+2, the Enzygnost F1+2, is commercially available. This is an enzyme immunoassay for the in vitro determination of human F1+2 and is based on the sandwich principle.

Free thrombin cleaves fibrinogen to fibrin monomer in a multi-step process that proceeds via a series of intermediate products. At an early stage of fibrin formation, soluble fibrin (SF) complexes are formed between fibrin monomers and fibrinogen molecules. Later, if thrombin remains active, more SF is created and forms a fibrin gel in which the subunits are cross-linked by FXIII. Plasmin, the most important fibrinolytic enzyme, may degrade fibrinogen, SF and cross-linked fibrin. When cross-linked fibrin is digested, fragments containing dimerized D-domains, including D-dimer, are formed. The concentration of D-dimer is primarily a measure of both intravascular and extravascular fibrin formation and, to a lesser extent, of fibrinolytic activity (Dempfle 2002, Dempfle 2004).

A large number of test kits based on different techniques are commercially available for both SF and D-dimer. There is no simple way to compare results from different assays. Correlation between different SF assays is weak (Dempfle et al 1995), as their degree of cross-reactivity with fibrin degradation products, varies considerably. However, a new monoclonal antibody, IL-43, used in the Iatro SF assay, has been found not to recognize cross-linked fibrin degradation products and it therefore has a lower correlation with D-dimer (Pearson's r = 0.26) (Ieko et al 2007). In a study, the Iatro SF assay was compared with two latex D-dimer assays; the SF assay identified more patients with overt disseminated intravascular coagulation (DIC), and exhibited the highest prognostic power concerning mortality (Dempfle 2004). One possible reason for this might be that D-dimer may stem from intravascular as well as extravascular sources, whereas SF appears to be more closely linked to acute intravascular fibrin formation.

Assays for D-dimer, using monoclonal antibodies against conformational epitopes of fibrin compounds containing dimerized D-domains, differ concerning specificity for cross-linked fibrin, with a preference for either high-molecular-weight cross-linked fibrin derivatives or for low- molecular-weight fibrin degradation products. There is no simple linear relationship between different assays (Dempfle 2003).

Therefore, only D-dimer assays tested in appropriate clinical trials should be used to rule out VTE. The main source of D-dimer antigen reactivity is not the proteolysis of clots but cross-linked fibrin in the circulating blood (Dempfle 2005). D-dimer enzyme-linked immunosorbent assays (ELISA) have been considered to be the gold standard. However, they are cumbersome, labour-intensive and unsuitable for routine emergency use and have thus been replaced by latex-based systems in the clinical setting.

APC resistance and the FVL mutation

APC resistance was first described by Dahlbäck in 1993. He was testing a new APTT-based coagulation assay with a fixed amount of added APC. When this test was run on plasma from a man suffering from recurrent episodes of venous thrombosis, prolongation of the clotting time was much shorter than expected. Several of the proband's relatives demonstrated a similar poor in vitro response to APC and family studies suggested that the disorder was inherited as an autosomal dominant trait with decreased penetrance. The term used for describing this condition was "APC resistance", and the quotient between the two clotting times was called the APC ratio. The molecular basis of this phenotype was identified as the FVL mutation (Bertina et al 1994), a single mutation G→A in position 1691 that converts arginine 506 of factor V to glutamine (FV:Q^{506}). FVa:Q^{506} is 10-fold less sensitive to APC-mediated degradation. However, it is slowly cleaved at Arg^{306} and Arg ^{679} (Kalafatis et al 1996), which explains the partial APC sensitivity of individuals with FVa:Q^{506}. Thrombi formed in a FVL carrier are more resistant to fibrinolysis due to sustained activation of TAFI by APC and subsequent inhibition of fibrinolysis (Bajzar et al 1996), another way the mutation may contribute to thrombosis.

More than 90% of patients with APC resistance are carriers of the FVL (Zöller et al 1994) mutation.

It is the most common thrombophilia among Caucasians and the prevalence of heterozygosity in Caucasian populations is reported at 2 - 15 %. The highest prevalences are found in northern Europe and the Middle East. Haplotype analyses by Zivelin support a single origin for FVL and the mutation is estimated to have arisen between 17 000 and 29 000 years ago, i.e. after the evolutionary divergence of Africans from non-Africans and of Caucasoid from Mongoloid subpopulations towards the end of the last glacial period. It has been suggested that the founders of both this and the prothrombin gene mutations lived in the Middle East because the current prevalences of both mutations are higher in ancient Middle Eastern populations than in other white populations (Zivelin et al 1997 and 2006).

The FVL mutation's widespread presence among Caucasians suggests that it may be a balanced polymorphism with some advantages conferred upon heterozygotes, especially in pre-modern times when death from bleeding associated with childbirth, trauma, or warfare was a significant risk and when scurvy was a common condition.

The increase in risk of a first VTE was found to be 7.9 (95 % confidence interval (CI) 4.1-13) in heterozygotes and 91 (95 % CI 26-322) in homozygotes (Bertina 1997). The presence of other thrombophilic mutations multiplies the risk of VTE.

Deficiency of protein Z leads to a bleeding tendency in non-carriers but raises the risk of VTE in FVL carriers (Kemkes-Matthes et al 2004).
Since 1994, a few other rare FV mutations leading to APC resistance have been found: Arg^{306} to glycine (FV Hong-Kong) or threonine (FV Cambridge) and mutations leading to low FV levels (FV R2 haplotype). Concomitant heterozygous FVL mutation in one gene and mutation in the other gene leading to FV deficiency is called pseudo-homozygous APC resistance and results in an APC ratio in the reference range for homozygosity of the FVL mutation. This is explained by the fact that FV is not only a procoagulant but also an anticoagulant, as it is a cofactor, together with protein S, in the degradation of FVIIIa.

APC resistance can be evaluated in plasma using different commercially available methods, many of which are derived from the original Coatest APC Resistance test method, available since 1993. These tests, classic APC resistance assays, measure the classic APC ratio as clotting time in the presence of APC, divided by clotting time in the absence of APC. These assays have been shown to have some shortcomings; many haemostatic variables can interfere because the test is based on the APTT. The assays are susceptible to platelet contamination and to clinical conditions such as age; gender; body mass index (BMI); smoking; blood group; APTT; fibrinogen, homocysteine, triglyceride and total cholesterol levels; all conditions giving rise to high levels of plasma FVIII.
Low classic APC ratios have been found among individuals with the metabolic syndrome (Tosetto et al 1997), during oral contraceptive use and pregnancy (Cumming et al 1995, Mathonet et al 1996, Peek et al 1997, Schlit et al 1996, Vasse et al 1994) and in individuals with lupus anticoagulants (LA) (Nojima et al 2009). In one study a correlation was found between the classic APC ratio and the protein C level (Maheiu et al 2007). The APC ratio is not useful during treatment with heparin or oral anticoagulants (OAC). It has also been shown that the APC ratio increases in plasma samples with only slightly reduced levels of FII, FVIII and FX (Ronde et al 1994). The condition with a classic APC ratio below the lower limit of the reference interval in the absence of a FV gene mutation is called acquired APC resistance. Studies have shown that there is a correlation between this condition, blood coagulation activation and subsequent incidences of VTE (de Visser et al 1999). The results from studies assessing the relationship between acquired APC resistance and the incidence of placenta-mediated complications are conflicting (Lindqvist et al 2006, Mimuro et al 1998, Paternoster et al 2002) but a relationship between a low ratio and thrombotic lesions in the placenta has been found (Sedano et al 2008).

Modified APC resistance tests are based on pre-dilution (1:4) of the sample plasma with FV-deficient plasma prior to analysis, which is then performed according to routine. This procedure makes the method much more specific for alterations in the FV molecule, resulting in a very high discrimination of the FV:R 506Q mutation as well as the ability to analyse plasma from OAC- and heparin-treated individuals and pregnant women (Jorquera et al 1996). The modified APC resistance test used in the research underlying this thesis has been reported to have 100% sensitivity and 100%

specificity for the FVL mutation and distinguishes homozygous from heterozygous individuals (Freyburger et al 1997, Gouault-Heilman et al 1996, Hall et al 1998, van Oerle et al 1997, Sifontes et al 1997). It cannot, however, be ruled out that analysis of plasma from patients with high LA titres and from patients with FV deficiency may yield misleading results.

FVL – mutation or polymorphism or what?

There is an ongoing debate concerning the nomenclature of variants in our genetic code. In biology, mutations (Latin: mutatio=change) are changes in the nucleotide sequence of the gebetic material of an organism. Less favourable mutations can be reduced in frequency in the gene pool by natural selection, while more beneficial or advantageous mutations may accumulate and result in adaptive evolutionary changes. Sometimes the term "mutation" is used to simply indicate "a disease-causing change". However, the fact that the term "mutation" has developed a negative connotation, associated with abnormality and terms with intense negative connotations such as "monstrosities" or "freaks", has resulted in the emergence of other terms for describing genetic processes. The term polymorphism (Greek: polys morphës=many forms) has been proposed to indicate a non-disease-causing change or a change found at a frequency of 1% or higher in the population. "Balanced polymorphism" is the suggested term for a condition that has advantages for the heterozygote but is hazardous for the homozygote. This definition is not unambiguous. FVL would be a mutation in some parts of the world and a polymorphism in others. Neutral terms such as "sequence variant alteration" or "allelic variant" have been suggested, instead of mutation and polymorphism, in order to prevent this confusion (Condit et al 2002).
However, without mutations we would have still been multi-layered microbial mats, so the term mutation has been chosen, in this thesis, to define a single base change.

