The protein C pathway, oral contraceptives and venous thrombosis

The protein C pathway, oral contraceptives and venous thrombosis

Thesis Universiteit Maastricht – with a summary in Dutch
ISBN 90-9015320-9

Cover: Painting by Irene Salemink
Printed by: Datawyse Universitaire Pers Maastricht

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Additional financial support for this thesis by the Dr Ir Van de Laar Stichting, Nodia BV/Chromogenix, Greiner Bio-one and Kordia BV is gratefully acknowledged.

The protein C pathway, oral contraceptives and venous thrombosis

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus Prof.Dr. A.C. Nieuwenhuijzen Kruseman,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen op
vrijdag 13 december 2001 om 12.00 uur

door

JOYCE CURVERS
Geboren 25 mei 1973 te Boxmeer

Promotor:	Prof. Dr. J. Rosing
Co-promotor:	Dr. G. Tans
Beoordelingscommissie:	Prof. Dr. H.A.J. Struijker-Boudier (voorzitter)
	Prof. Dr. H. Büller (Universiteit van Amsterdam)
	Prof. Dr. J. de Haan
	Prof. Dr. M.H. Prins
	Prof. Dr. C.P. van Schayck

The protein C pathway, oral contraceptives and venous thrombosis

List of Abbreviations

α_1-AT	α_1-antitrypsin
α_2-M(-IIa)	α_2-Macroglobulin(-thrombin complex)
APC	activated protein C
aPTT	activated partial thromboplastin time
AT	antithrombin
BSA	bovine serum albumin
Ca^{2+}	calcium
C4bBP	C4b binding protein
DSG	desogestrel
EE	ethinyl estradiol
ELISA	enzyme linked immunosorbent assay
EPCR	endothelial protein C receptor
ETP	endogenous thrombin potential
E2	17β-estradiol
FDP	fibrin degradation products
FSH	Follicle stimulating hormone
FVL	Factor V Leiden
F1+2	fragment 1 + 2
hCG	human chorionic gonadotropin
HRT	hormone replacement therapy
IIa	thrombin
IVF	in vitro fertilization
Lets	the Leiden thrombophilia study
LEV	levonorgestrel
OC	oral contraceptive(s)
OR	odds ratio
(n)APCsr	(normalised) activated protein C sensitivity ratio
NP	normal pool(ed plasma)
P	progesteron
PC(I)	protein C (inhibitor)
PK	prekallikriene
PL	phospholipid(s)
POP	progestagen only preparation
PS	protein S
$PS_{APC\ IND}$	APC independent anticoagulant activity of protein S
PT	prothrombin
S2238	D-Phe-(pipecolyl)-Arg-pNA, thrombin substrate
S2366	L-pyroGlu-Pro-Arg-pNA, APC substrate
SD	standard deviation
TAFI	thrombin activatable fibrinolysis inhibitor
TAT	thrombin antithrombin complex
TF(PI)	tissue factor (pathway inhibitor)
TM	thrombomodulin
TPA	tissue type plasminogen actovator
VTE	venous thromboembolism
vWF	von Willebrand factor

Chapter 1

General Introduction

Introduction

Blood flows through the vascular system as a carrier of nutrients, oxygen, metabolites and waste products as well as a transporter of proteins and hormones. When a blood vessel wall is damaged, leakage of nutrients and metabolites into the interstitium occurs, that lead to a loss of resources and to inflammatory responses. In order to prevent leakage at the wounded site, physiological mechanisms are available that turn the liquid blood into a solid mass that covers the injured part. The vessel wall also contracts, causing a reduction in blood flow that prevents excessive blood loss. The process of staying fluid and when necessary turning into a solid phase is regulated by many proteins in the blood and vessel wall. This process is called hemostasis. Preservation of the fluidity of blood is of great importance, since obstruction of a significant artery (e.g. coronary artery) by thrombi may be life threatening.

Under resting conditions the reactions of hemostasis are slowly ongoing, but once coagulation is initiated, the hemostatic system is rapidly activated. Although the various procoagulant and anticoagulant reactions are usually described in a stepwise manner, in reality many of these reactions occur parallel, with different reactions taking place simultaneously.

