Hemostatic risk factors for pregnancy-related venous thrombosis

Astrid Bergrem, MD

Department of Haematology, Oslo University Hospital, Oslo, Norway

Institute of Clinical Medicine, University of Oslo, Norway

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Selected Abbreviations

a – activated

aCL – anticardiolipin antibody

APA – antiphospholipid antibody

aPC – activated protein C

AT – antithrombin

ß2GP1 – beta-2-glycoprotein 1

CAT – calibrated automated thrombography

CI – confidence interval

DVT – deep vein thrombosis

ETP – endogenous thrombin potential

F – factor

F5 rs6025 – factor V Leiden polymorphism

F2 rs1799963 – prothrombin gene G20120A polymorphism

LA – lupus anticoagulant

OR – odds ratio

PC – protein C

PE – pulmonary embolism

PNP – pooled normal plasma

PS – protein S

TF – tissue factor

TFPI – tissue factor pathway inhibitor

VT – venous thrombosis

Summary

Background: Limited data exist on thrombophilia and the risk of pregnancy-related venous thrombosis (VT), and there are no information on differential risk factors for pregnancy-related pulmonary embolism (PE) and deep vein thrombosis (DVT).

Objectives: To investigate whether hemostatic parameters known to be risk factors for VT in the general population were associated with pregnancy-related VT and whether these risk factors were differentially associated with the risk of PE and DVT.

Methods: Blood samples were collected from a total of 313 cases with objectively verified first time VT and 353 controls with no history of VT originating from a source population of 613,232 pregnancies.

Results: Factor (f)VIII, normalized endogenous thrombin potential (n-ETP) and D-dimer values >90th percentiles were independent risk factors for pregnancy-related VT; adjusted ORs (aORs) 1.7 (95% confidence interval (CI) 1.1-2.8), 1.8 (95% CI 1.1-3.0) and 2.1 (95% CI 1.3-3.3), respectively. Reduced sensitivity to activated protein C (aPC), expressed as elevated normalized aPC sensitivity ratio (n-aPC-sr), was a risk factor for pregnancy-related VT in non-carriers of F5 rs6025 polymorphism (commonly known as factor V Leiden), aORs for VT for n-aPC-sr in the 4th quartile as compared with n-aPC-sr ≤ the 4th quartile was 2.6 (95% CI 1.7-4.0). Analysing only carriers of factor V Leiden the risk of VT increased, although not statistical significant, with higher n-aPC-sr, indicating a relation between an aPC resistance phenotype and risk of VT. The risk for PE as compared with controls was more than doubled in women with fIX >90th percentile; aOR 2.4 (95% CI 1.1-5.0) and three times increased in women with free protein S (PS) antigen ≤ 65%; aOR 3.1 (95% CI 1.3-7.2). Carriers of factor V Leiden, and non-carriers of factor V Leiden with n-aPC-sr in the 4th quartile had increased risk of DVT as compared with controls; OR 7.7 (95% CI 4.7-12.7) and aOR 3.3 (95% CI 2.1-5.2), respectively. 9 cases and none controls were positive for at least two antiphosholipid antibodies (APAs) (multi-positive). Excluding women with IgM antibodies, 7 cases were still APA multi-positive.

Conclusions: Elevated levels of fVIII, n-ETP, D-dimer and reduced sensitivity to aPC in absence of factor V Leiden were independent risk factors for pregnancy-related VT. APA multi-positivity was strongly associated with pregnancy-related VT. High levels of

fIX and low levels of free PS antigen was associated with increased risk of PE, and the risk of DVT was increased in women with reduced sensitivity to aPC in absence of factor V Leiden, and in carriers of factor V Leiden. Our data substantiate the hypothesis of differential pathophysiology between DVT and PE.

List of papers

Paper I

Bergrem A, Dahm AE, Jacobsen AF, Sandvik L, Sandset PM. Differential risk factors for pregnancy related deep vein thrombosis and pulmonary embolism - A population-based case-control study. ***Thrombosis and Hemostasis*** 2012; 108: 1165-71.

Paper II

Bergrem A, Dahm AE, Jacobsen AF, Mowinckel MC, Sandvik L, Sandset PM. Resistance to activated protein C is a risk factor for pregnancy-related venous thrombosis in the absence of the F5 rs6025 (factor V Leiden) polymorphism. ***British Journal of Haematology*** 2011; 157: 241-47.

Paper III

Bergrem A, Jacobsen EM, Skjeldestad FE, Jacobsen AF, Skogstad M, Sandset PM. The association of antiphospholipid antibodies with pregnancy-related first time venous thrombosis – a population-based case-control study. ***Thrombosis Research*** 2010; 125: e222-7.

1. Introduction

1.1. Hemostasis and coagulation

The hemostatic system maintains blood in a fluid state under normal conditions, but responds to vessel injury by rapid formation of a clot. Hemostasis is commonly described as *primary hemostasis* and *secondary hemostasis.* Primary hemostasis describes the process which begins immediately after vascular damage, and is characterized by vasoconstriction, and platelet activation, adhesion and aggregation, and culminates in the formation of a platelet plug. Secondary hemostasis concerns blood coagulation, thrombin generation, fibrin clot formation and fibrin dissolution.

In the early days, studying plasmas from patients with bleeding disorders was of vital importance for understanding the coagulation system. In 1947 Paul Owren, a Norwegian physician and scientist, described factor V deficiency[1]. In 1964 the "waterfall" or a "cascade" model of coagulation was proposed by two groups[2;3]. According to this model coagulation proceeded in a series of proteolytic reactions. Furthermore, the coagulation cascade was divided into two separate pathways; the *intrinsic* (contact) and the *extrinsic* (or tissue factor - TF) pathways, both converting into the common pathway (Figure 1).

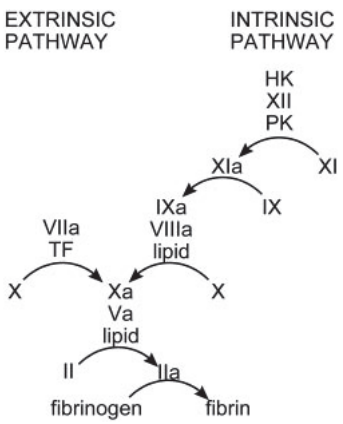

Figure 1. The *extrinsic* and *intrinsic* pathways as originally proposed by Macfarlane[3] and Davie and Ratnoff[4] in 1964.

In 1977 Osterud and Rapaport showed that the coagulation factor (f) VII(a)/TF complex beyond activation of fX also activated fIX[5]. These data indicated that the intrinsic and extrinsic pathways were linked rather than separated from each other. The functionality of the extrinsic and the intrinsic pathways can be tested by two simple and routine laboratory coagulation tests, i.e., the prothrombin time (PT) and the activated partial thromboplastin (aPTT) assays, respectively (the tests will be further discussed under section 1.3). The terminology "intrinsic" and "extrinsic" pathways are convenient to use when discussing laboratory assays and which coagulation factors they measure. However, the coagulation cascade model does not always correspond well with clinical observations, e.g., why cannot the activation of fX by the extrinsic pathway compensate for a lack of fVIII or fIX in hemophiliacs?

Today it is widely accepted that the intrinsic and the extrinsic pathways are two overlapping phases (Figure 2) that can be recognized in the kinetics of thrombin generation, the so-called initiation and propagation phases.

1.1.2. Initiation and propagation of coagulation

The clotting cascade can be triggered either via the extrinsic or TF pathway or the contact pathway (Figure 2 and Figure 3). The contact pathway of coagulation is initiated by the activation of fXII bound to an anionic surface in a reaction involving high molecular weight kininogen and plasma kallikrein. Recent studies have reported that inorganic polyphosphate (polyP), mostly released from platelets, binds to and activates fXII and can thereby directly trigger the contact pathway[6]. Initiation of coagulation by the contact pathway is not essential for hemostasis as people lacking fXII have no bleeding tendency[7], initiation by the TF pathway is, on the other hand, required for proper hemostasis[8].

TF is a transmembrane protein primarily expressed in extravascular tissues and coagulation is normally initiated when TF exposed to circulating blood binds to fVIIa. The fVIIa/TF (*extrinsic tenase*) complex then converts zymogens fIX and fX to their active enzymatic forms. FXa provides rapid feedback activation of fVII bound to TF, and converts small amounts of prothrombin (fII) to thrombin (fIIa). The small amounts of thrombin produced activates platelets[9], and activates tiny amounts of plasma cofactors V and VIII to active cofactors fVa and fVIIIa[10], and begins to cleave fibrinogen to fibrin.

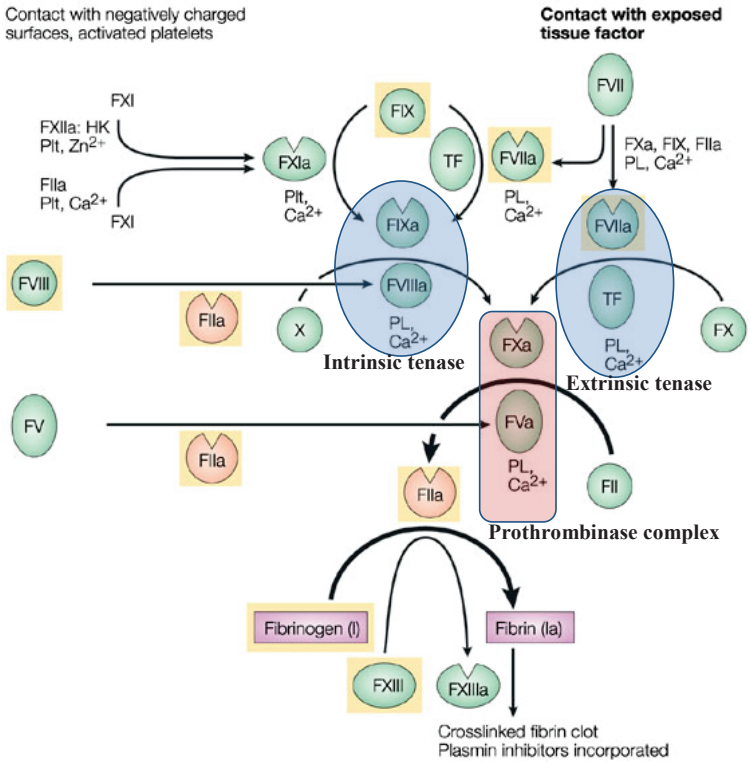

Figure 2. Illustration of the "modern" coagulation system with its overlapping phases. Thrombin (FIIa) is highlighted in red. Arrows indicate activation. Whole circles: proenzyms. Circles with a nick: activated coagulation factors. F−factor; a−activated; HK−high-molecular-mass kininogen; Plt−platelets; PL−phospholipids. Reprinted from Bishop and Lawson[11], with permission.