Other thrombophilias

The prothrombin G20210A mutation, leading to increased prothrombin generation, was reported in 1996 (Poort et al 1996). In that study the odds ratio (OR) for thrombosis was 2.8 (95% CI 1.4-5.6) in heterozygous carriers. Haplotyping suggests that the mutation arose 19 000-31 000 years ago and has the same geographic extent as the FVL mutation (Zivelin et al 1998 and 2006). However, prevalences (range 1.7-6.7) are highest in the Middle East and lowest in northern Europe (Rosendaal et al 1998, Zivelin et al 1998). The prevalence of the prothrombin mutation has been found to be doubled in FVL carriers, compared with non-carriers, suggesting that these mutations originated in the same area (Ehrenforth et al 1998). Concomitance of heterozygosity for these two mutations is often called double heterozygosity. A survival advantage based on a protective prenatal effect of this genotype, such as improved embryonic implantation, has been suggested (Hundsdoerfer et al 2003). Another effect can be a decreased bleeding tendency, comparable with the FVL mutation's effect.
AT, protein C and protein S are the most important downregulators of coagulation. Deficiencies of these plasma proteins are strong risk factors for VTE. There are

several different types of genomic abnormalities responsible for these conditions. If the mutation leads to a reduction in the amount of circulating protein it is called a type 1 or quantitative deficiency and if the mutated allele produces a variant protein with less activity it is called type II or qualitative deficiency. In clinical practice, an individual is said to have symptomatic deficiency if he or she has a low level and at least two relatives with low levels and a history of VTE. The condition is also called symptomatic phenotype. The respective prevalences of AT deficiency type I and type IIc (heparin binding site-related) have been estimated to be 1:4400 and 1:700 (Tait 1994). The prevalences of protein C deficiency type I and protein S deficiency type I have been estimated at 1:300 (Tait 1995) and 1:300-1:800 (Dykes 2001), respectively.

Placenta-mediated complications

The key feature in IUGR, preeclampsia and often in spontaneous abortion is poor placentation, leading to deficient uteroplacental blood flow. This is caused by two defects involving the spiral arteries. The first is a relative absence of trophoblast invasion of the spiral arterial walls during placentation between gestational weeks 8-18, caused by an excessive attack by the maternal inflammatory response on the zygote (Faas and Schuiling 2001), leading to a relative absence of trophoblast invasion of the spiral arterial walls during placentation. The second defect is "acute atherosis", i.e. aggregates of lipid-loaded macrophages, platelets and fibrin-creating placental infarction. Increased placental apoptopic or necrotic debris is shed from the syncytial surface of the placenta and reaches the maternal circulation, resulting in endothelial damage and activation of blood coagulation. There are increased levels of inflammatory cytokines, an increased number of natural killer cells and neutrophil activation both in the placenta and in the maternal circulation. These changes are compatible with a state of low-grade DIC. The process accelerates when pregnancy proceeds, and is more pronounced in pregnancies complicated by placenta-mediated complications (Redman and Sargent 2003).

It is possible that the fetus provokes the mother to compensate for poor placentation by increasing her blood pressure (Greer 2001). The maternal response may depend on the woman's genotype and/or phenotype. Women with insulin resistance or other risk factors for atherosclerosis and women with underlying inflammatory conditions may exhibit an exaggerated inflammatory response and those with congenital thrombophilia may exhibit an exaggerated coagulation response (Greer 2001, Wikström et al 2005).

The question of whether venous thrombophilia raises the risk of bad obstetric outcome is under debate. The association between the FVL variant and adverse obstetric outcome is controversial and data in the literature is inconsistent, due to major between-study heterogeneity, potential publication bias and sequential testing (Figure 2). The five largest case-control studies (GOPEC 2005, de Groot et al 1999, Kim et al 2001, Mello et al 2005, O'Shaughnessy et al 1999) and three prospective studies (Clark et al 2007, Dizon-Townson et al 2005, Lindqvist et al 1999) did not show any differences in the prevalences of gestational hypertension, preeclampsia or severe preeclampsia between FVL carriers and controls. Six large studies (Clark 2007, Mc-

Cowan et al 2003, Dizon-Townson et al 2005, Infante-Rivard et al 2002, Lindqvist et al 1999, Verspyck et al 2004) indicated no elevated risk for IUGR.
Statistical association has been said to be a matter of fact, whereas causation is a matter of opinion. Is the evidence extensive enough to claim that a causal association has been proven? Rodger and Paidas thoroughly discussed Hill's ten criteria for causation applied to the issue of thrombophilias causing placenta-mediated complications, concluding that the criteria were not met in this case (Rodger and Paidas 2007).
Several candidate markers, including the classic APC ratio and the PAI-1/PAI-2-ratio, have been proposed as markers for preeclampsia and small for gestational age (SGA); the optimal marker has yet to be discovered. Theoretically, markers of activation of coagulation such as F1+2, SF or D-dimer might be valuable for prediction of placenta-mediated complications.
For effective screening, rapid and reliable diagnostic tests are required that can predict the forthcoming onset of disease with precision. Doing so in the first trimester of pregnancy improves the test's prognostic value significantly because this provides time for prophylactic strategies to be implemented.

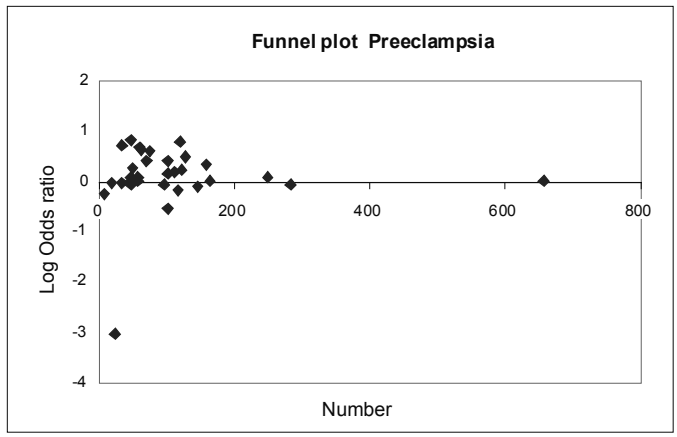


Figure 2. Funnel plot showing results from 32 case-control studies concerning preeclampsia and the FVL mutation.

Placental abruption

Placental abruption is often unpredictable and a serious condition with an incidence of 1 in 100-200 pregnancies, associated with high perinatal mortality and high maternal morbidity and mortality. Known risk factors are direct trauma to the uterus, hypertension (both essential and pregnancy-induced), severe fetal growth restriction, cigarette smoking, advanced maternal age and multiparity. The process begins with uterine vasospasm, followed by relaxation and consequently venous stasis. The arterioles rupture and the placenta separates from the maternal attachment by pressure. The clinical signs are frequent small contractions, elevated uterine tone, abdominal pain or backache and sometimes vaginal bleeding. Placental bed biopsies

after abruption show the same abnormal vascular structures as in preeclampsia, such as inflammation of the anchoring villi (Dommisse and Tiltman 1992, Eskes 1997), and placental abruption can probably be included among the placenta-mediated pregnancy complications. The role of thrombophilia as a risk factor for placental abruption is controversial (Figure 3). Two prospective studies failed to detect any association between FVL carriership and placental abruption (Dizon-Townson et al 2005, Lindqvist et al 1999), as did two of the three largest retrospective studies (Jääskeläinen et al 2004, Prochazka et al 2003, Prochazka et al 2007).

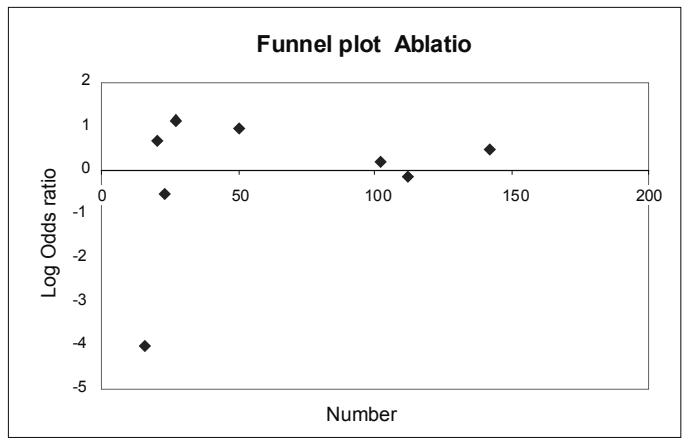


Figure 3. Funnel plot showing results from 8 case-control studies concerning placental abruption and the FVL mutation.

Fetal loss

Pregnancy loss is one of the leading women's health issues; 25% of reproductive-age women experience one clinically recognized loss, 5% experience two or more losses and about 1 % suffer three or more losses (Regan and Clifford 2001). Most (98%) pregnancy losses occur before 12-14 weeks' gestation (Goldstein et al 1994). Abnormal karyotype, infections, luteal phase defects and uterine abnormalities are the most common findings in connection with miscarriage; the problem of confounding is thus huge. The incidence of stillbirth (fetal loss after 22 gestational weeks) in our region is about 6‰. IUGR, lethal anomalies, abnormal karyotype and infections are well-known risk factors but about half of stillbirths are unexplained. The relationship between fetal loss and thrombophilia, including FVL mutation, has been questioned. As human fetal nutrition is mainly histiotrophic until at least 10 gestational weeks and as the maternal intraplacental circulation is not fully established until the third month of gestation, it is unlikely that thrombophilia can cause early miscarriage due to increased blood coagulation (Burton et al 2002). A population of 85 304 neonates (>98% of all newborns in Berlin) was tested for both the FVL mutation and the prothrombin mutation, identifying 5 338 heterozygous and 116 homozygous FVL infants (Hundsdoerfer et al 2003). According to the Hardy-Weinberg equation, 91 homozygous infants would have been expected in this population

(p=0.08). The difference indicates no major prenatal loss of fetal homozygous FVL carriers but does suggest a tendency toward significance in favour of carriership. Numerous early case-control studies with varying design assessing the relationship between the FVL mutation and recurrent miscarriage, first or second trimester miscarriage or IUFD have been published; low but statistically significant ORs are often found. However, the results are controversial and data in the studies are inconsistent, due to major between-study heterogeneity, potential publication bias and sequential testing. In some studies, the prevalences of FVL were surprisingly low. Numerous meta-analyses including these studies have also been published. However, the largest studies did not show any association between fetal loss and FVL (Dizon-Townson et al 2005, Gris et al 1997, Lindqvist et al 1999, Rai et al 2001, Roqué et al 2004, Völzke et al 2003). The largest case-control study found a significantly elevated risk (OR 4.8, CI 1.8 –12.4) for fetal loss after 22 gestational weeks (Gris et al 1999) but the largest cohort studies did not (OR 1.57, CI 0.76-3.25 (Lindqvist et al 1999, Tormene et al 1999, Völske et al 2003). 63 homozygous FVL carriers did not suffer more fetal losses than controls (Pabinger et al 2000).