1.1 Blood coagulation

The coagulation cascade consists of many proteolytic reactions in which inactive zymogens are converted into active enzymes. For most zymogens to become active in coagulation (FVII, FIX, FX and prothrombin, Protein S and C), specific glutamic acid (Glu) residues need to be carboxylated into γ-carboxyglutamic acid (Gla) residues by the vitamin K dependent γ-glutamyl carboxylase. Gla residues are necessary for calcium-mediated binding of coagulation factors to membrane surfaces. Calcium binding leads to conformational changes that promote the binding of coagulation factors to negatively charged phospholipid surfaces [1]. Inhibition of γ-carboxylation, e.g. by vitamin K antagonists, results in defective calcium binding and loss of interaction with phospholipids. This phenomenon forms the basis of anticoagulant treatment with warfarin and coumarin in patients suffering thrombotic events.

Historically two pathways of coagulation have been recognized: the intrinsic and extrinsic pathway (Fig 1.1). The extrinsic pathway that is triggered by tissue factor is regarded as the principal physiologic pathway. The intrinsic pathway of coagulation is initiated when blood is exposed to arteficial negatively charged surfaces (e.g. glass or kaolin). On these surfaces kallikrein activates FXII that, together with the cofactor high molecular weight kininogen (HMWK), subsequently activates FXI. The physiological role of this so-called contact activating system of hemostasis is likely associated with immunological reactions, since the immunological complement pathway and the contact activating system share the same inhibitors [2].

The primary plug

The extrinsic coagulation pathway is activated after injury of the vessel wall. Under normal conditions the vessel wall is covered with a thin layer of endothelial cells. These cells secrete or expose several substances that regulate the vessel wall motility (e.g. Nitric Oxide), and the activation of platelets (prostacyclin (PGI2), von Willebrand factor (vWF)) as well as the hemostatic system (tissue factor pathway inhibitor (TFPI), thrombomodulin (TM), tissue type plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1)). However, when endothelial cells are damaged or lost, collagen and fibronectin that are present in the sub-endothelium, become exposed. Platelets adhere to these proteins (via surface receptor GPIa/IIa, GPVI and GPIb/IX/V [3]) and become activated to form the primary plug. Activated platelets release a number of compounds contributing to platelet aggregation and to the activation of the coagulation system (*e.g.* FV, vWF, TXA_2). After activation platelets expose negatively charged phospholipids (phosphatidylserine) on their cell surface, which serve as a template at which coagulation factors efficiently interact [4], thereby ensuring optimal rates of coagulation factor activation.

Procoagulant reactions

Damaged or pathologic endothelial cells expose tissue factor on their surface [5, 6]. Tissue factor (TF) is a transmembrane cell surface receptor that binds activated FVII. The TF-FVIIa complex initiates the so-called extrinsic pathway of coagulation (Fig 1.1). The complex of TF-FVIIa will activate FX and FIX [7, 8]. The comples of

activated FIX (FIXa), together with its cofactor FVIIIa, negatively charged phospholipids and calcium ions produces more FXa. This Xa-forming complex of FIXa, FVIIIa, phospholipids and calcium ions is called the ***tenase complex***. In turn, FXa cleaves prothrombin (PT) to thrombin (FIIa). The conversion of prothrombin to thrombin by FXa is enhanced several orders of magnitude by its cofactor FVa, negatively charged phospholipids and calcium ions [9]. This complex of FXa, FVa, phospholipids and calcium ions is called the ***prothrombinase complex***. In both complexes the cofactors FVIIIa and FVa, two proteins that share 80% amino acid homology, are essential for efficient substrate activation. The subsequently formed thrombin is a key enzyme in haemostasis, which activates coagulation factors FVIII and FV, but also FXI [4, 10] and FXIII and most important, thrombin converts fibrinogen into fibrin. Fibrinogen is cleaved to form fibrin monomers, which are cross-linked into a fibrin network by FXIIIa. The fibrin clot stimulates the repair of the vessel wall, which takes 5 to 10 days. During this time the clot is very slowly lysed by plasmin (generated from plasminogen by tissue plasminogen activator (tPA)).