Thrombin generation also leads to activation of fXIII, which is responsible for covalent cross-linking of fibrin[12]. These initial catalytic events provide the components needed for the formation of the *intrinsic tenase complex* comprising cofactor VIIIa and fIXa, and the *prothrombinase complex* comprising cofactor Va and fXa (Figure 3).

Vigorous thrombin generation occurs during the propagation phase when fIXa generated by the fVIIa/TF complex combines with cofactor VIIIa on the activated platelet surface to form the intrinsic tenase complex, which becomes the major activator of fX. The assembly of fX, fIXa, cofactor VIIIa and Ca^{2+} on phospholipid membranes enhance the activation of fX several orders of magnitude[13;14]. Consequently, the fXa generated by the

intrinsic tenase complex supersedes the activation of fX by fVIIa/TF. The prothrombinase complex is formed by the assembly of activated fX and cofactor Va and Ca^{2+} on activated platelet membranes, and this complex accelerates the conversion of prothrombin to thrombin several hundred thousand times[15]. The tight assemblies of coagulation factors, cofactors and $Ca2^{+}$ on phospholipid membranes make very efficient catalytic complexes, resulting in a boost of thrombin formation.

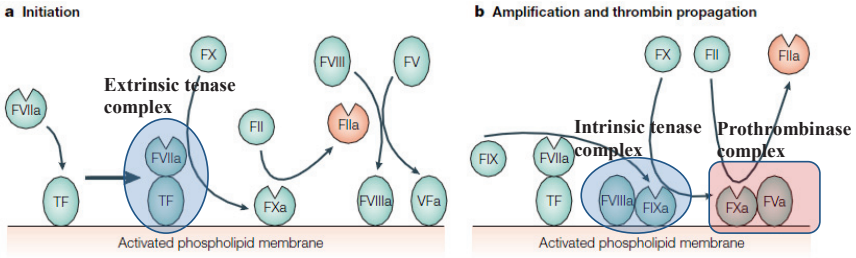

Figure 3. The primary event that initiates coagulation (**a**) and propagation of thrombin generation (**b**) via assembly of the tenase and the prothrombinase complexes. Thrombin (FIIa) is highlighted in red. Arrows indicate activation. Whole circles: proenzymes. Circles with a nick: activated coagulation factors. F-factor; a-activated; From Bishop and Lawson[11], with permission.

The major bolus of thrombin (>95%) is produced during the propagation phase after formation of the fibrin clot. Increased thrombin formation is necessary for the activation of thrombin activatable fibrinolysis inhibitor (TAFI), which is an inhibitor of fibrinolysis[16]. Fibrinolysis will be further discussed below. The major bolus of thrombin formation occurs within the fibrin mesh and it is believed that this last "bulk" of thrombin and the activation of TAFI are important to protect the clot from premature lysis[17].

In the absence of cofactor VIII or fIX, the intrinsic tenase complex cannot be assembled, and no amplification of fX activation occurs. This is the principal defect observed in hemophiliacs. The blood of individuals with either severe hemophilia A or hemophilia B has no ability to generate the intrinsic tenase complex and hence is unable to support the propagation phase of coagulation[18].

1.1.3. Fibrinolysis

A delicate balance between fibrin formation and fibrin dissolution is required to maintain adequate hemostasis and to prevent thrombus formation. Excess fibrin is removed by the fibrinolytic system. Plasminogen is the inactive precursor of the enzyme plasmin, which is the primary catalyst of fibrin degradation. Plasminogen is activated by tissue-plasminogen activator (t-PA) in the presence of fibrin[19] or by factors of the contact pathway[20]. The proteins involved in fibrinolysis are recruited to the site of injury simultaneously with initiation of fibrin formation, i.e., the fibrin clot serves as a substrate and a surface for activation and localization of fibrinolytic proteins. The natural inhibitors of the fibrinolytic system are; TAFI, plasminogen activator inhibitor type 1 (PAI-1) and type 2 (PAI-2), and α2-antiplasmin. The net result of ongoing coagulation and fibrinolysis is the formation of fibrin degradation products, such as D-dimer, which is specific for fibrin degradation. Overall reduced fibrinolysis is associated with increased risk for VT[21].

1.1.4. Anticoagulant pathways

Regulation of coagulation is exerted at each level of the coagulation pathway, either by enzyme inhibition or by modulating the activity of the cofactors. TF pathway inhibitor (TFPI), a glycoprotein mainly produced by vascular endothelium, and which exists in different forms in circulating blood, inhibits the fVIIa/TF catalytic complex in a fXa dependent manner. TFPI forms a complex with fXa and the TFPI/fXa complex then binds with high affinity to the fVIIa/TF complex, resulting in a fully inhibited quaternary TFPI/fXa/fVIIa/TF complex[22].

Antithrombin (AT) binds to and irreversibly neutralizes the coagulation enzymes generated during activation of coagulation. The activity of AT is greatly enhanced by glycosaminoglycans that are present on the surface of endothelial cells. The effect on AT is the molecular basis for the use of heparin as a therapeutic anticoagulant[23].

Protein C (PC) is a vitamin K-dependent zymogen, and is the key component of the PC anticoagulant pathway. Thrombin bound to the membrane protein thrombomodulin on intact endothelial cells activates PC. Protein S (PS) is a vitamin K-dependent cofactor protein that enhances the anticoagulant activity of activated PC (aPC). The accelerated activation of prothrombin (prothrombinase complex) and fX (tenase complex) are dependent on cofactors Va and VIIIa, respectively, and the activity of these two

cofactors is tightly regulated by the PC anticoagulant pathway. APC and PS form a membrane bound complex, which cleaves cofactors Va and VIIIa, even when they are assembled in the prothrombinase or tenase complexes. APC also cleaves intact cofactor V, resulting in anticoagulant cofactor V that functions in synergy with PS as cofactors for aPC in the degradation of cofactor VIIIa[24]. Recently, it has been shown that PS also has cofactor activity for TFPI and enhances the interaction between TFPI and fXa and thereby accelerates the feedback inhibition of fVIIa/TF by TFPI[25]. The PC and the TFPI pathways act in concert in down-regulating coagulation[26].

1.2. Reduced sensitivity to activated protein C

Functional defects of the PC pathway are quite common[27] and can be detected *in vitro* by aPTT or thrombin generation based assays (section 1.3) which probe the anticoagulant response of plasma to the addition of aPC. A poor anticoagulant response of plasma to exogenous aPC is known as aPC resistance[28]. The majority of people with an inherited cause of aPC resistance carry the F5 rs6025 polymorphism[29]. Because of a single nucleotide change in the fV gene, arginin is replaced by glutamin at the main aPC cleavage site in cofactor V and cofactor Va. Consequently, F5 rs6025 interferes with inactivation of both cofactor Va and cofactor VIIIa[27], resulting in an aPC resistant phenotype and increased risk of VT[30].

Given the complexity of the PC pathway, there are various possibilities of aPC resistance. Particularly, quantitative and qualitative alterations of the substrates and cofactors of aPC, as well as of the components of the tenase and the prothrombinase complexes, may influence the plasma response to aPC by affecting the rates of cofactor Va and/or cofactor VIIIa inactivation. Because the aPTT-based and the thrombin generation-based assays rely on different coagulation triggers and end-points, the assays probe different coagulation reactions and are sensitive to different plasma factors. The effect of cofactor VIII levels is specific for aPC resistance in the aPTT-based assay, whereas low levels of PS and/or TFPI are major determinants of aPC resistance in TF-triggered thrombin generation-based assays[31].

Several acquired risk factors for VT such as pregnancy[32], use of oral contraceptives[33], hormone replacement therapy[34] and canser[35] are associated with reduced sensitivity to aPC. During pregnancy[36], and use of oral contraceptives[37], the levels of PS and/or TFPI

decrease and since these proteins are major determinants of the thrombin generation-based assay but not the aPTT-based assay, hormone-induced aPC-resistance is best detected by the thrombin generation-based assay[38].

Of notice, from here and throughout the thesis, cofactors V and VIII are referred to as fV and fVIII.

1.3. Coagulation assays

1.3.1. Activated partial thromboplastin time

APTT measures the activity of the intrinsic and common pathways of coagulation. The term "partial" means there is phospholipids present but no TF. An activator, such as kaolin, silica, or ellagic acid, is added, and clotting is initiated when calcium is added to the solution. The aPTT is the time taken from the addition of calcium to the formation of a fibrin clot. Our laboratory uses an automated method for measuring aPTT and clot formation is considered to have occurred when the optical density of the mixture has reached a certain threshold. This threshold varies between laboratories.

1.3.2. Thrombin generation assay

While the end point of aPTT is the formation of a fibrin clot, which occurs when less than 5% of the total amount of thrombin is generated, the thrombin generation assay measures thrombin generation as a function of time, yielding a thrombin generation curve (*thrombogram*), which reflects all phases of coagulation. There are several methods to measure thrombin generation. We used the calibrated automated thrombography (CAT) assay[39], which is performed in a microtitre plate. Thrombin activity in plasma is continuously registered by fluorescence readings which are automatically converted into thrombograms by dedicated software. Figure 4 shows a typical thrombogram and the many parameters that can be derived from the thrombin generation curve.

The lag time is arbitrarily defined as the moment when 10 nM thrombin is formed and shows a good correlation to plasma clotting time measured by aPTT. The endogenous thrombin potential (ETP) is the area under the curve and represents the total amount of thrombin generated during the test. Of the parameters derived from the thrombogram, ETP is considered the most predictive parameter of thrombosis[39-41].

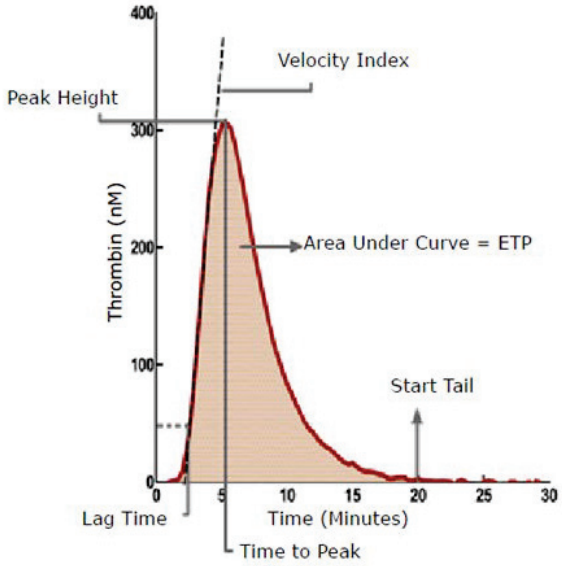

Figure 4. Thrombogram by the Calibrated Automated Thrombography assay. From The Calibrated Automated Thrombogram®, www.thrombinoscope.com

The assay outcome is dependent on the experimental conditions used (so-called assay determinants) as they influence which pro-and anticoagulant pathways contribute to the shape of the thrombogram. The TF concentration used to trigger coagulation and addition of aPC to the assay are such determinants. The thrombin generation assays are also influenced by biological variables such as age[42], pregnancy[43] and use of combined oral contraceptives[44], all of which affect the levels of certain coagulation factors and inhibitors. Furthermore, the thrombogram may be hampered by preanalytical variables such as blood collection and plasma preparation[45].