VTE during pregnancy

VTE is one of the major causes of morbidity and mortality among women during pregnancy and the puerperium (Andersgaard et al 2008). Long-term complications such as postthrombotic syndrome and pulmonary hypertension lead to high costs for society and major suffering for the affected women (The Swedish Council on Technology Assessment in Health Care 2002). The growing uterus alters venous blood flow in the legs and pelvis and hormonal changes cause decreased resistance in the veins, which increases venous stasis. Together, this leads to an increased risk of VTE. The incidence of VTE probably increases at least about fourfold when a woman becomes pregnant (Heit et al 2005) and incidence related to pregnancy is influenced by age, smoking, postpartum care, thromboprophylaxis regimens and the frequency of caesarean section. The presence of thrombophilias leads to a multifold increase in overall risk.

In two large register studies from Scandinavia, the incidence of pregnancy-related VTE was found to be 10 -13/10 000. Half of the episodes occurred antepartum and the other half postpartum (Jacobsen et al 2008, Lindqvist et al 1999). A meta-analysis initially selected 63 articles examining the association of FVL with first, or recurrent, pregnancy-related VTE. They found that the FVL mutation was associated with a 4.5- (95% CI 1.8-10.9) and 8.6- (95% CI 5.9-12.6) fold increased risk of a first VTE during pregnancy in cohort and case-control studies, respectively (Biron-Andreani et al 2006). The risk of VTE is further increased in women with heredity for VTE and in pregnancies complicated by preeclampsia, placental abruption or operative delivery. Although thromboprophylaxis is given to women with increased risk for VTE according to guidelines, this condition is still one of the major causes of morbidity and mortality during pregnancy and the puerperium. More reliable risk criteria would be of great value, provoking the question of whether prediction by markers of coagulation activation such as F1+2, SF or D-dimer might be feasible.

Aims

The aims of the studies were to improve our knowledge of haemostasis during pregnancy and the puerperium and to evaluate the possibility of using of coagulation and fibrinolysis markers to predict venous thromboembolism (VTE) and placenta-mediated complications.

The specific aims were:

- To study changes in haemostasis (blood coagulation and fibrinolysis) during normal pregnancy and puerperium in non-carriers of the FVL mutation and to determine reference intervals for haemostatic variables (Paper I).

- To analyse the relationship between the total change in the classic activated protein C (APC) ratio and the total changes in some haemostatic variables during pregnancy (Paper I).

- To identify ranges in the Sonoclot variables in healthy non-carriers during normal pregnancy (Paper II).

- To determine the prevalence of the FVL mutation in two subpopulations in Sweden (Paper III).

- To study the association between the FVL mutation and adverse obstetric outcome, such as major blood loss at delivery, prematurity, preeclampsia, eclampsia, IUGR, late miscarriage, intrauterine fetal death (IUFD), placental abruption and neonatal asphyxia and the risk of VTE (Paper III).

- To determine blood coagulation marker (F1+2 and SF) and fibrinolysis marker (D-dimer) levels in FVL carriers during pregnancy and the puerperium and to compare them with those in non-carriers (Paper IV).

- To evaluate if determination of F1+2, SF and D-dimer might be useful in predicting placenta-mediated complications or VTE during pregnancy and the puerperium (Paper IV).

Participants

Haemostatic variables and global assessment of haemostasis during pregnancy and postpartum (Papers I and II)
48 healthy Caucasian women, mean age 29 (range 23-40), 27 nulliparas and 21 paras, were recruited to the study at their first visit to the antenatal care unit. 42 of these women completed normal pregnancies and all infants were healthy with normal birth weights and Apgar scores > 6 at 5 min. No woman had any haemostatic complication during pregnancy, delivery or puerperium. Four women gave birth before the 38th week. Five women were excluded as they developed preeclampsia or pregnancy-induced hypertension and one woman was excluded because of heterozygosity for the FVL mutation.

Prevalence of the FVL mutation, pregnancy outcome and determination of F1+2, SF and D-dimer during pregnancy and postpartum in FVL carriers (Papers III and IV)
Healthy Caucasian pregnant women without previous VTE were given written information about the study concerning hereditary APC resistance at their first visit, in about the 12th gestational week, at 46 antenatal care centres. The only additional criterion for inclusion was that the participants were required to understand the Swedish language.
4 268 and 1 718 women in Gothenburg and Stockholm, respectively, accepted participation in the study and were screened for hereditary APC resistance. 483 tested positive for the FVL allele with polymerase chain reaction (PCR) technique.
Another 17 FVL carriers, previously genotyped because of heredity for VTE and without previous VTE, were enrolled and added to the carrier group. Every fifth woman with modified APC ratio above the lower reference limit served as a control (Paper III). The heterozygous FVL carriers, the homozygous FVL carriers, the women with double heterozygosity and the controls were equal regarding age, parity, BMI, smoking and essential hypertension. There were no differences between the study group and controls regarding mode of delivery or anaesthesia during delivery. The follow-up duration after delivery was at least six weeks.
The 500 women with the FVL mutation were followed regarding levels of haemostatic markers during pregnancy and the puerperium (Paper IV).

Methods

In **Paper I**, blood sampling was performed between 8-10 a. m. at gestational weeks 10-15, 23-25, 32-34 and 38-40, within one week after delivery and eight weeks postpartum. Haemoglobin (Hb), haematocrit and platelet count were analysed on the same day. Classic APC ratio, modified APC ratio, fibrinogen, FVIII, protein C, free protein S, t-PA, PAI-1, PAI-2, F1+2, SF and D-dimer GOLD were analysed later in series.

In **Paper II**, Sonoclot analysis was performed at gestational weeks 10-15, 32-34 and 38-40 and eight weeks postpartum. After taking the first 2 ml of whole blood

for other analyses, the cuvette was filled with 0.4 ml whole blood, using a plastic syringe, and a Sonoclot signature was performed with a Sonoclot® II analyser within 1 min The following variables were analysed: sonact time, clot rate, secondary rate, peak amplitude, peak time and downward rate.

In **Paper III**, blood samples for analysis of modified APC ratio were taken in gestational week 17 at the routine ultrasound scan appointment. Women with modified APC ratio <2.0 were genotyped for the FVL mutation.
In Gothenburg, according to clinical routine in connection with detection of any thrombophilia, extended testing was performed, consisting of analysis of prothrombin gene mutation, AT, protein C and free protein S activity. All FVL carriers were personally given information about the thrombophilia, including known consequences during pregnancy and other situations, and recommended to use compression stockings. According to local guidelines, low molecular weight heparin (LMWH) was recommended as thromboprophylaxis during pregnancy for conditions entailing increased risk of VTE. Women who contracted VTE or superficial thrombophlebitis were treated with LMWH in therapeutic doses.
The FVL carriers were compared with controls concerning gestational age at delivery, gestational hypertension, mild/moderate preeclampsia (MP), severe preeclampsia (SP), syndrome of haemolysis, elevated liver enzymes and low platelets (HELLP), eclampsia, IUFD, birth weight deviation, late miscarriage, placental abruption and neonatal asphyxia. Furthermore, blood loss at delivery as well as frequency of VTE and superficial thrombophlebitis were compared. The number of clinical consultations on suspicion of VTE was also recorded.
Women with hemorrhagic disorders, acetylsalicylic acid medication or additional thrombophilia (except the prothrombin gene mutation) were excluded after comparison of demography, clinical features and incidence of late miscarriage. Furthermore, women treated with LMWH for more than three days during pregnancy as well as women with multiple pregnancies and late miscarriages were excluded from comparisons of obstetric complications, blood loss and VTE. In addition to these exclusions, children with trisomy 18 and 21 and children who died before birth or neonatally were excluded when birth weight deviations were compared.

In **Paper IV**, blood samples were taken from the FVL carriers between 7.30 and 10.00 a. m. in gestational weeks 23-25, 32-34, 38-40, one day after delivery and four-five weeks and eight-ten weeks postpartum. Women with multiple pregnancies and late miscarriage were excluded. Seven women withdrew from the study.
F1+2 and D-dimer ELISA were analysed in all 464 women. SF Latex and D-dimer Latex were analysed in 56 women who were homozygous FVL carriers or who had additional thrombophilias, VTE or placenta-mediated complications (except gestational hypertension) and in 56 of the heterozygous women with uneventful pregnancies matched for age, BMI and parity with the former group.
The haemostatic marker levels in women with uneventful pregnancies and puerperia and in women with additional thrombophilia, homozygosity for FVL, placenta-mediated complications or VTE at the different stages of pregnancy and puerperium

were compared. Comparisons were also made between healthy carriers and non-carriers with uneventful pregnancies.

Laboratory methods

Blood samples were taken through careful cubital vein venipuncture following a 20-minute rest. Butterfly syringe (23G) and Vacutainer (Venoject®, Terumo, Leuven, Belgium) techniques were used. Blood was collected in tubes containing 0.04 ml EDTA (0.47 mol/l) for determination of Hb, haematocrit and platelet count, into Stabilyte® (Biopool, Umeå, Sweden) tubes for determination of t-PA and into tubes containing 0.5 ml trisodium citrate (0.13 mol/l) for the other analyses. The blood was centrifuged at 2 000 g for 20 minutes at room temperature within 20 minutes of sampling in Paper I and within one hour of sampling in Paper IV. The plasma was carefully removed without stirring the buffy coat and aliquotted into polystyrene tubes. The plasma samples for modified APC ratio in Paper III were stored at -20ºC until analysis, carried out within a week. The plasma samples for the other analyses were stored at -70ºC until analysed. In all studies each analysis was run simultaneously for all women and the same batches were used. If absorbance was above the top standard, the sample was retested after dilution so that the results were read on the linear part of the calibration curve.
All analyses were performed by accredited laboratories (ISO 15189).