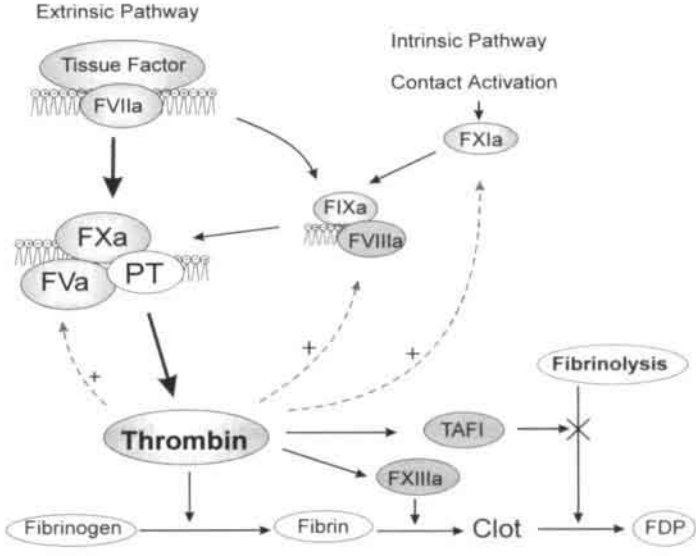


Figure 1.1 Initiation and continuation of coagulation. The activation of many proteolytic enzymes and cofactors (indicated with roman numbers) eventually leads to the formation of thrombin that cleaves fibrinogen to fibrin. Fibrin threads, together with platelets form a clot. Platelets are not in this figure, however, it is assumed that procoagulant surfaces (▨) stem from platelet origin. F= factor, PT=prothrombin, TAFI=thrombin activatable fibrinolysis inhibitor, FDP=fribrin degradation products.

Thrombin-activated FXI, which can activate more FIX, plays an important role in maintaining and amplifying thrombin formation and fibrin deposition. The additional supply of thrombin formed via FXIa also ensures that enough thrombin is formed to convert a protein called TAFI (thrombin activatable fibrinolysis inhibitor) [11, 12] into an active carboxypeptidase that releases carboxyterminal lysines from fibrin. This renders the fibrin less susceptible to fibrinolytic proteins like plasmin.

The reactions of coagulation and plug formation at the site of injury are very rapid and prevent loss of blood from the circulation, however coagulation should not be too effective and obstruct the whole vessel. Thus thrombus formation should be restricted to the site of the injured part of the vessel wall. This proces is regulated by the anticoagulant and fibrinolytic systems.

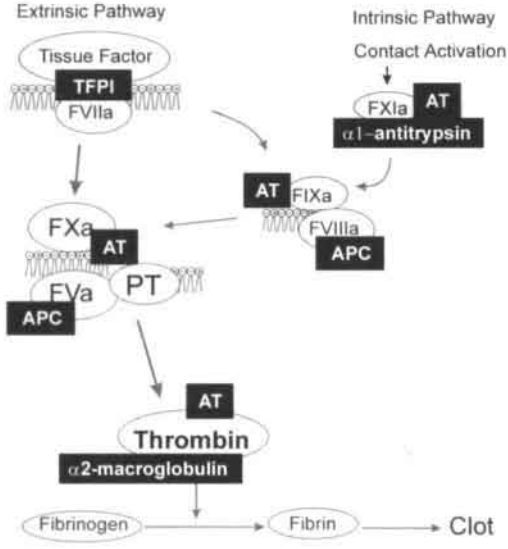


Figure 1.2 Inhibitors of activated coagulation factors represented in the black boxes. TFPI=tissue factor pathway inhibitor, AT=antithrombin, APC=activated protein C

Inhibition of coagulation

Blood contains several specific inhibitors that exert a one-to-one action with coagulation factors at each level of the coagulation pathway. The enzym-inhibitor complexes are no longer active and are rapidly cleared from the circulation. Important

inhibitors are antithrombin (AT), tissue factor pathway inhibitor (TFPI [13]), α2-macroglobulin (α2-M), α1-antitrypsin (α1-AT) and heparin cofactor II (Fig 1.2).

Tissue factor pathway inhibitor is exposed by the endothelium and rapidly binds the TF-FVIIa(-FXa) complex [14], leading to the formation of an inactive quarternary complex, thus inhibiting the formed FXa and preventing further activation of FX. Antithrombin inhibits several enzymes of the coagulation cascade such as Xa, IXa and thrombin. Heparin or heparin-like molecules stimulate the activity of antithrombin, increasing the rate of inhibition. Furthermore, thrombin is inhibited by α2-M, α1-AT and heparin cofactor II.