Normalization

The term "normalization" has a range of meanings. When we use the term "normalized" for a laboratory result, it refers to a simple calculation where the result from test plasma has been divided by the result obtained with reference plasma (Figure 5). Normalization of laboratory test results has become a valid method to increase reproducibility and

improve inter-center variability of results[46-48]. We used pooled normal plasma (PNP) (described in section 3.3.) as "normal plasma".

A.

$$\text{aPC sensitivity ratio} = \frac{\text{ETP with aPC}}{\text{ETP without aPC}}$$

B.

$$\text{Normalized aPC sensitivity ratio} = \frac{\text{ETP with aPC / ETP without aPC Test plasma}}{\text{ETP with aPC / ETP without aPC Normal plasma}}$$

Figure 5. A. Activated protein C (aPC) sensitivity ratio. **B**. Normalized aPC sensitivity ratio. ETP − endogenous thrombin potential.

1.4. Antiphospholipid antibodies

Antiphospholipid antibodies (APAs) comprise a heterogeneous group of antibodies and the major target antigen is beta-2-glycoprotein 1 (β2GP1)[49;50]. As illustrated by Figure 6, β2GP1 circulates in plasma in a circular conformation. When β2GP1 binds to a phospholipid surface, β2GP1 undergoes a conformational change and uncovers epitopes which are recognized by a substantial proportion of anti-β2GP1 antibodies. These antibodies are both cardiolipin-positive and have lupus anticoagulant activity[51;52]. Binding of the epitope by the antibody fixes β2GP1 in the "new" unfolded conformation. The antibody- β2GP1 complex can then interact with several surface receptors. APAs are thought to induce thrombosis by interfering with pro-coagulant, anticoagulant and fibrinolytic systems[53].

Diagnosing the antiphospholipid syndrome (APS) requires at least one positive laboratory test for APAs (lupus anticoagulant (LA), anticardiolipin antibodies (aCL) or β2GP-1 antibodies) performed on two occasions at least 12 weeks apart, and the presence of thrombosis and/or pregnancy morbidity i.e. unexplained death(s) of a fetus at or beyond the 10th week of gestation, premature birth(s) before the 34th week of gestation because of eclampsia or severe preeclampsia, or recurrent spontaneous abortions before the 10th week of gestation[54].

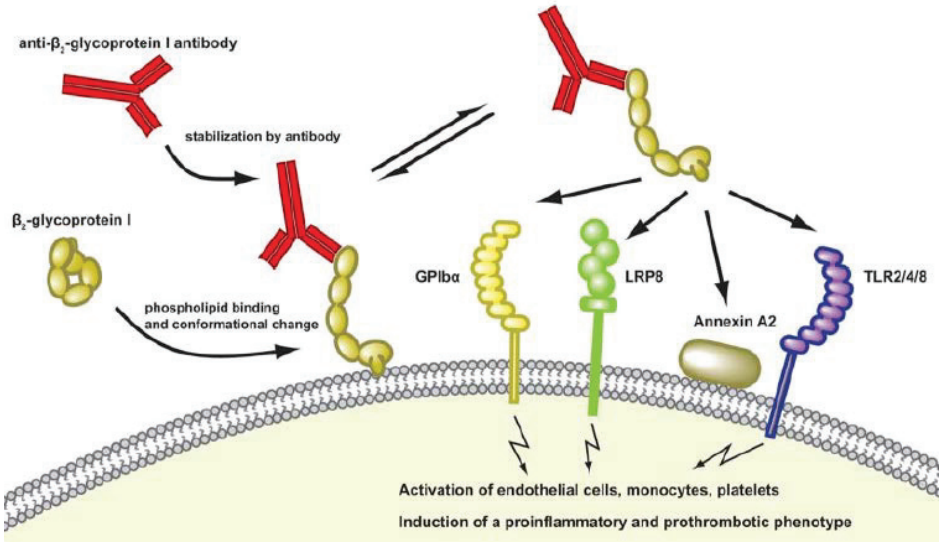

Figure 6. Sequence of events leading to cellular activation by beta-2-glycoprotein 1 (β2GP1) antibody complexes. From de Groot and Urbanus[55], with permission.

1.4.1. Detection of antiphospholipid antibodies

APAs are detected by functional assays and by enzyme-linked immunosorbent assays (ELISAs).

Functional assays for lupus anticoagulants

LAs are a heterogeneous group of antibodies which prolong phospholipid-dependent coagulation assays, among others; aPTT and the Russell's viper venom time (RVVT). The Russell's viper venom isolated from the snake Daboia russelii contains a potent activator of fX, which in the presence of phospholipids, prothrombin and calcium activates fibrinogen to fibrin. To optimize the sensitivity to LAs, Russell's viper venom is diluted to give a certain clotting time in normal plasma.

Detection of LAs includes three steps: screening, mixing and confirmation tests. The screening tests are most often LAs sensitive aPTT (low phospholipid concentration) or dilute RVVT (dRVVT) tests. The preparation of platelet-poor plasma is important as platelets provide phospholipids and residual platelets in plasma can cause false negative tests.

The second step involves mixing test sample with normal plasma, usually 1:1, before the test is repeated. This step is to differentiate between inhibitors of coagulation and coagulation factor deficiencies as cause for prolonged clotting times. The final confirmatory step demonstrates the phospholipid dependency of the antibody as the clotting time is normalized in the presence of excess phospholipids. The confirmation test must be performed with the same assay principle that was employed in the positive screening test[56]. In our laboratory, the principles of these three assays are integrated into one assay and the results are normalized against results obtained from PNP run in parallel.

Enzyme-linked immunosorbant assay (ELISA)

ACL antibody ELISA detects antibodies directed against β2GP1 dependent aCL, whereas β2GP1 ELISAs measure only antibodies against β2GP1. The antibody isotypes of interest for the diagnosis of APS are IgG and IgM present at a certain titre.

The epitopes uncovered by the β2GP1conformational change are recognized by anti-β2GP1 antibodies which are both aCL-positive and have LA activity[51;52]This means, that the same antibody might be recognized by the three different assay systems (the coagulation assays detecting LAs and the ELISA assays detecting aCL and β2GP1). Antibodies recognized by all three assay systems have the strongest association with VT[57].

1.5. Venous thrombosis

The overall incidence rate of a first event VT in the adult population is approximately 1.4 per 1000 person years[58]. The incidence of VT is strongly age dependent, the incidence rate being three times higher for persons aged 70 years or above than those aged 45-69 years, which again is three times higher the rates in persons aged 20-44 years[58]. The most common presentations of VT are DVT of the lower limbs and PE. Sometimes VT may occur in other deep veins, such as in the upper limbs, in the mesenteric veins including the portal vein, in the liver vein, and in cerebral sinuses. Two important sequalae may arise after DVT or PE; post thrombotic syndrome (PTS) and chronic thromboembolic pulmonary hypertension (CTPH). PTS occurs in 20-50% of VT patients and can result in chronic calf swelling which may lead to skin ulcer, sustained

itching or pain in the affected leg[59]. CTPH is defined as a mean artery pressure above 25 mm Hg that persists for >6 months after a diagnosis of PE. The disorder occurs in 2-4% of patients after acute PE and results in disabling dyspnea[60].

In 1856 Dr. Virchow postulated that thrombosis was due to stasis of the blood, changes in the vessel wall, or changes in the composition of the blood, now commonly known as the Virchow's triade[61]. Genetic risk factors for VT lead to hypercoagulability (changes in the composition of the blood), whereas the acquired causes are either associated with decreased flow (stasis) or related to hypercoagulability (Table 1). Increased risk of VT due to hypercoagulability is commonly referred to as thrombophilia. Thrombophilia are detectable in more than 50% of patients with a first unprovoked VT[62]. Contrary to arterial thrombosis, where structural changes of the vessel wall play a key role, much less is known about the role of the vessel wall in non-traumatic VT. Normally, endothelium has anticoagulant properties, whereas in response to variation in blood flow and/or oxygenation the endothelium can convert to a pro-coagulant phenotype[63].

1.5.1 Inherited and acquired risk factors for venous thrombosis

VT is a multicausal disease, i.e., several risk factors need to be present simultaneously to cause thrombosis. As illustrated by Figure 7, an individual's thrombotic potential may be described as being dynamic age-dependent with inherited and acquired risk factors increasing the risk for VT in an additive or synergistic fashions[64].

The strongest risk factor for VT is being affected by a previous VT. After 5 years of follow-up a recurrence rate of approximately 25% have been reported[65]. Age is another strong risk factor for VT, illustrated by Figure 7. The risk of VT increases approximately 50 times from 20 to 70 years of age. Other acquired risk factors for VT are listed in Table 1.

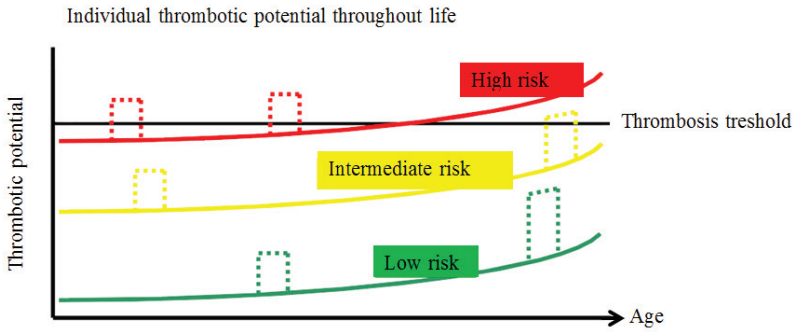

Figure 7. Each solid line (red, yellow, green) represents an individual's thrombotic potential. The stippled boxes represent situations where the persons are disposed to increased risk of venous thrombosis, e.g. pregnancy, surgery or use of combined oral contraceptives. Modified after Rosendaal FR[64].

Genetic thrombophilia (Table 1) is commonly divided into loss-of-function and gain-of-function mutations. The loss-of-function mutations are limited to genes that encode proteins with anticoagulant properties, whereas gain-of-function mutations encode pro-coagulant proteins.

In the general population the prevalence of deficiencies of the natural anticoagulants are rare, probable due to loss of mutant alleles from the gene pool through critically ill homozygous individuals[66]. AT deficiency is found in approximately 1:2000-1:5000, and the prevalence of PC deficiency is between 1:200-1:500[67;68]. The prevalence of PS deficiency in the general population is uncertain[69]. Inherited deficiencies of these natural anticoagulants represent strong risk factors for VT with relative risks ranging from 5-50 according to the defect and the population investigated[69-73].