Hb, **haematocrit**, **platelet count** and **AT** were analysed according to routine.
APTT was determined with APTT Coatest (Chromogenix, Mölndal, Sweden).
The classic APC ratio was determined as the ratio between the time (APTT) with and without adding activated protein C (Coatest®, APC Resistance, Chromogenix, Mölndal, Sweden). An ACL 300R (Instrumentation Laboratory, Milan, Italy) was used for clot time determinations.
The modified APC ratio was determined with the method described above but with the plasma sample pre-diluted 1+4 with lyophilised, stabilised diluent (V-DEF Plasma, Chromogenix, AB, Mölndal, Sweden) (Paper I) and (COATEST® APC™ Resistance V, Chromogenix AB, Mölndal, Sweden) (Paper III).
FVIII was analysed with an amidolytic assay using the chromogenic substrate S-2765 (Coamatic®, Factor VIII, Chromogenix).
Fibrinogen concentration was analysed using the Clauss method (Fibri- Prest®, Automate, Diagnostica Stago, Asnières-sur-Seine, France).
Protein C was determined with a chromogenic assay using the substrate S-2366 (Coamatic®, Protein C, Chromogenix).
Free protein S antigen was determined with ELISA (Asserachrom®, Free Protein S, Diagnostica Stago).
The **activity of t-PA** in plasma was determined by a solid phase enzyme bioimmunoassay (Coatest®, BIA t-PA, Chromogenix).
PAI-1 was determined with ELISA (Coaliza®, PAI-1, Chromogenix).
PAI-2 was determined with ELISA (Tint Elize®, PAI-2, Biopool).
F1+2 was analysed with a quantitative ELISA (Enzygnost® F1+2 micro, Dade Behring, Marburg, Germany).

SF was analysed with an assay based on the ability of SF to stimulate the t-PA-catalysed conversion of plasminogen to plasmin. The amount of produced plasmin was determined with the chromogenic substrate S-2403 (Coatest®, Soluble Fibrin, Chromogenix).
SF Latex was analysed with an automated latex immunoturbidometric assay using a monoclonal antibody, IF-43 (LPIA-Iatro, Mitsubishi Kagaku Iatron Inc, Japan).
D-dimer GOLD was measured as cross-linked fibrin degradation products using an ELISA (Dimertest®, GOLD EIA kit, Agen, Acacia Ridge, Australia).
D-dimer ELISA was analysed with a quantitative ELISA technique (Asserachrome® D-Di/ Diagnostica Stago, France).
D-dimer Latex was analysed with a quantitative latex agglutination assay (STA-Liatest® D-DI / Diagnostica Stago, France).
The **FVL mutation** (Zöller et al 1994) and the **prothrombin 20210G>A mutation** (Poort et al 1996) were detected by PCR coupled with restriction fragment length polymorphism analysis (PCR-RFLP). Known wild-type, heterozygous, homozygous and blank controls were included in each run.
Sonoclot analyses were performed using Red Top cuvettes #800±0420 (SIENCO, Morison, Colo., USA. The sensitivity was set at 60 and the paper chart speed was set at 30 cm/h.

Statistics

Sample size
In order to determine the reference intervals for haemostatic variables (Papers I and II) we chose a sample size of 50 women, based on common practice and for practical reasons. This number provides a good estimate of the mean in order to study changes over time. However, these reference intervals are uncertain in the tails of the distributions with CI widths, for the limits of the 95% reference intervals, exceeding the SD (Papers I and II).
Power analysis was undertaken to determine the required number of participants for the study on hereditary APC resistance. The incidences of pregnancy-induced hypertension (including preeclampsia), premature delivery and SGA in Sweden are reported at 5.0, 6.0 and 2.5 %, respectively (Hanson et Persson 1993). In order to achieve 80 % power in finding a doubled relative risk of the complication with the lowest of these incidences in a two-tailed test, 430 cases and 860 controls were required; 500 cases and 1000 controls were recruited (Paper III).

Descriptive statistics
The mean, SD, median and range were calculated for each variable and time (Papers I, II and III).

Change over time
Linear regression was used to determine the change in each variable over time for each individual, as plotting the data showed that changes over time existed and that they were close to linear. The sample taken eight weeks postpartum was used as a non-pregnant baseline value and was set at time zero. The assumption was that

random error contribution added up proportionally to time during pregnancy. A 5% sign test was performed for each variable based on the hypothesis of median 0 for the change in individuals during pregnancy, with the alternatives increase and decrease. (Paper I).
A two-sided paired t-test was used to compare values from samples taken at 10±15 weeks of gestation with those taken at 32±34 weeks of gestation, and to compare samples taken at 32±34 weeks of gestation with those taken at 38±40 weeks of gestation. This test was used to obtain a more precise comparison, as plotting the data revealed small differences between the different trimesters. The two-sided paired t-test was also used to compare values from samples taken at 38±40 weeks of gestation with those taken eight weeks postpartum. Multiple testing was taken into consideration by using the Bonferroni correction. (Paper II).
The paired t-test was chosen for comparisons between marker levels at the different sampling occasions as there was some missing data at the different occasions. The statistical analyses were performed on logarithmic transformed data, as the variables showed a positive skewed distribution. Multiple testing was taken into consideration by using the Bonferroni correction. (Paper IV).

Comparison of independent groups
In Paper III the two-sample t-test was used for comparing demographic and clinical features as well as outcome, such as frequency of thromboprophylaxis, birth weight deviation and blood loss between groups. Occurrence probability was compared for analyses of differences in smoking and essential hypertension. Blood loss at delivery is a positive variable with considerable relative variation and was thus analysed on a logarithmic scale.

Comparison of incidences of complications in different groups
The relative risks for complications were calculated by cross-tabulation and with a 95 % CI. Multiple testing was taken into consideration by using the Bonferroni correction. (Paper III).

Correlation between levels of different variables
The Spearman rank correlation test, with the significance level adjusted for multiple testing with a total level of 5%, was used to see if the total changes in the variables covaried. The slopes of the linear regression lines from thirty complete series of signatures were used as data in these correlation tests, since they were equal to the estimated changes over the whole pregnancy period from time zero to 40 weeks of gestation. This value is referred to as the total change during pregnancy. A nonparametric test was chosen since normal distribution could not be assumed (Paper I).
The Spearman rank correlation test, with the significance level adjusted for multiple testing with a total level of 5%, was used to ascertain if any of the Sonoclot variables covaried with any of the routine coagulation analyses using all samples (n = 154). A nonparametric test was used since normal distribution could not be assumed. (Paper II).
Pearson's correlation test was used to measure the degree of association between

the levels of F1+2 and SF and between the levels of SF and D-dimer ELISA at the different sampling occasions. If $r \geq 0.5$ (determination coefficient ≥ 0.25), the association is regarded as high. Pearson's correlation test was also calculated in order to characterise the correlation between D-dimer ELISA and D-dimer Latex. In this case the same variable is compared using two different assays; $r \geq 0.9$ is thus regarded as acceptable. The calculations were performed on logarithmic transformed data (Paper IV).

Funnel plot
Log ORs were plotted against the number of participants in the respective studies. The results of large studies are expected to be the closest to real and those of small studies are expected to be more widely scattered. Overall, the plots should produce an inverted funnel. Asymmetry of the plots proves that a publication bias is present.

Hill's criteria
The existence of covariance is often interpreted as implying the existence of a causal relationship. Two variables (A and B) can be associated for several reasons. Excluding the possibility that the association can be attributed to chance, it may exist because A influences (or causes) B or because B influences (or causes) A or because both A and B are influenced by one or more other variables. Hill's Criteria of Causation (Hill 1965) provides a valuable instrument with which to evaluate theories and explanations proposed in medical science. The criteria are: strength of association, consistency of association, temporal relationship, specificity, biologic plausibility, dose-response relationship, coherence, analogy and experimentation.

RESULTS

Haemostasis in FVL non-carriers during pregnancy and the puerperium (Paper I and II)

Haemostatic variables during pregnancy and the puerperium (Paper I)

Hb, haematocrit, APTT and platelet count were within the reference intervals for pregnant women.

Compared with non-pregnant individuals, FVIII, fibrinogen, PAI-1 and PAI-2 were increased, and free protein S levels were decreased during pregnancy. Eight weeks postpartum, 15% of the women still had a free protein S level below the reference interval for non-pregnant individuals. There was no change in protein C. t-PA increased but remained within the reference interval. Analyses from the samples taken before the women left hospital, at mean 2.4 days postpartum, showed that PAI-1 had already decreased to the non-pregnant level and that PAI-2 was reduced to 28% of the level in the third trimester. Eight weeks postpartum, 11 of the 48 women had PAI-1 levels below the lower reference limit for non-pregnant individuals.

F1+2, SF and D-dimer increased during pregnancy. F1+2 and D-dimer were above the reference intervals for the majority of patients in the last trimester, while SF remained within the reference interval in 80% of the women (Figure 4). All the variables except the classic APC ratio and free protein S were within the reference intervals for non-pregnant individuals eight weeks postpartum.

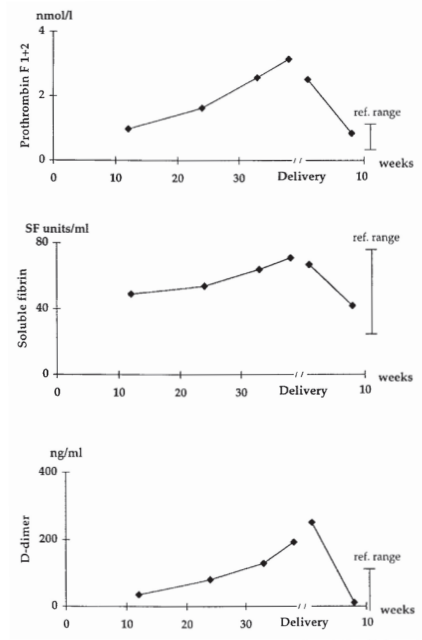


Figure 4. Mean values for prothrombin fragments 1+2, soluble fibrin and D-dimer during pregnancy, within one week after delivery and eight weeks postpartum, respectively. Reference ranges apply to non-pregnant individuals.