The protein C pathway

Besides the protease inhibitors, there is an anticoagulant pathway, the protein C pathway that puts a stop to thrombin formation (Fig 1.3). Thrombin bound to thrombomodulin (TM), a protein that is anchored in the membrane of endothelial cells[15], efficiently converts protein C into activated protein C (APC) [16, 17] which can inactivate FV(a) and FVIIIa [18]. Activation of protein C by thrombin is facilitated by the endothelial cell protein C receptor (EPCR[19, 20]). The EPCR brings protein C in close range of TM to which thrombin is bound. Inactivation of FV(a) and FVIIIa by APC is accelerated by negatively charged phospholipids and by an additional cofactor, protein S [21]. Protein S is reported to increase the affinity of APC for phospholipid surfaces, where the Va and VIIIa are located [22].

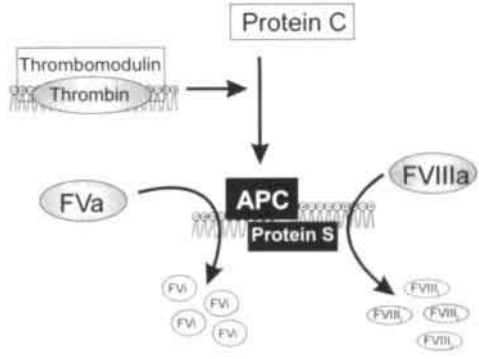


Figure 1.3 The anticoagulant Protein C-Protein S pathway. APC = activated protein C.

The inactivation of factor V(a) by APC involves three cleavage sites (Arg306, Arg506 and Arg679). Cleavage at Arg506 occurs rapidly and results in a FV molecule with approximately 40% cofactor activity in the prothrombinase complex[23]. Total loss of cofactor activity occurs after cleavage at Arg306. The cleavage of Arg679 is

reported to have no major role in the inactivation of FVa[24]. Inactivation of FVa is inhibited by FXa, since FXa protects the cleavage at the Arg506 position. This effect is, however, overcome by the presence of protein S, which stimulates the cleavage at Arg306 about 20-fold, resulting in full inactivation of FV(a) [21]. The proteolytically cleaved FV(a) no longer binds to prothrombin and FXa[25]. FV is also directly cleaved and inactivated by APC, resulting in a loss of procoagulant activity. Inactivation of FVIII does not occur, as it is protected through binding to vWF. Upon activation, however, FVIIIa dissociates from vWF and the FVIIIa-inactivation occurs in a similar manner as in FV(a) inactivation with cleavages located at Arg336, Arg562 and Arg740. The first two cleavages are considered important in inactivation by APC. Free protein S enhances cleavage at Arg562 two-fold.

Protein S

Protein S circulates through plasma both in a free form (40%) and in 1:1 complex with C4b binding protein (C4bBP) (60%), a protein of the acute phase classical complement system. *In vivo*, all C4bBP binds to protein S thus the free protein S is determined by the molar excess of protein S in plasma [26]. Protein S has anticoagulant properties by hampering thrombin formation but it is also a cofactor of APC in the inactivation of FV(a) and FVIIIa (see above). It is reported that free protein S and thrombin-cleaved protein S can bind FVa and independently of APC inhibit prothrombin activation [27-29]. Furthermore free protein S and protein S bound to C4bBP, but not thrombin-cleaved protein S, can bind and inhibit FXa in prothrombinase. In the tenase complex C4bBP-protein S inhibits FX activation by binding to FVIIIa [30, 31]. Thus, protein S can exert an APC-independent inhibitory effect on coagulation through interaction with FVIIIa, FVa, FXa and phospholipids [32]. Furthermore, only the free protein S is reported to have APC cofactor activity.

It can be concluded from these paragraphs that thrombin exerts a dual action in hemostasis: It acts as a potent procoagulant enzyme by activating many other pro-coagulant enzymes and it is a key regulator of the anticoagulant pathway by activating the protein C-protein S pathway and by modulating fibrinolysis via TAFI.

1.2 Thrombosis

Most people may be familiar with the classical bleeding disorder hemophilia (A or B), resulting in extreme low levels of factors VIII or IX. Bleeding problems may, nonetheless, also result from rare cases of other deficiencies in clotting factors (factor V, VII, X, XIII, or prothrombin deficiency). However, in this thesis I will focus on (coagulation) disorders that lead to venous thrombosis.