F5 rs6025 polymorphism, phenotypically expressed as reduced sensitivity to aPC, is the most common thrombophilia among Caucasians. In Europe the frequency of F5 rs6025 polymorphism is between 2-15% in the general population, whereas it is rare in the rest of the world[74]. In heterozygous carriers, the risk of VT is increased 3 to 8-fold, and in homozygous 50 to 80-fold[75;76]. F5 rs6025 is a weaker risk factor of VT than deficiencies of the natural anticoagulants, but being far more common, it has greater impact as a risk factor in the general population.

Another common genetic polymorphism in Caucasians is the F2 rs1799963 polymorphism (commonly known as the prothrombin gene G20210A mutation), resulting in elevated plasma levels of prothrombin. The prevalence of carriers varies between 1-4% in the general population with a higher prevalence in southern than in northern Europe[77]. Carrying this polymorphism increases the risk of VT about a 3-fold[78]. Because of the rarity of homozygous carriers of F2 rs1799963 information is uncertain regarding homozygous carriers and risk of VT[77;79].

As both the F5 rs6025 and the F2 rs1799963 polymorphisms are relatively common in the Caucasian population, their combined presentation is not rare. Family members, who are heterozygous for both polymorphisms, have six times increased risk of VT as compared with non-affected family members[80].

There is evidence that the levels of coagulation factors are regulated by genetic factors[81], and if so, familial thrombophilia may not be restricted to deficiencies in the natural anticoagulants or the F5 rs6025 or F2 rs1799963 polymorphisms. Interestingly, having a positive family history of VT (i.e., at least one 1st degree relative with VT) independently of inherited thrombophilia increases the risk of VT more than 2-fold. Moreover, individuals with VT more often have a positive family history than individuals without VT, even when known risk factors are similar. This finding suggests that other, but yet unknown genetic factor(s), may be involved[82].

Table 1. Risk factors for venous thrombosis in the general population.

Inherited	**Aquired**	**Mixed/ Unknown**
Antithrombin deficiency[70]	Older age[83]	High levels of factor VIII[84]
Protein C deficiency[70]	Previous VT[85]	High levels of factor IX[86]
Free protein S deficiency[70]	Surgery[87]	High levels of factor XI[88]
F5 rs6025 polymorphism[30]	Trauma[87]	High levels of fibrinogen[89]
F2 rs1799963 polymorphism[78]	Plaster cast[90]	High levels of TAFI[91]
	Minor injuries of the leg[92]	Low levels of TFPI[93]
	Acute medical illness[87]	Hyperhomocysteinemia[94]
	Cancer[35]	aPC resistance in absence of F5 rs6025[95]
	Immobilization[96]	
	Overweight[97]	
	Antiphospholipid antibodies[49]	
	Pregnancy[32]	
	Puerperium[32]	
	Combined oral contraceptives[33]	
	Hormonal replacement therapy[98]	
	Air travel[99]	
	Central venous catheter[100]	

1.5.2. Differential risk factors for deep vein thrombosis and pulmonary embolism

There has been a consistent reporting that DVT patients more often are carriers of F5 rs6025 polymorphism as compared with PE patients. This phenomenon is often referred to as the *factor V Leiden paradox*[101-105]. In a recent review by van Langvelde et al on differential risk factors for DVT and PE, obesity, use of combined oral contraceptives, and pregnancy and puerperium were found to increase the risk for DVT[106]. The authors concluded that the differential effects of these risk factors could be explained through reduced sensitivity to aPC in these conditions. Pulmonary inflammatory diseases and sickle cell disease increased the risk of PE. Conflicting results have been reported regarding the F2 rs1799963 polymorphism and different risk for DVT or PE. Van Langevelde et al found no difference in risk for DVT and PE in carriers and non-carriers of the F2 rs1799963 polymorphism. These observations support the comprehension that DVT and PE may be partly different diseases. Investigations of differential hemostatic risk factors for DVT and PE, besides the F5 rs6025 and F2 rs1799963 polymorphisms, have not previously been published.

1.6. Physiological changes in coagulation during pregnancy

Normal pregnancy is associated with increased concentrations of most clotting factors, decreased or unchanged concentrations of natural anticoagulants and reduced fibrinolysis (Table 2). These changes are interpreted as mainly being due to increased estrogen levels[107]. After delivery, the changes in blood coagulation and fibrinolysis reach levels seen outside pregnancy within 3-6 weeks postpartum[108].

Table 2. Physiological changes of coagulation factors, natural anticoagulants and parameters of fibrinolysis during normal pregnancy. ⇧– Increased levels; ⇩– decreased levels; ⇔– unchanged levels.

Coagulation factors	Natural anticoagulants	Fibrinolytic parameters
fibrinogen[107] ⇧	AT[109] ⇔	tPA[110] ⇩
fII[109] ⇧	PC[109] ⇔	uPA[111] ⇧
fV[107] ⇧	PS[109] ⇩	PAI-1[112] ⇧
fVII[107] ⇧	TFPI[36] ⇩	PAI-2[112] ⇧
fVIII[107] ⇧	aPC sensitivity[109] ⇩	D-dimer[113] ⇧
fIX[107] ⇧		
fXI[114] ⇩		
fXII[109] ⇧		
fXIII[109] ⇧		

1.6. 1. Venous thrombosis in pregnancy and postpartum

Virchow's triade of underlying factors in VT are all present in pregnancy. During vaginal or abdominal delivery endothelial damage to pelvic vessels can take place. Approximately 85% of pregnancy-related DVTs affect the left leg and this lateralization might be due to the compression of the left iliac vein by the right iliac artery which cross the vein on the left side only (May-Thurner syndrome)[115;116]. The hemostatic changes that occur in normal pregnancy (Table 2) steer hemostasis in a "coagulable" direction. This protects pregnant women from severe bleeding during delivery but at the same time leads to a hypercoagulable phenotype that disposes for VT[117]. The incidence of pregnancy related VT is approximately 1 per 1000 pregnancies[118], which is 5-10 times higher than would be expected for women in that age group[119], and fatal PE accounts for 1.1 deaths per 100000 deliveries[120]. In a previous study by our group, the incidence of fatal VT was found to be 0.48/100000 deliveries[118]. Although rare, VT is one of the main causes of maternal mortality in developed countries[117;121-123].

Pregnancy-related VT is equally distributed during pregnancy and within 3 months after delivery. The highest risk is found in the third trimester and the first 3 weeks postpartum (Figure 8). Furthermore, DVT is the most common presentation during pregnancy whereas PE is most common diagnosed after delivery[118].

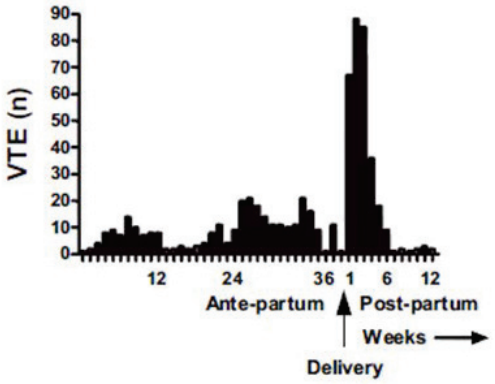

Figure 8. Distribution of VT in pregnancy and postpartum. VTE − venous thrombo-embolism. From Jacobsen et al[118], with permission.

1.6.2. Risk factors for pregnancy related venous thrombosis

Our group has previously reported on clinical risk factors for VT during pregnancy or postpartum[118]. Immobilization during pregnancy and obesity were important risk factor for both antenatal- and postnatal VT. Women who smoked cigarettes had increased risk for both antenatal and postnatal VT. Furthermore, assisted reproductive therapy and small maternal weight gain during pregnancy were risk factors for antenatal VT, whereas preeclampsia, intrauterine growth restriction and complications such as surgery, heavy bleeding and infection, associated with labor and Cesarean section were risk factors for postnatal VT.

Whether or not alterations in hemostatic factors associated with VT in the general population affect the risk of pregnancy-related VT is incompletely described. Carrying the F5 rs6025 or the F2 rs1799963 polymorphisms increases the risk for pregnancy-related VT approximately 5- and 9-fold, respectively[124-126]. Reviewing 9 studies, of which 4 studies investigated deficiencies of the natural anticoagulants and risk of VT during pregnancy or postpartum, Robertson et al concluded that deficiencies of AT, PC and PS increased the risk of pregnancy-related VT approximately 3-5 fold as compared with non-carriers of the deficiencies[127]. A possible association between the levels of fibrinogen, fVIII, fIX, and D-dimer, and the risk of pregnancy-related VT has not previously been described.

2. Aims

We hypothesized that abnormal levels of hemostatic parameters known to increase the risk of VT in the general population also increase the risk of a first time VT during pregnancy or the postpartum and furthermore, that some of these hemostatic parameters are associated with differential risk for pregnancy-related DVT and PE. Given our hypotheses, the aims for the present thesis are:

- To investigate whether high or low levels of selected hemostatic parameters increase the risk of VT during pregnancy or the postpartum (Paper I).
- To study differential risk for DVT and PE for hemostatic parameters associated with pregnancy-related VT (Paper I).
- To investigate whether reduced sensitivity to aPC in both non-carriers and carriers of the F5 rs6025 polymorphism is associated with increased risk of VT during pregnancy or the postpartum (Paper II).
- To investigate whether APAs either alone or in combination, and in relation to the F5 rs6025 and the F2 rs1799963 polymorphisms, increase the risk of pregnancy-related VT (Paper III).

3. Materials and Methods

3.1. The original VIP study-population

The study-populations defined in the registry-based and the hospital-based studies published by Jacobsen et al[118;128] are briefly described below. For the studies included in this thesis, eligible participants from the hospital-based study met for further investigations as described under section 3.2.

3.1.1. Case ascertainment

From January 1st 1990 to December 31st 2003, 1231 women with a first time VT in pregnancy or 3 months postpartum (cases) were identified in 18 hospitals localized in 11 of 19 Norwegian counties[118]. The cases were identified by search for selected international classification of diseases (ICD) codes in the Norwegian Patient Register using the ICD versions 8, 9 and 10 codes (Table 3).

In the hospital-based VIP study-population[128], eligible hospital records from the cases identified in the Norwegian Patient Register were reviewed and each case was validated for VT. The hospital-based study finally comprised 559 cases (Figure 9).