APC ratio during pregnancy and the puerperium (Paper I)
The classic APC ratios were significantly lower during pregnancy than eight weeks postpartum, although 92% were above the lower reference limit in the third trimester. Two women still had a classic ratio below the reference range for non-pregnant individuals eight weeks postpartum. All modified APC ratios during pregnancy were above the lower reference limit for non-pregnant women, although linear regression showed that there was a small but statistically significant decrease over time.
The Spearman rank correlation test showed no correlation between the total change during pregnancy in the classic APC ratio and the changes in FVIII (r_s = 0.041), fibrinogen (r_s = 0.028), protein S (r_s = -0.36), F1+2 (r_s = 0.12) or SF (r_s = -0.032); nor between the change in SF and changes in F1+2 (r_s = 0.27), fibrinogen (r_s = 0.41) or D-dimer (r_s = 0.35). We found no correlation between SF in the late third trimester and the classic APC ratio in the first trimester.

Sonoclot signature during pregnancy and the puerperium
Sonoclot analysis indicated hypercoagulability during pregnancy, compared to eight weeks postpartum. Sonact time and peak time were unchanged during pregnancy and shorter during pregnancy than eight weeks postpartum. Clot rate and secondary rate were unchanged but higher during pregnancy than eight weeks postpartum. There was no difference in peak amplitude or downward rate in pregnancy, compared to eight weeks postpartum. The signatures were easy to interpret and had the same general shape for each patient over time. A Sonoclot signature reflecting the late third trimester and one reflecting eight weeks postpartum have been drawn using the mean values of the variables (Figure 5). We found a small but significant correlation between APTT and sonact time (r = 0.25) and between APTT and clot rate (r = -0.25). There was no correlation between platelet count and secondary rate, haematocrit or peak amplitude, or between fibrinogen and peak amplitude.

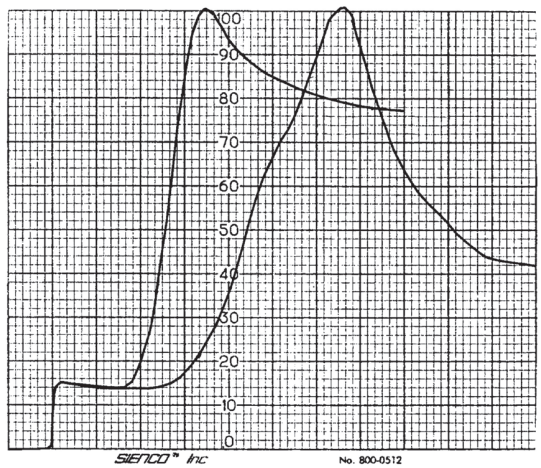


Figure 5 Sonoclot signature during third trimester (left) and eight weeks postpartum (right), drawn based on the mean values of the variables

Prevalence of FVL mutation and of additional thrombophilias (Paper III)

5 986 women were tested for APC resistance. 483 with a modified ratio <2.0 were genotyped for the FVL mutation and tested positive. The prevalence of heterozygous FVL mutation was 7.9 %. In Gothenburg the prevalence of homozygous FVL carriership was 0.26 %. None of the FVL carriers in Stockholm was found to be homozygous. In addition to the FVL mutation, five women (1.4 %) tested positive for the heterozygous prothrombin G20210A mutation in Gothenburg. One woman had concomitant AT deficiency type I and one woman had AT deficiency type IIc. No deficiencies of protein C or protein S were detected. One heterozygous FVL carrier with an APC ratio in the reference range for homozygosity was found to have partial type 1 quantitative FV deficiency (pseudo-homozygous APC resistance).

FVL carriership during pregnancy and the puerperium (Paper III)

Obstetric outcome

There was no significant difference regarding placenta-mediated complications.
The gestational age at delivery was the same. There was no difference in the birth weight deviation in percent. However, the incidences of SGA and large for gestational age (LGA) were higher in FVL carriers, but the difference was not significant.
There was one unexplained IUFD among the controls and none among the carriers. Furthermore, one intrapartum death occurred in a control with severe preeclampsia. One FVL carrier and two controls suffered unexplained late miscarriages.
There were no complications, except one premature birth in gestational week 36, among the homozygous FVL carriers or women with double heterozygosity.

Blood loss at delivery

The total blood loss at delivery was lower in the carriers, but the difference was not significant. The incidence of blood loss exceeding 1 000 ml was lower among the FVL carriers.

Incidence of VTE

Two heterozygous FVL carriers contracted DVT in the left iliac vein in gestational week 28 and 30, respectively. The diagnoses were confirmed by magnetic resonance venography and triplex ultrasonography (combination of venous compression, colour Doppler and pulsed Doppler ultrasonography), respectively. One heterozygous FVL carrier had a PE, diagnosed by s-CT scan, two weeks after vaginal delivery. Two of these three carriers had no other risk factors for VTE, while one woman with a DVT had heredity for VTE. None of these women had been given thromboprophylaxis. Another heterozygous FVL carrier with PE, confirmed by s-CT scan, five weeks postpartum also tested positive for both LA and anticardiolipin antibodies at follow-up. She was excluded from the study.
The number of clinical consultations on the women's own suspicion of VTE was fourfold higher among the FVL carriers than among controls. The proportion of objective testing, however, was the same in carriers and controls.

Haemostatic markers in FVL carriers during pregnancy and the puerperium (Paper IV)

F1+2, SF and D-dimer in heterozygous FVL carriers with normal pregnancies and puerperia

F1+2 increased gradually during pregnancy, but was not higher one day postpartum than in the late third trimester. F1+2 was lower eight weeks postpartum than during pregnancy. One third of the carriers had F1+2 levels exceeding the reference interval for non-pregnant adults eight weeks postpartum. Compared to non-carriers, carriers had significantly higher levels of F1+2 eight weeks postpartum, but not during pregnancy.

SF Latex was mainly unchanged during pregnancy and at the same level as eight weeks postpartum, but there was an increase one day postpartum. Carriers did not have significantly higher levels of SF Latex during pregnancy or eight weeks postpartum, compared to non-carriers, and the levels were in the same reference intervals as those in non-pregnant adults.

D-dimer ELISA increased gradually during pregnancy and was higher one day postpartum than in the late third trimester. D-dimer ELISA was significantly lower eight weeks postpartum than during pregnancy. One third of the carriers had a D-dimer ELISA level exceeding the reference interval for non-pregnant adults. Carriers had significantly higher D-dimer Latex levels during both pregnancy and puerperium than non-carriers. The correlation coefficients between D-dimer Latex and D-dimer ELISA were high during pregnancy and one day postpartum but low four and eight weeks postpartum.

The correlations between F1+2 and SF Latex during the second and early third trimester were low but significant but they were not significant in the late third trimester, after delivery or postpartum.

The correlations between SF Latex and D-dimer ELISA during pregnancy and one day postpartum were high but not significant four or eight weeks postpartum.

F1+2, SF and D-dimer in homozygous FVL carriers, carriers with additional thrombophilias or with complications during pregnancy or the puerperium

The levels of the markers in homozygous FVL carriers, carriers with additional thrombophilias or with complications during pregnancy or the puerperium were in the same ranges as in the heterozygous women with normal pregnancies and puerperia. The ratio SF Latex /D-dimer ELISA was not higher in women with complications than in women with uneventful pregnancies or puerperia.

Discussion

Study design

In Papers I and II, a sample size of 50 women was chosen to determine reference intervals for haemostatic variables, based on common practice and for practical reasons. Reference intervals are most often used in clinical chemistry to provide a standard reference against which to assess substance levels in patients under investigation. The reference interval is simply the estimated range of values that includes a certain percentage (most often 95%) of the values among the relevant population. It is either estimated directly from observation or from the antilog values of the mean ±1.96 SD of the logged values (geometric mean), as clinical data are never normally distributed. Choosing 50 as a sample size yields a good estimate of the mean in order to study changes over time. However, these reference intervals are uncertain in the tails of the distributions; the widths of the CI, for the limits of the 95% reference intervals, exceed the SD of the observations. Furthermore, it is not advisable to use the reference intervals as a basis for decision-making, as it is uncertain whether values outside them indicate clinical health problems or whether values inside them are unassociated with health problems. The more appropriate prediction intervals are wider than the reference intervals.

Eight weeks postpartum was chosen to represent the non-pregnant state. All haemostatic variables had returned to the reference interval for non-pregnant individuals except the classic APC ratio, PAI-1 and free protein S in some women. Perhaps it would have been better to wait until after the breast-feeding period to undertake the final sampling. However, that might have entailed some women moving out of the region, some becoming pregnant again and some having started to use hormonal contraception, which can also influence haemostatic variable levels.

In Papers III and IV, our intention was to investigate the prevalence of the FVL mutation, plasma levels of haemostatic markers and the incidences of placenta-mediated complications and VTE. This required a prospective study. In order to find about 500 women with the FVL mutation, we selected a cohort of about 6 000 women. With a sample size of about 500 the CI of the limits of the resulting reference intervals are about 0.35 SD, but the above-mentioned caveat concerning clinical application of reference intervals must still be taken into consideration.

In order to decide if the pregnancy was normal or if the criteria for a complication were met, it was necessary to scrutinize the medical records. The diagnoses in medical records are very often inadequate. We thus chose the case-cohort study design and selected every fifth non-carrier as a control. This design allows calculation of the risk ratio without the need for the rare-disease assumption (Sato 1994).

The prospective design is not useful in finding elevated relative risks of rare conditions like placental abruption and IUFD. The absolute risk is more informative and most clinicians find it more appealing to discuss the chance of an uneventful pregnancy than the risk of contracting a rare condition with a patient (Paper III).

Haemostasis in FVL non-carriers during pregnancy and postpartum (Paper I and II)

Haemostatic variables during pregnancy and the puerperium (Paper I)

Haemostatic variables were analysed in order to obtain reference intervals for FVL non-carriers. There was a change towards a hypercoagulable state with increased concentration of blood coagulation factors and fibrinolysis inhibitors, decreased t-PA activity and decreased or unchanged concentration of blood coagulation inhibitors, which is in agreement with many other studies concerning haemostasis during pregnancy.