When normal hemostasis is disturbed *e.g.* the balance between pro- and anticoagulant reactions is pushed towards the procoagulant side, thrombosis may occur. The triad of Virchow (1859) represents three factors involved in thrombosis: changes in the vessel wall, reduction in blood flow and changes in blood composition. A distinction between arterial and venous thrombosis can be made based upon the blood vessel in which the thrombus arises. Arterial thrombosis occurs in arteries e.g. cerebrovascular accident (CVA) or myocardial infarction (MI). Venous thrombosis occurs in the veins (pulmonary embolism, sinus thrombosis and deep venous thrombosis). Due to differences in "initiation" mechanisms and variation in fibrin content of the plug arterial and venous thrombosis are quite different processes that lead to different types of thrombi.

In arteries the shear force and flow are high and platelets are easily activated. Also activated platelets secrete and bind to von Willebrand Factor (vWF) multimers under high shear [33]. In these larger arteries, the formation of a thrombus is often the result of a rupture in the vessel wall. This is most pronounced in arteriosclerotic lesions [34] where tissue factor containing macrophages and monocytes[35] are present and the subendothelium is packed with collagen. After damage of the vessel wall, activated platelets easily adher and fibrin deposition occurs[36], resulting in a thrombus that is rich in platelets and fibrin.

In veins a different process is thought to occur. Under normal conditions a low level of coagulation factor activation probably occurs continuously. However, inhibitors of coagulation and the protein C pathway are capable of counterbalancing this. In patients with so-called prothrombotic states, this *in vivo* activation presumably is increased and is in disbalance with the anticoagulant capacity. Decreased blood flow or stasis in veins leads to local accumulation of activation products of the coagulation system and fibrinolysis, which can induce endothelial damage that in turn leads to further activation of the hemostatic system. Locally this may result in

concentrations of activated clotting factors that rise above a certain threshold at which anticoagulant proteins are no longer able to withhold the procoagulant force. Accordingly, venous thrombosis can be regarded as the clinical symptom of hypercoagulable blood.

Risk factors for venous thrombosis

A subdivision can be made in two major classes of risk factors for venous thrombosis, hereditary and acquired risk factors (Table 1.1). Hereditary factors that lead to the development of venous thrombosis are often associated with mutations in proteins that are involved in the down-regulation of coagulation. Such mutations can lead to deficiencies in anticoagulant proteins like protein S (PS), antithrombin (AT) or protein C (PC), which result in impaired down-regulation of the clotting system. In 8% of VTE patients these genetic alterations are the probable cause of venous thrombosis. These mutations follow an autosomal dominant trait and thrombosis develops at a relatively young age (below 50 years). In general there are two types of deficiencies in AT, PC and PS, type I and type II deficiency. In type I deficiency both antigen and activity are reduced, a quantitative deficiency. In type II deficiency, a qualitative deficiency, functional defects lead to a reduced activity, while the antigen level of the protein is normal. For AT and PC several subtypes of deficiency type II are recognized, depending on the site of the mutation. For protein S deficiency an additional type III is recognized in which only the free protein S antigen and activity is reduced and total protein S antigen and activity is normal. More recently, mutations in pro-coagulant coagulation factors have been reported as a probable cause of venous thrombosis. These mutations lead to proteins that are either insensitive to inactivation (FV_{Leiden}) or are over-expressed (PT20210,[37]). Moreover, elevated levels of several other clotting factors have been identified as risk factors for venous thrombosis (factors VIII[38], IX[39], XI[40] and TAFI[41]). These latter defects are found in approximately 60% of patients that develop venous thrombosis.

Acquired factors that lead to an increased risk for thrombosis are generally transient states of hypercoagulability that occur during surgery, immobilization, use of oral contraceptives and hormone replacement therapy or underlying diseases. Pregnancy and the post-partum period are also periods during which women are at high risk for the development of venous thrombosis (Table 1.1).

Table 1.1 Risk factors for venous thrombosis

Hereditary risk factors	*Acquired risk factors*
Antithrombin deficiency	Immobilisation
Protein C deficiency	Surgery / Trauma
Protein S deficiency	Pregnancy / post-partum
FV-Leiden (FV R506Q)	Oral contraceptive use
PT20210 (G20210A, high levels of prothrombin)	Lupus anticoagulans (ACA, anti-ß$_2$GPI)
	Cancer
Presumably Hereditary	
High levels of Factor VIII	
High levels of Factor IX	
High levels of Factor XI	
High levels of TAFI	
APC resistance (without FV-Leiden)	

It is well accepted that venous thrombosis is a multifactorial disease[42, 43] and the so-called second hit hypothesis states that more risk factors have to be present to cause a thrombotic event. 73% of the patients with co-segregation of FV_{Leiden} and protein C deficiency suffered from venous thrombosis, compared to 31% and 13% resp. in the patients with either protein C deficiency or FV_{Leiden} [44]. This indicates that co-existence of two or more mutations may increase the risk for venous thrombosis several-fold. The interaction of environmental factors and genetic status might also predispose individuals to an increased risk for thrombosis [42]. Additionally, more polymorphisms and mutations are being identified that may render a person more vulnerable to thrombosis (e.g. TFPI-polymorphism [45], R2-Factor V [46], protein C [47]).