Table 3. International classification of diseases (ICD) codes for cases selection

ICD 8	
433	Cerebral vein thrombosis
450	Pulmonary embolism
452	Portal vein thrombosis
453	Venous thrombosis
671	Venous thrombosis in puerperium
673	Pulmonary embolism during pregnancy and puerperium
ICD 9	
325	Phlebitis and thrombophlebitis of intracranial venous sinuses
415.1	Pulmonary embolism
451	Venous thromboembolism
452	Portal vein thrombosis
453	Other vein thrombosis
671.3 ,4 ,5 ,9	Deep phlebothrombosis, antepartum, postpartum and other thrombosis during pregnancy
673.2 , 3	Obstetric blood clot embolism, puerperal pulmonary
ICD 10	
G 08	Phlebitis and thrombophlebitis of intracranial venous sinouses
I 26	Pulmonary embolism
I 80	Venous thromboembolism
I 82	Other venous thrombosis
O 22.3,5,8,9	Venous complications in pregnancy

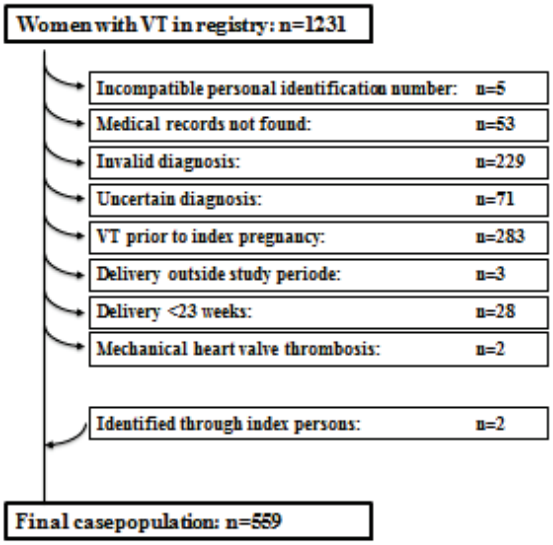

Figure 9. Flow-chart describing the selection of cases from the registry-based to the hospital-based study-population.

3.1.2. Ascertainment of the diagnosis of VT

Reviewing the hospital records, the diagnosis of DVT was accepted when it was confirmed by compression or color ultrasonography or by venography. The diagnosis of PE was accepted when objectively verified by perfusion lung scanning, computed tomography, magnetic resonance imaging or by angiography. Patients with suspected but not objectively verified VT were excluded.

3.1.3. Control selection

All pregnancies within the same area (18 hospitals) and period of time (1990-2003) were possible controls, in total 616236 pregnancies. The controls had no history of VT and were identified by the Medical Birth Registry (MBR) of Norway[118].

In the hospital-based study[128] all controls were selected from Oslo university hospital, Ullevål (formerly Ullevål University Hospital). The selection of controls was done by a software program run by the MBR of Norway. First all cases from the participating hospitals were identified and from this data file a list of cases was prepared by the

11-digit personal identification number, which is unique for all Norwegian citizens, and date of delivery. By date of delivery, MBR of Norway selected 4 women who gave birth at the same time as a case as possible controls, regardless of hospital where the case delivered. The first two controls were selected, and then the 3rd and 4th control, in that order, if records were not available.

3.2. The VIP study-population used in the studies included in this thesis

As we wanted to study hemostatic biomarkers and the risk for pregnancy-related VT, we invited eligible cases and controls from the hospital-based VIP study-population to answer a questionnaire and donate blood during the year 2006.

3.2.1. Case ascertainment

531 eligible cases were invited for blood sampling and answering a questionnaire at the hospital the VT was diagnosed. After two reminders, 316 cases agreed to participate in the study. Blood sampling failed for one case and the questionnaire revealed that two cases had been treated for previous VT. The final study population comprised 313 cases (Figure 10).

3.2.2. Control selection

1092 eligible controls from the hospital-based study population were invited to donate blood sample and answering a questionnaire at Oslo University Hospital at Ullevål (Figure 10). After two reminders 356 controls agreed to meet at Oslo University Hospital at Ullevål. As 3 controls did not complete the questionnaire the final control population comprised 353 controls (Figure 10).

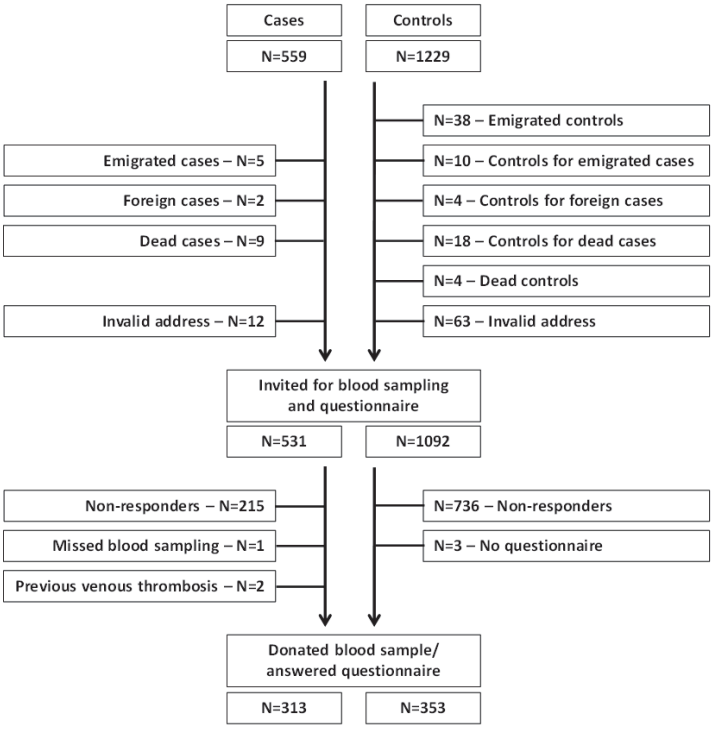

Figure 10. Flow-chart showing the final selection of cases and controls participating in the studies presented in the present dissertation.

3.3. Blood sampling and analysis

For the controls, blood samples were drawn at Oslo university hospital at Ullevål. The cases met at the hospital they had had their index VT and two investigators from the VIP study met at the respective hospitals and draw and prepared blood samples from the cases as described below.

Venous blood samples were drawn from fasting women at a single time point during 2006, 3-16 years (median 8 years) after index pregnancy, and citrated blood was kept at room temperature and centrifuged at 2000g for 15 minutes within 1 hour to prepare platelet poor plasma. Plasma aliquots were stored at -70 °C until assayed. All analyzes were performed by skilled technical staff with long experience in our research laboratory.

At time of blood sampling, self-reported morbidity was low, and none of the women had hematologic disease, active cancer, thyroid disorder, or inflammatory bowel disease. Anti-allergy drugs were used in approximately 10% of both cases and controls. Less than 5% of the cases and the controls used other daily medications, such as antihypertensives, insulin, and antidepressants.

Citrated plasma was used to measure levels of coagulation factors and inhibitors, markers of activated coagulation and fibrinolysis and APAs. DNA was analyzed from whole blood collected in containers with EDTA. Details on the specific analysis are given in Papers I, II and III. All analyses were performed examiner blind, and the samples were run in batch using a balanced set-up with equal number of cases and controls in each run.

The functionality of the PC system was analyzed by testing the effect of aPC on the ETP as determined by the CAT assay (Figure 11). Coagulation was triggered by recalcification of citrated plasma in the presence of 5 pM recombinant human TF. We used 6.5 nM aPC which gave a residual thrombin generation activity of 15-16% in PNP. The aPC sensitivity ratio (aPC-sr) was defined as the ETP in presence of aPC divided by ETP without aPC (Figure 5 section 1.3.2.). In order to increase reproducibility, all test results were expressed as normalized aPC-sr (n-aPC-sr). High levels of n-aPC-sr reflect an aPC resistant phenotype.

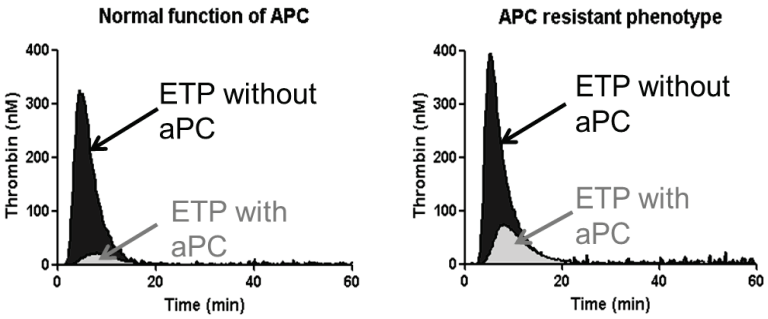

Figure 11. Functionality of the protein C system with and without exogenous aPC. ETP − endogenous thrombin potential; aPC − activated protein C.

The assays were performed at the Haematological Research Laboratory at the Department of Haematology, Oslo University Hospital. Intra-assay coefficients of variation (CV) were: factor VIII 2.7%, factor IX 5.3%, AT 2.9%, PC 1.8%, free PS antigen 4.4%, free TFPI 4.1%, and D-dimer 7.7%, all consistent with good internal validity.

PNP was prepared from 21 healthy blood donors. Citrated blood was centrifuged at 2000 g for 15 min. within 1 hour, pooled and stored at -70^0C. Residual platelet count in individual plasmas was $<5{\cdot}10^9$ /L before pooling. The men and women from whom the plasmas were prepared did not have thrombophilia. None of the women used oral contraceptives, hormone replacement therapy, and none were pregnant. We used the same PNP in Papers I-III.

3.4. Statistics

The distribution of continuous variables was evaluated by inspecting their histograms. For approximately normally distributed variables, parametric statistics were used and data were reported as mean and standard deviation (SD), whereas non-parametric statistics were applied for skewed variables and data were reported as medians, quartiles and percentiles. When exploring associations between two dichotomized variables, unadjusted odds ratios (OR)s were calculated using 2x2 cross-tables. Adjusted ORs were calculated using multiple logistic regressions. ORs with 95% CI and a significance level of 5% were used in all three papers.

In Papers I and II women being pregnant, using oral contraceptives or anticoagulation at time of blood sampling were excluded when we analyzed the phenotypic hemostatic variables.

Cut-offs for high or low levels of the hemostatic variables and APAs were defined in the control group.

In Paper I the ORs for DVT and for PE as compared with controls were calculated for hemostatic and genetic variables by using logistic regression. High sensitivity (hs) C-reactive protein (CRP) values were logarithmically transformed for calculations of mean hsCRP levels.

In Paper II the association between n-aPC-sr and VT was analyzed in two subpopulations: 1) in non-carriers of F5 rs6025 and 2) in carriers of F5 rs6025. The n-aPC-sr quartile and percentile limits were defined in the controls in the two subpopulations, respectively. Unadjusted and adjusted ORs were studied by using logistic regression models. Spearman's correlation coefficient was used when associations between two continuous variables were analysed and a two-sided Mann-Whitney test was used when comparing n-aPC-sr in two groups. When comparing n-aPC-sr in more than two independent groups, the Kruskal-Wallis test was used.

In Paper III the data were analyzed using chi-square test or Fisher's Exact Test.

The statistical analysis was performed by using the statistical software program SPSS, versions 16, 18 and 19.

3.4. Approvals

The research protocol was approved by the Regional Committee for Research Ethics in Health Region East and the Norwegian Ministry of Health and Social Affairs. The Norwegian Data Inspectorate approved the processing of data files with sensitive personal health information and merging of clinical data and register data. Informed consent was collected from the participants at the time of blood sampling. The study was registered at www.clinicaltrials.gov, unique identifier number NCT00856076, with acronym Venous Thromboembolism in Pregnancy (VIP) study.