The mean values of FVIII and fibrinogen were almost doubled during the last trimester, compared to the non-pregnant state. Other studies (Hathaway and Bonnar 1987, Hellgren and Blombäck 1981, Mathonet et al 1996, Schlit et al 1996, Stirling et al 1984) have shown a similar increase. The protein C level was within the reference interval for non-pregnant women and did not change during pregnancy. This finding corresponds to those from other studies (Bremme et al 1992, Faught et al 1995, Hathaway and Bonnar 1987, Stirling et al 1984). The concentration of free protein S dropped early during the first trimester and decreased continuously during pregnancy, as described previously (Bremme et al 1992, Faught et al 1995, Stirling et al 1984). Eight weeks postpartum 15% of the women still had levels below the reference interval for non-pregnant women, which is in accordance with another study (Bremme et al 1992). Thus, the free fraction of protein S does not seem to reach the non-pregnant value within eight weeks postpartum, which might be taken into consideration when evaluating thrombophilia. This is probably also why a remarkably high prevalence of protein S deficiency is found in many case-control studies of pregnancy complications, both among the cases and controls. Furthermore, some functional protein S assays and functional protein C assays yield false low values in cases of FVL mutation (Faioni et al 1994, Jennings et al 2000).

The results of studies on fibrinolysis alterations in pregnancy are difficult to interpret, as decreased levels of t-PA and increased levels of PAI-1 would suggest impaired fibrinolysis concomitant with increasing levels of D-dimer. Thus, fibrinolysis compensates for increased fibrin formation during pregnancy and the micro-circulation in the placenta is maintained during normal pregnancy.

t-PA activity decreased during pregnancy, as previously described (Ishii et al 1994). This is partly an effect of increased production of PAI-1 in the placental endothelium. Other studies show increasing PAI-1 and PAI-2 levels during pregnancy (Bremme et al 1992, Dalby Sörensen et al 1995, Estellés et al 1990, Halligan et al 1994). However, the mean levels of PAI-1 and PAI-2 found in this study were within these previously published intervals. PAI-1 and PAI-2 levels normalised rapidly after delivery. PAI-2 levels that might have been due to remaining trophoblast were found eight weeks postpartum in two patients, although neither patient had any clinical symptoms.

Classic and modified APC ratio during pregnancy and the puerperium (Paper I)

The classic APC test showed decreasing ratios during pregnancy, similar to the development reported in other studies (Cumming et al 1995, Mahieu et al 2007, Mathonet et al 1996, Peek et al 1997, Schlit et al 1996, Vasse et al 1994). The modified

APC resistance test showed a minimal, albeit significant, decrease during pregnancy. However, all the modified ratios during pregnancy and the puerperium were above the reference limit for non-pregnant women. This result agrees well with three other studies (Benedetto et al 2002, Cumming et al 1996, Schlit et al 1996). Two women had a classic APC ratio below the reference interval for non-pregnant individuals eight weeks postpartum. The modified APC test is consequently more useful in order to detect the FVL mutation during pregnancy, during which the classic APC test may yield false positive results.
Our intention was to study the influence of haemostatic variables on the classic APC ratio. We found no explanation for the "acquired" APC resistance, as there was no correlation between the total change in the classic APC ratio and the total changes in FVIII, fibrinogen or free protein S during pregnancy, in agreement with other studies (Mahieu et al 2007, Vasse et al 1994). The results concerning the correlation between the classic APC ratio and FVIII are conflicting (Cumming et al 1995, Mahieu et al 2007, Mathonnet et al 1996). In an experimental study, in which the classic APC ratio was measured in series of pooled normal non-pregnant plasma diluted in protein S-deficient plasma, the ratio remained constant when free protein S was above 20% (Ronde and Bertina 1994).

Sonoclot signature during pregnancy and the puerperium (Paper II)
We found the Sonoclot® analyser rapid and simple to handle and the Sonoclot signature simple to interpret. Compared to the signatures eight weeks postpartum, the signatures throughout pregnancy were altered in a manner indicating hypercoagulability. We chose to present the 90% ranges for all values during pregnancy, as normal distribution could not be assumed, and as we found no changes during pregnancy. We recommend that the ranges be used with caution, especially their peripheral areas. Very few studies have been performed assessing the correlation between Sonoclot variables and routine coagulation analyses, and only rarely has a statistically significant correlation been found. This could be due to the fact that, as in this study, the materials were homogeneous and included patients with normal haemostasis. However, Spearman's rank correlation test showed a low but significant correlation between APTT and sonact time and between APTT and clot rate. Other studies have shown low or absent correlation between these variables (Ekbäck et al 1995, Goldman et al 1993, Schött et al 1995, Steer and Krantz 1993). We found no correlation between platelet count and secondary rate, concurring with another study (Horlocker and Schroeder 1997). The platelet count provides a quantitative but not a qualitative index of platelet status, both of which influence the secondary rate. Furthermore, the secondary rate is influenced by fibrin formation and polymerisation. We found no correlation between haematocrit and peak amplitude or between fibrinogen concentration and peak amplitude, perhaps due to the fact that peak amplitude is a measure of maximal viscosity during the coagulation process. Lack of correlation between fibrinogen concentration and peak amplitude has been reported previously (Goldman et al 1993).

Prevalence of the FVL mutation and of additional thrombophilias (Paper III)

The prevalence of FVL carriership was 8.1 %, which is lower than the 11 % prevalence previously reported from Malmö in the south of Sweden (Lindqvist et al 1999). Women with heredity for VTE might have been more prone to participate and some women did not want information concerning an increased risk of contracting a disease. These circumstances might have led to a volunteer bias leading to both under- or overestimation of the prevalence. The fact that the women with previous VTE were not enrolled may have led to a slight underestimation of the prevalence (estimated at 0.2 %).

The prevalence of homozygosity was calculated on the material from Gothenburg and found to be 0.26 %; according to Hardy-Weinberg's equation, a prevalence of 0.16 % (95 % CI 0.11-0.41 %) would have been expected. The prevalence of heterozygous prothrombin G20210A variant was 1.4 %. Other studies have reported a prevalence of 1.7 % in a general population in northern Europe (Rosendahl et al 1998). The six women with double heterozygosity were not excluded from the study, as the same prevalence of the prothrombin G20210A variant is assumed to exist in the control group. Three of the 360 FVL carriers in Gothenburg had other additional thrombophilias. Due to the very low prevalences of these thrombophilias (AT deficiency type I and type IIc and pseudo-homozygous APC resistance) in the present study and in general populations, their presence was assumed to be very low in the control group (Simioni et al 2005, Tait et al 1994) and would not, we presumed, influence the results.

FVL carriership during pregnancy and the puerperium (Paper III)

Obstetric outcome

The incidences of placenta-mediated complications were low, but concurred with data from the Swedish Medical Birth Register (Hanson and Persson 1993).

Cross-tabulation showed no difference between the FVL carriers and controls regarding placenta-mediated complications. This finding might be due to the fact that pathological changes in the placental vessels in the studied conditions are more similar to pathology in atherosclerotic diseases such as myocardial infarction, ischemic stroke and peripheral arterial disease (Redman and Sargent 2003). The FVL variant is a thrombophilia entailing increased risk of VTE. The current literature contains no solid evidence to support an important relationship between the FVL variant and atherosclerotic diseases (Boekholdt and Kramer 2007). However, when recruitment to this study commenced, there was scarce published data either confirming or refuting any such relationships. The prevalences of gestational hypertension, MP and SP were not higher in FVL carriers than in controls, and were in accordance with the five largest case-control studies (GOPEC 2005, de Groot et al 1999, Kim et al 2001, Mello et al 2005, O'Shaughnessy et al 1999) and three prospective studies (Clark et al 2007, Dizon-Townson et al 2005, Lindqvist et al 1999). The assumption that women with congenital thrombophilia might exhibit an exaggerated coagulation response to endothelial damage could not be confirmed in this study.

We found no significant difference in birth weight deviation between FVL carriers and controls, indicating no elevated risk for IUGR, in agreement with other large

studies (Clark et al 2007, McCowan et al 2003, Dizon-Townson et al 2005, Infante-Rivard et al 2002, Lindqvist et al 1999, Verspyck et al 2004). The weight distribution of the FVL carriers' neonates had a higher kurtosis than that of the controls' neonates, resulting in more cases of SGA and LGA among the carriers. However, these differences were not significant if multiple testing is taken into consideration. The mean gestational age at delivery was equal in the two groups, indicating that there was no higher rate of conditions requiring induction of delivery or spontaneous preterm delivery. If the FVL and the prothrombin G20210A mutations had been associated with bad obstetric outcome, lower birth weight would have been expected in neonates born to the women with homozygous FVL or double heterozygosity.
Adverse outcome such as eclampsia, HELLP syndrome, placental abruption, late miscarriage, low Apgar score, IUFD and intrapartum death were rare events. Our results did not support the assumption that FVL carriership raises the relative risk of these obstetric complications. Some of these outcomes can be assumed to be prevented by good obstetric care and are therefore hard to evaluate related to risk factors. Furthermore, a prospective study of rare conditions requires an unmanageably large number of enrolled patients in order to show significant differences and the costs would not be justifiable. It is more important to consider the absolute risks.
Two prospective studies (Dizon-Townson et al 2005, Lindqvist et al 1999) and two of the three largest retrospective studies (Jääskeläinen et al 2004, Procházka et al 2003, Procházka et al 2007) failed to detect any association between FVL carriership and placental abruption. The largest case-control study found a significantly elevated risk (OR 4.8, CI 1.8 –12.4) for fetal loss after 22 gestational weeks (Gris et al 1999) but the largest cohort study did not (OR 1.57, CI 0.76-3.25) (Völske et al 2003).
The question of whether LMWH lowers the risk of placenta-mediated complications in women is under debate. Many authors suggest that women with placenta-mediated complications in previous pregnancies should be given thromboprophylaxis if their venous thrombophilia work-up is positive. As placenta-mediated complications seem to be associated with atherosclerotic diseases and since LMHW is useful in treatment of other arteriosclerotic complications, it would be a pity if prophylaxis is withheld from the women without venous thrombophilia.

Blood loss at delivery
Estimation of blood loss at delivery is uncertain, but as the same method was used in all parturients, we considered a comparison to be appropriate. There was no difference in blood loss volume at delivery. However, the fact that the number of women with blood loss exceeding 1 000 ml was lower among the FVL carriers suggests a possible survival advantage of FVL carriership in times when bleeding complications could not be treated appropriately and scurvy was a common condition. This finding concurs with a previous report (Lindqvist et al 1999) but was not confirmed in a more recently published study (Clark et al 2007).