1.3 APC resistance

The discovery of mutations and polymorphisms in the human genome has contributed a great deal to understanding the molecular basis of many diseases. Until 1993 the genetic causes for thrombophilia were inherited deficiencies of protein S, protein C or antithrombin, although these only accounted for 8% of VTE cases. In 1993, Dahlbäck and coworkers were the first to report that in a population of patients with venous thrombosis, the clotting time was poorly prolonged after the addition of APC [48]. These patients were called APC resistant. One year later, APC resistance was reported to result from a single point mutation in the FV-molecule at a predominant cleavage site of APC (the G to A base substitution at position 1691

leading to an Arg to Gln mutation at position 506) [49-52]. This mutation renders the FV-molecule less susceptible for cleavage and inactivation by APC [53-55]. Moreover, FV_{Leiden}, or FV^{R506Q} as it is called, is reported to have full procoagulant activity but a reduced cofactor activity in the inactivation of factor FVIIIa by APC[56]. As a result of this, down-regulation of thrombin formation is impaired. This defect may lead to a higher level of ongoing thrombin formation, which could induce venous thrombosis in these patients. Furthermore, the increased thrombin formation may result in higher levels of activated TAFI, impairing fibrinolysis in these subjects. The FV_{Leiden} mutation appears to be present in 20-40% of the patients with deep-venous thrombosis and its prevalence is 5% in the Caucasian population [57].

The discovery of this important risk factor for venous thrombosis led to the development of various clotting assays to quantitate APC resistance. Most of these are based on quantitation of the effect of activated protein C, either exogenously added APC or by activating the protein C present in plasma, on clotting times initiated via the intrinsic pathway. An alternative approach which is used throughout this thesis concerns assessment of the efficacy by which APC downregulates the generation of thrombin in tissue factor initiated coagulation. In this approach the total amount of thrombin formed is assessed by measurement of the amount of thrombin that becomes trapped in the α2M-thrombin complex in the absence and presence of exogeneously added APC.[58, 59]

Oral contraceptives and APC-resistance

Ever since their use, oral contraceptives (OC) are associated with an increased risk for venous thromboembolism. However the relative small increase in risk associated with the low dose estrogen containing second generation pills was generally considered acceptable as the benefits outweighed the disadvantages. With the marketing of so-called third generation OC (low dose estrogen with desogestrel or gestodene) it was expected that this risk would be decreased even further. However in 1995 several epidemilogical findings indicated that women using third generation OC (desogestrel or gestodene as progestagen) had a 2-3 fold higher risk for venous thrombosis compared to older oral contraceptive preparations containing second generation progestagens (levonorgestrel)[60-62]. These data were highly disputed not in the least for want of a biological explanation. In the same year two

other reports appeared that mentioned the occurrence of a mild APC resistant phenotype in plasma of oral contraceptives users in an aPTT-based clotting assay [63-65]. Moreover, in 1997, using the assay developed in Maastricht it was reported that plasma of women who were using OC, was found to exhibit an APC resistant phenotype similar to that observed for FV_{Leiden} carriers [59]. The results obtained with the thrombin generation-based APC resistance assay showed similar trends as the risks observed by epidemiologists for different OC preparations and FV_{Leiden} carriers. Thus it was proposed that OC-induced APC resistance might explain the risk increase reported for OC users [66] (Table 1.2).