4. Summary of results

4.1. Paper I

Differential hemostatic risk factors for pregnancy-related deep vein thrombosis and pulmonary embolism - A population-based case-control study

In paper I we investigated whether high or low levels of hemostatic parameters known to be risk factors of VT in the general population were associated with VT during pregnancy or the postpartum, and whether these parameters showed differential risk for pregnancy-related DVT and PE. We adjusted each hemostatic variable for high-sensitivity CRP (hs-CRP), age and the time interval between index pregnancy and the time for blood sampling. FVIII, n-ETP and D-dimer levels above the 90th percentile were independent risk factors for pregnancy-related VT; aORs 1.9 (95% CI 1.2−3.1), 2.1 (95% CI 1.3−3.3) and 2.3 (95% CI 1.4−3.7), respectively. After adjusting for hsCRP, fIX above the 90th percentile was no longer associated with VT. However, fIX above the 90th percentile was an independent risk factor for PE, aOR 2.4 (95% CI 1.1-5.0). Low levels of free PS antigen (at or below 65% in controls) increased the risk for PE three times as compared with controls, aOR 3.1 (95% CI 1.3-7.2), whereas the risk for DVT was not increased. The risk for DVT was increased in both carriers and non-carriers of F5 rs6025 polymorphism (commonly known as factor V Leiden). aOR for DVT as compared with controls for n-aPC-sr above the 75th percentile in non-carriers of F5 rs6025 polymorphism was 3.3 (95% CI 2.1-5.2). For women carrying F5 rs6025 polymorphism, OR for DVT as compared with controls was 7.7 (95% CI 4.7−12.7). Reduced sensitivity to aPC was not associated with increased risk for PE.

In conclusions, high levels of fVIII, n-ETP and D-dimer were independent risk factors for pregnancy-related VT. Reduced sensitivity to aPC increased the risk for DVT in absence of F5 rs6025 polymorphism and in carriers of F5 rs6025 polymorphism, whereas high levels of fIX and low levels of free PS antigen increased the risk of pregnancy-related PE.

4.2. Paper II

Resistance to activated protein C is a risk factor for pregnancy related venous thrombosis in the absence of the F5 rs6025 (factor V Leiden) polymorphism

In the second paper we investigated the association between reduced sensitivity to aPC, expressed as n-aPC-sr, and the risk of VT in pregnancy or the postpartum in non-carriers and carriers of the F5 rs6025 polymorphism (commonly known as factor V Leiden). Women, who were pregnant or used oral contraceptives or anticoagulation therapy at time of blood sampling, were not included in the analyses. The association between n-aPC-sr and VT was analyzed in two subpopulations: 1) in non-carriers of F5 rs6025 polymorphism and 2) in carriers of F5 rs6025 polymorphism. When we analyzed aPC resistance in women not carrying F5 rs6025 polymorphism, n-aPC-sr quartiles were defined in F5 rs6025 polymorphism negative controls. In F5 rs6025 polymorphism non-carriers, the risk of VT was more than doubled in women with n-aPC-sr in the 4th quartile as compared with women having n-aPC-sr in the 1st quartile; OR 2.3 (95% CI 1.4-3.9). Adjusting for age, free TFPI, free PS antigen and the time interval between index pregnancy and blood sampling had little influence on the association between VT and n-aPC-sr.

To investigate whether an aPC resistant phenotype increased the risk of VT in carriers of F5 rs6025 polymorphism, we defined n-aPC-sr quartiles in F5 rs6025 polymorphism positive controls. Including only F5 rs6025 polymorphism carriers in the analyses, the risk of VT was increased with higher n-aPC-sr, but not statistical significant. The OR for VT for n-aPC-sr above the 90th quartile as compared with n-aPC-sr at or below the 90st quartile was 3.7 (95% CI 0.8-17.1).

In conclusions, aPC resistance in absence of F5 rs6025 polymorphism is a risk factor for pregnancy-related VT, and there is a relation between an aPC resistant phenotype and risk of VT in carriers of F5 rs6025 polymorphism.

4.3. Paper III

The association of antiphospholipid antibodies with pregnancy-related first time venous thrombosis - a population-based case-control study

In paper III we investigated the association between LAs, aCLs and anti-ß2GP1 antibodies and pregnancy-related VT. According to international consensus, the 99th percentiles for the different APAs in the control group were used as cut-off values. There was no association between pregnancy-related VT and being positive for at least one APA. Nine cases and no controls had two or more positive tests for APA (=multi-positive). Excluding women with IgM (aCL and anti-ß$_2$GP 1) antibodies from the analyses, seven cases were still APA multi-positive. 6 cases were still multi-positive for APA after carriers of the factor V Leiden or the prothrombin polymorphisms were excluded from the analyses. Separate analyses for women with index pregnancy during 1990 through 1996 and during 1997 through 2003 revealed no significant difference between the numbers of women positive for any APA in the two time periods.

In conclusion, multi-positivity for APAs is strongly associated with pregnancy-related VT.

5. General Discussion

5.1. Methodological considerations, strengths and limitations

5.1.1. Study design and study population

Our study has a case-control design. This design was chosen because the incidence of pregnancy-related VT is low, and because we aimed at identifying risk factors for pregnancy-related VT.

The large number of cases included in this study and the population based design are strengths of the studies presented in this thesis. Furthermore, the diagnoses of VT were validated by reviewing hospital records, ensuring that all VTs were objectively diagnosed and they were the first lifetime VT.

Control selection

The selection of controls from a single hospital was done because of administrative matters and limited funding. Nevertheless, this selection is a potential weakness of the study. Because the controls were selected from a more restricted geographic area than the cases, the controls do not fully represent the population from which the cases were selected (the source population). In case-control studies the controls are used to estimate the distribution of potential risk factors in the source population and the controls should therefor ideally be selected from the entire source population. To limit this weakness, the study was planned in such a way, that it is possible to describe the distributions of potential risk factors for VT within the different study populations. Columns B and C of Table 4 display the distribution of risk factors for pregnancy-related VT among the controls selected from one hospital (n=1229) and the controls selected from all 18 hospitals (n=613232), respectively. Overall, the differences were nominally small, but the controls in population B were older, had lower parity and had more often Cesarean section than the controls in population C. As described earlier, the risk of VT increases progressively with increasing age, but age was not associated with pregnancy-related VT in our previous study on clinical risk factors[128] and age did not affect our risk estimates when we adjusted for maternal age in the multivariate analyses. This may be explained by the participants being too young to demonstrate an “age-effect” on the risk of VT. The hemostatic variables we studied were not associated with parity or Cesarean section. If the controls are selected in such a way that they become more similar to the cases, estimates for the variables might be biased towards null and thereby we might not detect a

difference for the variables between cases and control that actually exists (Type II errors). If, on the other hand, the controls are selected in such a way that they become more different from the cases, risk estimates will be overestimated (Type I errors).

Table 4. Distribution of potential risk factors for pregnancy-related VT among the cases (Column A), among controls selected from Oslo University Hospital, Ullevål (Column B) and among controls selected from the source population (Column C).

Risk factor		**Cases** **A** **N=559**	**Controls - one hospital** **B** **N=1229**	**Controls – all births 18 hospitals** **C** **N=613232**
		%	%	%
Age (years)	17-24	19.1	13.5	18.3
	25-29	31.5	29.9	35.5
	30-34	28.8	32.1	31.4
	35-54	20.6	24.6	14.8
Parity	0	53.0	48.3	43.5
	1	27.0	33.4	35.1
	2	13.2	12.5	15.9
	3+	6.8	5.8	5.6
Conceived after ART		5.0	1.9	0.8
Multiple pregnancy (twins)		4.7	2.1	1.6
Preeclampsia		11.4	4.7	3.5
Diabetes 1		0.2	0.4	0.6
Gestational diabetes		1.3	1.1	0.5
Premature rupture of membranes		2.9	0.2	5.2
Placenta abruption		2.1	1.0	0.6
Vaginal delivery		68.0	80.6	87.3
Planned Cesarean		8.9	9.2	4.8
Acute Cesarean		23.1	10.2	7.9

Vaginal delivery comprises vaginal, vacuum and forceps delivery. Immobilization is defined as strict bed rest one week or more prior to the diagnosis of VT or prior to delivery, in controls strict bed rest one week or more at any time during pregnancy. ART - assisted reproductive therapy; BMI - body mass index.

Drop-outs

A common problem in epidemiological studies, and also a concern in the studies presented in this thesis are potential selection bias caused by drop-outs. Selection bias is a systematic error which appears when the association between the risk factor and the disease are different for those participating and those not participating in the study. We provided information on clinical risk factors from medical files both for participants and non-participants and have, in contrast to many other studies, presented the source population and the selection of cases and controls. All eligible women from the hospital-based study were invited to participate in the study presented in this thesis, but a substantial number, approximately 40% of the cases and 70% of the controls declined. Table 5 shows the distribution of potential risk factors for pregnancy-related VT between eligible cases and controls participating and not participating. As showed by table 5, participating controls were older than non-participating controls and older than participating cases. Except for age, potential clinical risk factors were fairly distributed between participating and non-participating women and the direction of the selection bias of clinical risk factors was mainly the same for the cases and controls participating and not participating (Table 5). Thus our study appears to suffer from some degree of selection bias, but we still consider our results to have good external validity.

Table 5. Distribution of possible risk factors for pregnancy-related VT among eligible cases and controls who agreed/did not agree to participate in the study.

	Eligible Cases		Eligible Controls	
Risk factors	**Not participating N=215**	**Participating N=313**	**Participating N=353**	**Not participating N=736**
	%	**%**	**%**	**%**
Antenatal VT	50	50	na	na
Postnatal VT	50	50	na	na
Age (years) 17-24	24.3	15.7	4.8	16.6
25-29	34.4	30.4	26.1	31.7
30-34	23.9	34.2	37.7	29.6
35-54	17.4	19.8	31.4	22.1
Parity 0	52.3	55.9	49.9	49.5
1	26.6	28.8	39.7	29.1
2	12.4	12.5	8.2	14.7
≥3	8.7	2.9	2.3	6.6
ART	2.8	5.8	2.3	1.5
Cigarett smokers	28.9	19.8	9.9	15.0
Immobilization	5.0	7.7	0.8	1.1
BMI >25	30.2	27.1	17.8	22.6
Weight gain < 7.0 kg*	14.2	11.8	8.2	8.4
7-21 kg**	47.7	44.7	53.8	50.6
>21.0 kg***	38.1	43.5	38.0	41.0
Premature rupture of membranes	2.3	1.9	0.3	2.8
Placenta abruption	2.3	1.6	0.8	0.9
Caesarian section	32.6	31.0	21.0	19.4
Bleeding >1000 ml	13.3	13.4	2.0	4.2

Weight gain *less than the 10 percentile, **between the 10th and 90th percentile and *** more than the 90th percentile; ART−assisted reproductive therapy; BMI−body mass index; Immobilization−strict bed rest one week or more prior to the diagnosis of VT, in controls strict bed rest one week or more at any time during pregnancy.