Incidence of VTE
One weakness of the study is that the risk of VTE in unknown FVL carriers during

pregnancy might have been underestimated, due to the non-blinded design. More FVL carriers than controls were given thromboprophylaxis and recommendations for compression stockings, according to clinical routines, which might have prevented some VTE complications.
The incidence of superficial thrombophlebitis did not differ between the FVL carriers and controls, in agreement with another study (McColl et al 1998).
The number of clinical consultations on suspicion of VTE was fourfold higher among the FVL carriers than among controls, possibly due to heightened anxiety resulting from knowledge of the thrombophilia and its risks. The issue of whether to screen pregnant women for the FVL mutation is under debate. The patients and their relatives may benefit from genotyping but they may also be harmed both psychologically and economically, e.g. by jeopardized health insurance coverage.

Haemostatic markers during pregnancy and the puerperium (Paper I and IV)

Activation of blood coagulation in vivo can be determined by analysis of F1+2, SF and D-dimer. During uncomplicated pregnancy, the increase in SF may reflect increased fibrin formation, presumably in the uteroplacental unit. D-dimer reflects both blood coagulation activation and ongoing fibrinolysis. To estimate the degree of blood coagulation activation, fibrin formation and fibrin degradation, the ideal tests would measure the rate of formation of the actual markers. Instead, the concentrations of the markers are used as proxies. These are intermediate products in multistep processes and are dependent on both the formation rate and the reaction rate of the next step in the reaction chain and elimination from the body. Perhaps this is more prominent in the case of SF, which might explain the lower variation in the levels of this substance. The importance of increased levels of coagulation activation markers is uncertain but increased levels are found in cases of VTE and in patients with thrombophilia (Ginsberg et al 1995). Our results were only compared with results from studies using the same or very similar assays, as there is no simple way of transforming the data. Even if the differences were significant, there was always a considerable overlap, in this study as well as in the studies to which we refer.

Haemostatic markers in FVL non-carriers during pregnancy and the puerperium (Paper I)

F1+2, SF and D-dimer were used in order to obtain reference intervals for FVL non-carriers. We found gradual increases of F1+2, and D-dimer ELISA levels, up to those found in acute thromboembolic events, as well as higher levels during pregnancy than in the non-pregnant state, in agreement with other studies (Bellart et al 1997, Bremme et al 1992, Cerneca et al 1997, Comeglio et al 1996, Eichinger et al 1999, Francalanci et al 1995, Lindoff et al 1993, Nolan et al 1993, Schambeck et al 2001, Schlit et al 1996).

F1+2, SF and D-dimer in heterozygous FVL carriers with normal pregnancies and puerperia (Paper IV)

When blood sampling for Paper IV was completed, the assays for SF and D-dimer used in the first study were not available. We therefore used other assays, with other

reference intervals, in comparisons of SF and D-dimer in carriers and non-carriers.
F1+2 and D-dimer ELISA levels in the carriers increased gradually during pregnancy and were significantly lower eight weeks postpartum than during pregnancy. These results concur with the only previously reported longitudinal study of D-dimer in 11 carriers (Eichinger et al 1999). There were no major alterations in the SF Latex levels during pregnancy, in agreement with a cross-sectional study of SF in non-genotyped women, in which levels in most of the women were in the interval for non-pregnant women (Onishi et al 2007). No other study has been published concerning SF levels in normal pregnancy in FVL carriers. The low-grade DIC occurring in normal pregnancy does not result in increased SF levels, in contrast to fulminant DIC (Dempfle et al 2004).
Both SF and D-dimer ELISA were significantly higher the day after delivery than in the late third trimester, indicating more pronounced activation of haemostasis and fibrinolysis in connection with delivery. As in other studies (Cerneca et al 1997, Reber et al 1998) this was not the case for F1+2, which may be an effect of the shorter plasma half-life of F1+2. The plasma half-life of F1+2 is reported at 90 min and that of SF and D-dimer in plasma is reported at 5-6 hrs and 8 hrs, respectively (Bauer 2006, Nieuwenhuisen 1993). However, another plausible explanation is that F1+2 molecules, which remain attached to the phospholipids on the surface of the platelets, escape detection, as our assay was performed on platelet-poor plasma (Cadroy et al 1993).
A high percentage of the women still had levels of F1+2 and D-dimer ELISA above the reference interval eight weeks postpartum. This is probably because carriers have a higher grade of coagulation activation than non-carriers, but also perhaps because eight weeks postpartum is not representative for the non-pregnant state and because the limits of the reference intervals are uncertain.
Comparisons between carriers and non-carriers in the non-pregnant state revealed small but significant differences in the levels of F1+2 and D-dimer, although the overlap was substantial (Leroy-Matheron et al 1996, Martinelli et al 1996, Simioni et al 1996, Zöller et al 1996).
Our study showed that women carrying the FVL mutation had higher levels of F1+2 than non-carriers eight weeks postpartum, but not during pregnancy. This was also found in two other longitudinal studies (Eichinger et al 1999, Schambeck et al 2001).
We found no difference between SF Latex levels in carriers and non-carriers during pregnancy or eight weeks postpartum, perhaps because SF Latex is an intermediate product and the turnover is high.
Compared to women not carrying the FVL allele, carriers had higher levels of D-dimer Latex both during pregnancy and eight weeks postpartum, in concordance with another study (using D-dimer ELISA) (Eichinger et al 1999). We found a high correlation between the D-dimer assays during pregnancy and one day postpartum. The correlation coefficients were on the same levels as in another study (Dempfle 2001). The low correlation postpartum between D-dimer Latex and D-dimer ELISA can be explained by the low levels found with the respective assays at that time.

Correlation between markers (Papers I and IV)
The correlation between F1+2 and SF was low or absent. This can be explained by the fact that the level of F1+2 is a measure of thrombin generation. The formed thrombin is partly inhibited (AT, α_2-macroglobulin, and $\alpha_1$antitrypsin). SF is a measure of fibrin products produced by the remaining thrombin.
In Paper I there was no correlation between the total change in SF and D-dimer Gold. In Paper IV the correlation between SF Latex and D-dimer ELISA was high during pregnancy but absent four and eight weeks postpartum. These conflicting results can be explained by the fact that different assays for SF and D-dimer were used in these two studies. Perhaps the two assays in Paper I had a lower degree of cross-reactivity than those in Paper IV. The SF assay is a global indicator of thrombin action on fibrinogen and is based on an antibody detecting epitopes on fibrin monomers, fibrin polymers and FXIIIa-induced cross-linked fibrin derivatives; it is not, however, intended to detect plasmin-degraded SF products. The D-dimer assay is based on an antibody detecting epitopes on the D-D domain in fibrin compounds such as D-dimer, plasmin-degraded SF products and, above all, FXIIIa-induced cross-linked fibrin derivatives. A positive correlation between SF and D-dimer could be due to the fact that they detect the same products. The low correlation between this SF assay and D-dimer ELISA (r=0.26) was taken as proof that this antibody, which detects SF, does not recognize cross-linked fibrin degradation products (Ieko et al 2007, Soe et al 1996). However in another study, Spearman's rho was 0.75 between the SF assay, used in this study, and a D-dimer assay (Dempfle et al 2001).

F1+2, SF and D-dimer in homozygous FVL carriers and carriers with additional thrombophilias (Paper IV)
The homozygous FVL carriers and carriers with additional thrombophilia had markers in the same intervals as the heterozygous carriers with normal pregnancies and puerperia. Only one study is reported concerning markers in non-pregnant individuals who were homozygous for FVL or who had additional thrombophilia; their mean level of F1+2 was in the lower part of the reference interval (Harbrecht et al 1998).
In Paper IV, log SF Latex was plotted against log D-dimer ELISA in order to prove the theory that women with placenta-mediated complications or VTE might have an imbalance between fibrin formation and fibrinolysis. The plots for women with complicated pregnancies were scattered, without any discernible pattern, among the plots for matched women without complications.
If the balance had shifted towards increased fibrin formation or decreased fibrinolysis the plot for the women with complications would have been scattered above the regression line.

F1+2, SF and D-dimer in FVL carriers with pregnancy-mediated complications during pregnancy and the puerperium (Paper IV)
There were no differences in marker levels in women with normal pregnancies or in women with pregnancy-mediated complications, either before or after onset. Previous studies have shown significantly higher levels of F1+2 and D-dimer in non-

genotyped women with SP, early SGA or placental abruption at the time of diagnosis than in women with normal pregnancies (Belo et al 2002, Francalanci et al 1995, Freeman et al 2008, Nolan et al 1993, Schjetlein et al 1997, Sucak et al 2006). One study found no difference between SF levels in non-carriers with SP and women with normal pregnancies (Schjetlein et al 1997), while another study found higher levels in the former group (Östlund et al 1998). However, even when differences were significant in these studies, there were always substantial overlaps between the levels in women with normal pregnancies and in those with placenta-mediated complications. Furthermore, the tests were performed in women already diagnosed with the respective complication.
The results of this study may be explained by the fact that the DIC was low-grade. Studies have shown increased marker levels in manifest DIC (Dempfle et al 2004, Kontny et al 1999).

F1+2, SF and D-dimer in FVL carriers with VTE during pregnancy and the puerperium (Paper IV)
The women who contracted VTE had markers at the same levels as those without complications. In another study, D-dimer was analysed every two to three weeks during pregnancy in women with increased risk of thrombosis. They were subdivided into a low-risk, a high-risk and a thrombosis group. No differences were found between the low-risk and high-risk groups but levels were higher in women with VTE in the current pregnancy (Bombeli et al 2001).

CONCLUSIONS

During normal pregnancy in women who do not carry the FVL mutation, the levels of FVIII, fibrinogen, PAI-1 and PAI-2, Fl+2, SF and D-dimer increased and the free protein S levels and t-PA activity decreased as pregnancy progressed. There was no change in protein C. These results may serve as a reference database, useful for evaluation of haemostasis disorders during pregnancy (Paper I).