Table 1.2 Relative risk for venous thrombosis and results from tissue factor-based assay

	Relative risk	APC resistance value[59]
Women no FV_{Leiden}		
no OC	1	1.22
2nd generation OC	3.4[60-62]	1.81
3rd generation OC	6-8[61]	2.59
Heterozygous FV_{Leiden}		
no OC	7[67]	3.10
using OC	30-50[66,68]	5.41
Homozygous FV_{Leiden}	>50[69]	4.75

Aim of this thesis

The work presented in this thesis was initiated to follow up the original observations published in 1997 [59]. The first part of this thesis is devoted to the details of the tissue factor-based or thrombin generation-based APC resistance assay. Both pre-analytical and analytical variables that can affect the outcome of the assay are described in Chapter 2. We have investigated the effects of different molar concentrations of citrate, the time of processing of blood, different centrifugation steps, storage temperature and thawing on APC resistance in normal individuals, FV-Leiden carriers and OC users. Also the effects of analytical variables, such as the concentrations of tissue factor, calcium and APC are described. In chapter 3 the classical clotting test and the tissue factor-based assay are compared. Both tests are able to determine with reasonable specificity the FVLeiden mutation, but a difference

is observed between the two assays in screening for acquired APC resistance developed during OC use. Furthermore Chapter 4 describes how the results obtained with the tissue-factor based test are influenced by the presence of hereditary and acquired risk factors for venous thrombosis.

The second part concerns the mechanism(s) underlying oral contraceptive-induced APC resistance. Since the original pill study [59] concerned a non-cycle controlled, cross-sectional set-up, a second study was performed in a cycle-controlled randomized clinical setting, in which the effect of OC use on a large number of hemostatic parameters were measured (Chapter 5). Additionally, in vitro fertilization treatment was used to determine the effects of changes in endogenous estrogen and progesterone on APC resistance (Chapter 6). The final chapter (Chapter 7) comprises a study that evaluates the effects of second and third generation oral contraceptives in non-carriers and carriers of the FV_{Leiden} mutation using combined preparations, as well as progestagen-only preparations.

Chapter 8 contains a summary and general discussion of the results presented in this thesis, on hereditary and acquired APC resistance measured in the tissue factor based assay.

References:

1. Nelsestuen GL, Kisiel W, Di Scipio RG. Interaction of vitamin K dependent proteins with membranes. Biochemistry 1978; 17:2134-8.
2. Kaplan AP, Ghebrehiwet B, Silverberg M, Sealey JE. The intrinsic coagulation-kinin pathway, complement cascades, plasma renin-angiotensin system, and their interrelationships. Crit Rev Immunol 1981; 3:75-93.
3. Fressinaud E, Baruch D, Girma JP, Sakariassen KS, Baumgartner HR, Meyer D. von Willebrand factor-mediated platelet adhesion to collagen involves platelet membrane glycoprotein IIb-IIIa as well as glycoprotein Ib. J Lab Clin Med 1988; 112:58-67.
4. Oliver JA, Monroe DM, Roberts HR, Hoffman M. Thrombin activates factor XI on activated platelets in the absence of factor XII. Arterioscler Thromb Vasc Biol 1999; 19:170-7.
5. Nawroth PP, Stern DM. A pathway of coagulation on endothelial cells. J Cell Biochem 1985; 28:253-64.
6. Stern D, Nawroth P, Handley D, Kisiel W. An endothelial cell-dependent pathway of coagulation. Proc Natl Acad Sci U S A 1985; 82:2523-7.
7. Osterud B, Rapaport SI. Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. Proc Natl Acad Sci U S A 1977; 74:5260-4.
8. Marlar RA, Kleiss AJ, Griffin JH. An alternative extrinsic pathway of human blood coagulation. Blood 1982; 60:1353-8.
9. Rosing J, Tans G, Govers Riemslag JWP, Zwaal RFA, Hemker HC. The role of phospholipids and factor Va in the prothrombinase complex. J Biol Chem 1980; 255:274-283.
10. von dem Borne PA, Meijers JC, Bouma BN. Feedback activation of factor XI by thrombin in plasma results in additional formation of thrombin that protects fibrin clots from fibrinolysis. Blood 1995; 86:3035-42.
11. Von dem Borne PA, Bajzar L, Meijers JC, Nesheim ME, Bouma BN. Thrombin-mediated activation of factor XI results in a thrombin- activatable fibrinolysis inhibitor-dependent inhibition of fibrinolysis. J Clin Invest 1997; 99:2323-7.
12. Bajzar L, Morser J, Nesheim M. TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex. J Biol Chem 1996; 271:16603-8.
13. Sandset PM, Abildgaard U. Extrinsic pathway inhibitor--the key to feedback control of blood coagulation initiated by tissue thromboplastin. Haemostasis 1991; 21:219-39.
14. Rao LV, Rapaport SI. Studies of a mechanism inhibiting the initiation of the extrinsic pathway of coagulation. Blood 1987; 69:645-51.
15. Owen WG, Esmon CT. Functional properties of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. J Biol Chem 1981; 256:5532-5.
16. Esmon CT, Owen WG. Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. Proc Natl Acad Sci U S A 1981; 78:2249-52.
17. De Cristofaro R. Effect of thrombomodulin on the molecular recognition and early catalytic events in thrombin-protein C interaction. Thromb Haemost 1996; 76:556-60.
18. Esmon CT. The roles of protein C and thrombomodulin in the regulation of blood coagulation. J Biol Chem 1989; 264:4743-6.
19. Fukudome K, Esmon CT. Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. J Biol Chem 1994; 269:26486-91.
20. Stearns-Kurosawa DJ, Kurosawa S, Mollica JS, Ferrell GL, Esmon CT. The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. Proc Natl Acad Sci U S A 1996; 93:10212-6.
21. Rosing J, Hoekema L, Nicolaes GAF, et al. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor VaR506Q by activated protein C. J Biol Chem 1995; 270:27852-8.
22. Yegneswaran S, Wood GM, Esmon CT, Johnson AE. Protein S alters the active site location of activated protein C above the membrane surface. A fluorescence resonance energy transfer study of topography. J Biol Chem 1997; 272:25013-21.
23. Nicolaes GAF, Tans G, Thomassen MCLGD, et al. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor VaR506Q by activated protein C. Journal of Biological Chemistry 1995; 270:21158-66.