Ethnicity

The participants who donated blood samples were mainly Caucasian, 90.1% of the controls and 95.8% of the cases had parents who were both from north-western Europe. A larger portion of the controls than the cases had other ancestors than north-western Europeans, probably reflecting the greater multiplicity of ethnic groups living in Oslo compared with the source population. The F5 rs6025 and F2 rs1799963 polymorphisms which increase the risk of pregnancy related VT approximately 5- and 9-fold, respectively[124;127] are rarely detected in other populations than Caucasians. The larger proportion of non-Caucasians in the control group might have underestimated the results regarding F5 rs6025 and F2 rs1799963 polymorphisms. Deficiencies of AT, PC and PS

have been reported to be more common related to VT in Caucasian, but these observations might be biased by the more intensively investigation of the Caucasian population[129]. Moreover, the incidence of first time VT is higher in African-Americans and lower in Hispanics and Asians as compared with Caucasians[130].

Taken these assessments about the study population presented in this thesis into consideration, we do believe our results on the risk of pregnancy-related VT for hemostatic factors are generalizable for a Caucasian population of pregnant and postpartum women.

5.1.2. Measurements

Blood samples and analyzes

Two sets of blood samples, including one collected during index pregnancy VT, might have given us valuable information whether abnormalities of the hemostatic parameters were sustained over time. Furthermore, two sets of blood samples at least 12 weeks apart would have given us the possibility to diagnose APS in women with positive APAs.

In our study design blood sampling was performed at a single time point and several years after index pregnancy. The levels of coagulation and fibrinolytic factors return to non-pregnant values within 3-6 weeks after delivery[108]. The time it takes for normalization of coagulation factors after an acute VT is more uncertain. Persistent low or high levels of coagulation factors 3-16 years after an episode of VT are unlikely. We have reason to believe that hemostatic parameters normalize to pre-pregnant values after pregnancy[107;131] and we do not have reason to believe that transient elevation of the hemostatic variables or false positive or negative tests should be different among cases and controls 3-16 years after index pregnancy. In conclusion, we believe blood sampling at a single time point and several years after index pregnancy provide valid information on the role of the variables analyzed as sustained risk factors for VT.

We minimized potential pre-analytical variables and thereby strengthened the validity of our results by using a few dedicated and experienced persons for blood sampling, keeping blood samples in room temperature before centrifugation, centrifugation within 1 h after venepuncture and aliquotation and freezing the samples shortly after centrifugation. Platelet poor plasma ($< 10 \cdot 10^9$ /L) was used for all the analyses. The frozen blood samples have been thawed only once.

Thrombin generation assay

The thrombogram is dependent on so-called assay determinants such as the source and concentration of TF used to trigger coagulation and pre-analytical variables. When low TF concentration (<1 nM) is used to trigger coagulation, the system is more sensitive to coagulation factors involved in the contact pathway of coagulation, whereas for higher TF concentrations the thrombogram becomes more dependent on the TF pathway. To trigger coagulation we used an intermediate concentration of TF which creates a system sensitive for free TFPI and free PS[31]. As we tested the functionality of the PC system by probing the reactions with aPC, the thrombogram became sensitive to free PS antigen, free TFPI and fX[40].

A potential pre-analytical variable in thrombin generation assays is activation of the contact pathway. An option to avoid contact activation is to add corn trypsin inhibitor (CTI) to the blood sample tubes before venous puncture, as we did not. A recent published study showed that addition of CTI was not needed if coagulation was trigged by TF concentrations was above 1 pM and caution was paid to pre-analytical variables[132].

Median n-aPC-sr in our controls was lower than expected, i.e. lower than 1, which means that our PNP was less sensitive to aPC than plasma from the controls. Differences in preparing PNP and preparing single plasmas may explain the more aPC resistant PNP as compared with plasma from the controls. When preparing PNP, plasmas from both men and women were mixed and furthermore, the time between centrifugation and freezing was delayed compared to the time spent when preparing single plasmas. We have used the same PNP in all analyzes and have no reason to doubt that our results are valid.

Antiphospholipid antibodies

Variability on detection of APAs and determination of cut-offs are known challenges concerning the laboratory classification criteria for APS. We analyzed aCL- and anti-β2GP 1 antibodies based on methods and cut-offs described by the 2006 Sidney update of the classification criteria for APS[54], and LAs was mainly detected as described by Brandt et al in 1995[56].

For the detection of LAs we used assays integrating screening and confirmatory steps and 1:1 mixing of test plasma and PNP. The use of integrated assays increase sensitivity and specificity compared to the use of separate assays[133] and the results were normalized against results from PNP run in parallel.

Questionnaire

Information from the questionnaire that was completed at time of blood sampling has not been a source for main analyzes in any of the three papers included in the present thesis. Information from the questionnaire has been used to investigate 1) clinical characteristics at time of blood sampling such as morbidity, age, BMI, daily cigarette smoking and parity that might influence the variables analyzed, 2) the association between pregnancy-related VT and different hemostatic factors in the absence and presence of women who were pregnant, used oral contraceptives or anticoagulation at time of blood sampling and 3) information on the use of daily medications other than anticoagulation therapy.

5.1.3. Statistical aspects

Pre-study power calculations were done for the original VIP-study, but not for hemostatic variables as investigations of these variables were sub-studies of the original study. However, we performed post-hoc power analysis for the ability to detect differences in prevalence of the hemostatic variables analyzed. If we expected 10% of the controls to have a risk factor, and 20% of the cases to have the same risk factor, our material was estimated to have a test power of 95%. The test power increased to 99.9% if 10% of the controls had the exposure and 30% of the cases. For rare exposures such as deficiencies of the natural anticoagulants, our population was too small to calculate risk of VT.

The distribution of n-aPC-sr was relatively skewed and in Paper I we interpreted the residuals to be close enough to normal distribution to use parametric methods in the statistical analysis. In paper II we categorized n-aPC-sr into quartiles and percentiles.

Similar to other studies assessing the risk of VT for hemostatic factors[84;88;91;134-136] we defined high or low levels of the hemostatic variables by defining percentiles in the control group. This is not a method to screen for deficiencies of pro-or anticoagulant coagulation factors, but a way to investigate whether or not high or low levels of the hemostatic variables are associated with VT.

5.2. Discussion of main findings

All three papers included in the present thesis describe different biological risk factors for pregnancy-related VT, paper I additionally explore differential risk factor for DVT and PE. We have confirmed that risk factors for VT in the general population are risk factors also for pregnancy-related VT. Although it is impossible to directly compare risk for VT between different studies as laboratory assays and cut-offs are different, our risk estimates for pregnancy-related VT are mainly similar to risk estimates for VT in the general population. Our studies give valuable insight about the pathophysiology of pregnancy-related VT, and of special interest is our data concerning differential risk factors for pregnancy-related PE and DVT.

Similar to the acute phase reactant C-reactive protein (CRP), plasma levels of several coagulation factors are influenced by ongoing inflammation. High-sensitivity CRP (hs-CRP) is a sensitive method to measure low levels of CRP. We evaluated whether abnormal levels of coagulation parameters described in paper I were caused by inflammation by statistically controlling the coagulation parameters for hs-CRP.

5.2.1. Hemostatic factors associated with pregnancy-related venous thrombosis

Elevated levels of fVIII is an established risk factor for VT in the general-population[84;134;137]. We describe for the first time elevated levels of fVIII as an independent risk factor for pregnancy-related VT, not influenced by an ongoing inflammation or the sequelae after the VT itself. Similar to us, Kamphuisen et al described fVIII as an independent risk factor in the population-based Leiden Thrombophilia Study[138].

The determinants of elevated plasma levels of fVIII are largely unknown[139]. Bank et al found elevated levels of fVIII in near one-half of first-degree relatives of patients with VT and suggested a genetic basis of elevated plasma fVIII activity[140]. Other known determinants of fVIII plasma levels are von Willebrand factor (VWF) and the AB0 blood group[141]. We did not analyze VWF or the AB0 blood groups, so we don't know whether these variables would affect the association between fVIII and VT in our study, but previous reports have shown that corrections for VWF and AB0-blood groups did not change the relation between fVIII and the risk for VT[84;137].

The mechanism by which elevated levels of fVIII exerts its thrombotic effect is unclear. One explanation may be that elevated levels of fVIII lead to increased activation of fX resulting in excessive thrombin formation. High levels of fVIII have recently been shown to mediate enhancement of thrombin generation[142]. Another mechanism may be that high levels of fVIIIa override the anticoagulant effect of aPC and thereby increase the susceptibility to VT. High levels of fVIII has been shown to enhance the aPC resistant phenotype in carriers of F5 rs6025[143]. Furthermore, the Leiden Thrombophilia Study found a high correlation between high levels of fVIII and reduced sensitivity to aPC[144]. Finally, a recent report from two population-based case-controls studies showed that increased risk of VT in people with increased BMI was partly explained by fVIII induced aPC resistance[145]. We did not control for BMI as BMI was evenly distributed between the cases and the controls.

To our knowledge we are the first to describe independent associations between elevated levels of n-ETP and D-dimer and pregnancy-related VT. Similar associations between ETP and D-dimer and risk of VT have been described in the general population in the Leiden Thrombiphilia Study[135;146]. Lowe et al found increased risk of VT in elderly women with increased levels of D-dimer[147]. Increased levels of theses global hemostatic parameters several years after index pregnancy may reflect a sustained activation of the coagulation system in these individuals, making them more susceptible to VT.

5.2.2. Reduced sensitivity to activated protein C

APC resistance is the phenotypic expression of the F5 rs6025 polymorphism[30]. We investigated reduced sensitivity to aPC and the risk for pregnancy-related VT in two separate groups; in non-carriers and in carriers of the F5 rs6025 polymorphisms. To investigate the effect of aPC resistance on the risk of pregnancy-related VT in absence of known contributors to aPC resistance, we excluded women who were pregnant or used oral contraceptives at time of blood sampling.

Analyzing only non-carriers of F5 rs6025 polymorphism, women who had n-aPC-sr in the 4th quartile had a three-fold increased risk of pregnancy-related VT as compared with women with n-aPC-sr in the 1st quartile. Apart from the study presented in this thesis, there has, to our knowledge, been only one publication on aPC resistance in absence of F5 rs6025 and pregnancy-related VT and they did not find an association between reduced sensitivity to aPC and pregnancy-related VT[148]. Our results presumably differ

because they used an aPTT-based method to analyze the function of the PC system, and their sample size was relatively small.