Sonoclot analysis indicated a state of hypercoagulability during pregnancy. We found the Sonoclot® analyser simple to handle and the signatures easy to interpret. The same intervals for the Sonoclot variables can be used throughout pregnancy (Paper II).

The prevalence of FVL carriership was 8.1 % (Paper III).

Carriership of the FVL mutation did not influence gestational age at delivery, the incidence of gestational hypertension or preeclampsia or intrauterine growth. Adverse outcomes such as HELLP syndrome, neonatal asphyxia, eclampsia, placental abruption, late miscarriage, IUFD and intrapartum death were rare events. The incidence of blood loss at delivery exceeding 1000 ml was significantly lower in the carriers. There were three VTEs among FVL carriers and none among the controls. Thromboprophylaxis may have influenced the prevalence of VTE. Genotyping for the FVL allele in healthy pregnant women without heredity for VTE is doubtful, nor can genotyping be justified in women with obstetric complications (Paper III).

If a test is to serve as a clinically useful predictor, the overlap between levels in healthy and sick individuals should be minor. The results of our study do not support the assumption that F1+2, SF and D-dimer are useful in prediction of placenta-mediated complications or VTE in FVL carriers (Paper IV).

SAMMANFATTNING

Bakgrund

Under graviditet förändras hemostasen i riktning mot hyperkoagulabilitet. Detta medför att blödningsrisken vid förlossning minskar, men medför också en ökad risk att insjukna i djup venös trombos eller lungemboli, det vill säga i venös tromboembolism (VTE). Vid ärftlig resistens mot aktiverat protein C (APC), som beror på en mutation (FVL) i genen för faktor V, ökar denna risk ytterligare. Denna trombofili upptäcktes och beskrevs 1993 och är vanligt förekommande bland kaukasier. Den kan påvisas genom att beräkna kvoten av APTT med och utan tillsats av APC (klassisk APC kvot). Genom att tillsätta FV-brist plasma kan analysresultatet göras oberoende av koncentrationen av övriga koagulationsfaktorer (modifierad APC kvot). Den kan också fastställas genom genotypning med polymeraskedjereaktion.
Under graviditet och förlossning kan stora blödningar uppstå, dels beroende på komplikationer såsom placentaavlossning, förlossningsskador eller uterusatoni, men också beroende på akuta hemostasrubbningar såsom disseminerad intravasal koagulation (DIC) eller trombocytopeni. För att kunna normalisera hemostasen, genom att kirurgiskt åtgärda blödningar eller korrigera blodkoagulationen, krävs snabba och säkra analysmetoder. Medan konventionella laboratorieanalyser är tidskrävande ger Sonoclotanalys inom femton minuter en uppskattning av de olika stegen i hemostasen. Dock saknas erfarenhet av och referensintervall för Sonoclotvariabler vid graviditet.
Vid placentamedierade komplikationer som preeklampsi, intrauterin tillväxthämning, placentaavlossning, vissa missfall och intrauterin fosterdöd ses trombotiska förändringar i moderkakan. Studier har visat motsägande resultat angående sambandet mellan FVL och dessa komplikationer.
Vid aktivering av hemostasen ökar koncentrationen av markörer för aktivering av blodkoagulation och fibrinolys. Att analysera dessa har visat sig värdefullt vid diagnostik av DIC och VTE men kunskap saknas om huruvida höjda nivåer kan förebåda placentamedierade komplikationer eller VTE.

Frågeställningar

Hur stor är prevalensen av APC resistens i våra geografiska områden (Göteborg och Stockholm)?
Kan modifierad APC kvot användas under graviditet för att påvisa bärarskap av FVL?
Är Sonoclotanalysen användbar som en snabb bed-side metod för att identifiera hemostasrubbningar?
Påverkas incidensen av tromboemboliska eller placentamedierade komplikationer av bärarskap av FVL?
Är koagulationen mer aktiverad under graviditet hos bärare av FVL än hos icke-bärare?
Kan man genom att mäta markörer för aktivering av koagulation och fibrinolys bedöma risken för tromboemboliska eller placentamedierade komplikationer, för att

underlätta ställningstagande till profylaktisk behandling?

Metodik

Hemostasvariabler mättes hos friska kvinnor med normal graviditet, vilka var icke-bärare av FVL-mutationen. 48 kvinnor provtogs sammanlagt fyra gånger under graviditeten, inom en vecka efter förlossning samt åtta veckor efter förlossning. Följande analyser utfördes: klassisk och modifierad APC kvot, markörer för aktivering av koagulation och fibrinolys (protrombinfragment 1+2 (F1+2), lösligt fibrin (SF) och D-dimer) samt faktor VIII, fibrinogen, protein C, protein S, plasminaktivator inhibitorer 1 och 2 (PAI-1, PAI-2) och vävnadsplasminaktivator (t-PA) (Studie I).
Sonoclotanalys utfördes på samma kvinnor som i första studien, tre gånger under graviditet samt åtta veckor efter förlossning, för att få en totalbild av koagulationen under graviditet och puerperium (Studie II).
5 986 gravida kvinnor i Stockholm och Göteborg provtogs för bärarskap av FVL. 500 bärare följdes under graviditet och puerperium. Var femte icke-bärare användes som kontroll. Bärare jämfördes med kontroller avseende incidens av placentamedierade komplikationer och VTE (Studie III).
Bärare av FVL provtogs sammanlagt tre gånger under graviditeten och tre gånger efter förlossning för analys av F1+2, SF och D-dimer. Nivåerna av dessa markörer hos bärarna med komplikationsfri graviditet jämfördes med nivåerna hos dem som hade komplikationer. Dessutom studerades förändringar över tid under graviditet och efter förlossning. Jämförelse gjordes även mellan bärare och icke-bärare (Studie IV).

Resultat

Klassisk APC kvot visar på APC resistens hos icke-bärare i sådan utsträckning att metoden inte kan användas för bestämning av hereditär APC resistens under graviditet. Modifierad APC kvot låg dock över referensnivån under hela graviditeten. Ökning av F1+2, SF och D-dimer indikerade aktivering av koagulationen under normal graviditet. Faktor VIII, fibrinogen, PAI-1 och PAI-2 ökade, protein S och t-PA minskade medan protein C var oförändrat (Studie I).
Sonoclotanalysen var lätt att utföra och tolka. Sonoclotanalys indikerade hyperkoagulabilitet under graviditet (Studie II).
Prevalensen av heterozygot and homozygot FVL-bärarskap var 7,9 resp. 0,26 %. Det var ingen skillnad i incidenser av graviditetshypertoni, lätt och svår preeklampsi, eklampsi, placentaavlossning, intrauterin fosterdöd, oförklarligt missfall eller avvikelse i födelsevikt mellan bärare och icke-bärare. Bärare hade mer sällan blodförlust överstigande 1000 ml vid förlossning. Bärare hade troligen ökad risk för VTE (Studie III).
F1+2 och D-dimer steg successivt under graviditeten men SF låg inom referensområdet för icke- gravida. De kvinnor som utvecklade komplikationer, var homozygota för FVL eller hade annan trombofili utöver FVL hade ej högre markörnivåer än heterozygota kvinnor utan komplikationer. SF och D-dimer var positivt korrelerade under graviditet men ej åtta veckor efter förlossning (Studie IV).

Slutsatser

Hemostasen är aktiverad under graviditet.
Modifierad APC kvot kan användas för att diagnostisera FVL under graviditet.
Samma intervall för Sonoclotvariabler kan användas under hela graviditeten.
Bärare av FVL har troligen högre incidens av VTE men ej av placentamedierade komplikationer.
F1+2, SF eller D-dimer kan ej användas för att förutsäga tromboemboliska eller placentamedierade komplikationer.

Acknowledgements

I wish to express my gratitude to all the women who participated in our studies and to everyone else who has contributed to this thesis in different ways.

I would like particularly to thank:
Associate Professor **Margareta Hellgren**, my supervisor, colleague and friend, for generously sharing her great knowledge of research and for introducing me to the exciting field of haemostasis.

My co-authors in the studies: **Nils-Erik Andersson**, Master of Science and Engineering; **Steffen Rosén**, PhD, Haemochrome; Associate Professor **Lilian Tengborn**, the Department of Haematology, Malmö University Hospital; Associate Professor **Katarina Bremme** and Dr **Marianne van Rooijen**, PhD, the Department of Woman and Child Health, Karolinska Institutet.

Associate Professor **Margareta Wennergren**, Head of the Department of Obstetrics, Sahlgrenska University Hospital, my colleague and friend, for encouragement and positive support.

The midwives at the antenatal care units for enrolling women in the study.

The research midwives at Kvinnohälsan, Karolinska Hospital and the midwives at the Departments of Obstetrics, for technical assistance.

Professor **Göran Larson**; Dr **Marianne Dahlberg**; **Susanne Samuelsson**, laboratory engineer, and **Barbro Christiansson**, laboratory engineer, the Department of Clinical Chemistry, Sahlgrenska University Hospital; **Lena Weikum**, laboratory engineer, Chromogenix AB, for expert technical assistance, and Associate Professor **Andreas Hillary**, the Department of Clinical Chemistry, Lund University, for laboratory supervision.

Bo Wibergh, Sjukhus Teknik AB, Färgelanda, for placing a Sonoclot® analyser at our disposal.

Professor **Sture Holm**, Biostatistic, Göteborg University, for assistance with the statistical calculations.

Dr **Joy Ellis**, for excellent language review and for the transformation of my Swenglish to a higher level of linguistic perfection.

My dear husband **Bengt**, for support with hardware- and software-related issues and for preventing many nervous breakdowns.

My dear family **Bengt, Lisa, Johan, Olive**r and **Oskar,** for providing me with perspective on what is really important in life.

Disclosure of Interest

I declare that I have no conflict of interest. The sponsors did not influence the design of the studies or the interpretation of results.

Contribution to Authorship

All co-authors have been involved in planning the studies, enrolling patients, reading their records, evaluating the results and writing the papers.

Funding

The studies were supported by grants from the Västra Götaland Region, the Göteborg Medical Society, Lind's Foundation and Chromogenix AB, which is gratefully acknowledged.

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