When we analyzed women carrying the F5 rs6025 polymorphism, we found near a 4 times increased risk of VT in women with high levels of n-aPC-sr as compared with carriers with lower levels of n-aPC-sr. Although not statistically significant, there was a clear dose-response relationship between an aPC resistant phenotype and the risk of VT in carriers of F5 rs6025, suggesting that even in carriers of F5 rs6025, the degree of aPC resistance is an important determinant of the risk of VT.

Given the complexity of the protein C system, there are multiple possible explanations for the association between aPC resistance and increased risk of VT. We found that PC, free PS antigen and free TFPI were negatively correlated with n-aPC-sr in absence of F5 rs6025, but only free PS antigen was associated with pregnancy-related VT. When we adjusted n-aPC-sr for free PS antigen in non-carriers of F5 rs6025, the association between n-aPC-sr and VT was not lost, indicating that reduced sensitivity to aPC and increased risk for pregnancy-related VT was not explained by low levels of free PS antigen. In our population, n-aPC-sr was not correlated with fVIII. Single gene mutations in the F5 gene apart from F5 rs6025 polymorphism or subtle regulation of multiple clotting factor or inhibitor genes may be mechanisms responsible for aPC resistance in absence of F5 rs6025.

5.2.3. Anti-phospholipid antibodies

As described in paper III we did not find an association between having at least one positive test for APAs and pregnancy-related VT. However, we found that 9 cases and none controls were positive for at least two APAs. When we excluded women with IgM antibodies, still 7 cases were APA multi-positive.

The prevalence of APAs depends on the definition of a positive test. We used the 99th percentile defined in the control group as reference for each of the 6 APA assays, and thereby 1% of the controls had positive results for each test. In our study, 6.8% of the controls were positive for at least one APA, which is a result of the definition of cut-offs. Utilizing the 99.7th percentile as cut off, 4.8% (15/313) of the cases and 1.7% (6/353) of the controls were positive for any APA, whereas 9.3% (29/313) of the cases and 6.8% (24/353) of the controls were APA positive when the 99th percentile was used as cut-off.

This finding shows that a higher percentage of the APA-positive cases as compared with controls were still positive for any APA when we increased the cut-off level, which indicate that the cases had higher APA titers. Increasing the cut-off would probably increase the specificity of the tests, possible at the cost of decreasing the tests sensitivity. We used cut-off levels as suggested by the 2006 APS consensus report[54].

Beside titer levels, the number of positive APA tests is important for the risk of VT. It is now believed that β2GP1 is the main antigen for APAs and a substantial part of anti-β2GP1 antibodies have LA activity and are aCL positive[51;52]. In paper III we applied the statements of the 2006 APS consensus report[54], which assigned the same role and importance for each of the three types of APAs when deciding cut-offs and interpreting results. Over the last years the understanding of the importance of APA multi-positivity has been enlightened and several studies have shown that triple APA positivity are more strongly associated with thrombosis than the presence of single or double positivity[57;149-152]. Of notice, the association between APA multi-positivity and VT involve the IgG isotype, whereas the clinical relevance of the IgM isotype is unsettled[153]. We only found APA multi-positivity among the cases in this population of otherwise healthy women and with a long time range between the thrombotic event and blood sampling. When we excluded women with IgM antibodies (aCL and anti-β2GP1), still 7 women were APA multi-positive. Our data confirm the importance of APA multi-positivity and risk of VT. As venipuncture was performed only once we don't know if any of these APA multi-positive women fulfilled the criteria for APS.

5.2.4. Differential risk factors for pregnancy-related DVT and PE

The possibility of DVT and PE being partly different diseases are interesting concerning the understanding of the pathophysiology of VT and it would have important clinical implications regarding risk assessments and treatment. Therefore we investigated potential differential risk factors for pregnancy-related DVT and PE.

A recent systematic review found several risk factors for VT that had differential risk for DVT and PE, but reports on separate risks for DVT and PE for coagulation factors or the natural anticoagulants were not found[106]. The authors concluded that DVT and PE not always should be considered as two presentations of the same disease.

Hemostatic factors associated with increased risk of PE

After adjusting for hsCRP, fIX above the 90th percentile was no longer associated with pregnancy-related VT, but the risk for PE was more than doubled as compared with controls. For women with free PS antigen at or below 65% the risk of PE was increased 3 times as compared with controls. The risk of DVT as compared with controls for high levels of fIX or low levels of free PS antigen was not significantly increased. Differential risk for PE and DVT for abnormal levels of fIX and free PS antigen has not previously been reported and need to be confirmed in other studies. Interestingly, in a family study, Zoller et al found to their surprise that individuals with a combination of PS deficiency and a polymorphism in the PAI-1 promotor gene had increased risk of PE, whereas the risk for DVT was not increased[154].

We found that high levels of fibrinogen increased the risk of PE, whereas the risk for DVT was not increased. The association might partly be explained by a concomitant inflammation at time of blood sampling as the risk was lowered and no longer statistical significant when we adjusted for hsCRP. On the other hand, this adjustment might hide an interesting effect as chronic low-grade inflammation in these women might lead to elevated levels of fibrinogen which increase the risk of VT.

The association between high levels of fibrinogen and PE may be explained by clot structure as fibrinogen levels have been shown to be a predictor of the clot architecture[155]. Structural integrity of the clot is provided by fibrin and reduced clot stability might dispose for embolization. Homozygous carriers of the Ala allele in the α-fibrinogen Thr312Ala polymorphism, a common genetic variant, have been shown to have increased risk for PE[156]. The Thr312Ala polymorphisms might influence fibrin cross-linking and thereby clot stability, making the clot more unstable and susceptible to embolization. To further explore this finding the same group investigated clot formation and structure in an in vitro system for both Thr312 and Ala312, and they found that the α-fibrinogen Thr312Ala polymorphism affects clot structure and stiffness[157].

Hemostatic factors associated with increased risk of DVT

Reduced sensitivity to aPC in non-carriers of F5 rs6025 polymorphism and carriers of F5 rs6025 had increased risk of pregnancy-related DVT, whereas the risk for PE was not increased. APC resistant phenotype appears to be the key mechanism in the different presentations of DVT and PE. As initially suggested in 1996[101] it seems that the «factor V

Leiden paradox» in fact is an "aPC-resistant paradox». Van Langevelde et al[106] found in their systematic review a stronger effect on DVT than PE for women using combined oral contraceptives and for pregnant women. The association between reduced sensitivity to aPC and use of oral contraceptives and pregnancy presented in paper II support their proposal that the increased risk of DVT in oral contraceptive users and in pregnancy might be explained by acquired aPC resistance.

To investigate possible mechanisms behind the different presentations of DVT and PE in carriers of F5 rs6025, van Stralen et al studied differences in the location of VT, the number of affected veins, the time between provocation of thrombus formation and the actual diagnosis, growth speed of the thrombus and last, clot structure in carriers and non-carriers of the F5 rs6025 polymorphisms[158]. They confirmed the factor V Leiden paradox, but none of the investigated candidates to explain the phenomenon were explanatory.

APC has in an in vitro system shown to have indirect pro-fibrinolytic effects, mainly by reducing thrombin formation and thereby indirectly inhibit TAFI activation[159]. Increased levels of TAFI have been found in aPC resistant patients[160]. APC resistance promotes sustained thrombin generation and thereby stimulate TAFI activation and subsequent inhibition of fibrinolysis, which increases clot stability[161]. Hypothetically, increased TAFI activation and more stable clots might partly explain the increased risk of DVT in aPC resistant individuals. In a case report of a 45 year old woman with aPC resistance and who experienced several episodes of DVT in both legs, the pro-fibrinolytic effect of aPC was markedly reduced[162].

5.2.5. Clinical implications

Our reports on hemostatic risk factors associated with increased risk for pregnancy-related VT and differential risk of DVT and PE are pieces in the puzzle of the understanding of the mechanisms of VT, in particular pregnancy-related VT. Furthermore, our findings challenge the opinion that DVT and PE are two manifestations of the same disease. Improved understanding of the mechanisms of VT, and DVT and PE, is the key to be able to recognize who are at risk of VT and especially at risk of PE. A better understanding of the mechanisms of VT is necessary for improving treatment and prophylaxis of VT. Studying pregnancy-related VT itself is important as it increases the awareness of the disease.

We have investigated risk factors for pregnancy-related VT, but whether patients with VT or individuals with a family history of VT should be screened for thrombophilia is controversial, and our study does not try to give an answer to this question.

6. Conclusions

The studies included in this thesis explored the association between hemostatic biomarkers and a history of a first lifetime VT during pregnancy or the postpartum, and we found that known risk factors for VT in the general population were risk factors for pregnancy-related VT. We also investigated differential risk for pregnancy-related DVT and PE for hemostatic parameters, and our data strengthen the hypothesis of differential pathophysiology between DVT and PE. Having in mind the possible selection bias of the present studies, our results must be interpreted with caution. Still, our conclusions are as follows:

- High levels of fVIII, n-ETP and D-dimer are risk factors for pregnancy-related VT.
- Women with inherited aPC resistance and women with reduced sensitivity to aPC in absence of factor V Leiden have increased risk of pregnancy-related DVT, whereas women with high levels of fIX or low levels of free PS antigen have increased risk of PE.
- Reduced sensitivity to aPC in absence of factor V Leiden was a risk factor for pregnancy-related, and an aPC resistant phenotype was related to increased risk of VT in carriers of factor V Leiden.
- APA multi-positivity was highly associated with pregnancy-related VT.

7. Future perspectives

Pregnancy-related VT is relatively rare, but as it is a major cause of increased morbidity and mortality in young women, a future goal must be to decrease the incidence of VT during pregnancy and postpartum. How can this goal be reached? First of all it is important that doctors are aware of the risk of pregnancy-related VT, and especially the increased risk of PE at the end of the third trimester and the first 3 weeks postpartum. Shortness of breath is not always explained by pregnancy or being an exhausted mother.

To identify women at risk of pregnancy-related VT it is essential to know about the risk factors, and to increase our knowledge about the risk factors of pregnancy-related VT, we must understand the pathophysiology of VT. Much of the knowledge of the mechanisms of VT is based on mechanisms of arterial thrombosis. Little is for example known about the contribution of venous endothelial cells in the development of VT, and further investigations are needed to explore the PC anticoagulant pathways in VT. Increased knowledge about the interactions between venous endothelial cells and pro-and anticoagulant pathways may enlighten the understanding why some VTs embolize and others do not. Clot structure and fibrinolysis probably also play a central in the understanding of why some thrombi embolize and this need to be further investigated.

The association between PE and high levels of fIX and low levels of free PS antigen needs to be confirmed in other studies. Likewise, the relation between F5 rs6025 polymorphism and aPC resistant phenotype and risk of VT needs to be further investigated.

To better compare reports on risk factors of VT, standardization of laboratory assays and standardization of cut-off values are needed.

8.References

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9. Errata

10. Papers I-III

I

II

III