24. Thorelli E, Kaufman RJ, Dahlback B. Cleavage of factor V at Arg 506 by activated protein C and the expression of anticoagulant activity of factor V. Blood 1999; 93:2552-8.
25. Guinto ER, Esmon CT. Loss of prothrombin and of factor Xa-factor Va interactions upon inactivation of factor Va by activated protein C. J Biol Chem 1984; 259:13986-92.
26. Griffin JH, Gruber A, Fernandez JA. Reevaluation of total, free, and bound protein S and C4b-binding protein levels in plasma anticoagulated with citrate or hirudin. Blood 1992; 79:3203-11.
27. Hackeng TM, Hessing M, van 't Veer C, et al. Protein S binding to human endothelial cells is required for expression of cofactor activity for activated protein C. J Biol Chem 1993; 268:3993-4000.
28. Heeb MJ, Mesters RM, Tans G, Rosing J, Griffin JH. Binding of protein S to factor Va associated with inhibition of prothrombinase that is independent of activated protein C. J Biol Chem 1993; 268:2872-2877.
29. Heeb MJ, Rosing J, Bakker HM, Fernandez JA, Tans G, Griffin JH. Protein S binds to and inhibits factor Xa. Proc Natl Acad Sci USA 1994; 91:2728-32.
30. Koppelman SJ, Hackeng TM, Sixma JJ, Bouma BN. Inhibition of the intrinsic factor X activating complex by protein S: evidence for a specific binding of protein S to factor VIII. Blood 1995; 86:1062-71.
31. Koppelman SJ, van't Veer C, Sixma JJ, Bouma BN. Synergistic inhibition of the intrinsic factor X activation by protein S and C4b-binding protein. Blood 1995; 86:2653-60.
32. van Wijnen M, Stam JG, van't Veer C, et al. The interaction of protein S with the phospholipid surface is essential for the activated protein C-independent activity of protein S. Thromb Haemost 1996; 76:397-403.
33. Goto S, Ikeda Y, Saldivar E, Ruggeri ZM. Distinct mechanisms of platelet aggregation as a consequence of different shearing flow conditions. J Clin Invest 1998; 101:479-86.
34. Sakariassen KS, Barstad RM. Mechanisms of thromboembolism at arterial plaques. Blood Coagul Fibrinolysis 1993; 4:615-25.
35. McGee MP, Foster S, Wang X. Simultaneous expression of tissue factor and tissue factor pathway inhibitor by human monocytes. A potential mechanism for localized control of blood coagulation. J Exp Med 1994; 179:1847-54.
36. Weiss HJ, Turitto VT, Baumgartner HR. Role of shear rate and platelets in promoting fibrin formation on rabbit subendothelium. Studies utilizing patients with quantitative and qualitative platelet defects. J Clin Invest 1986; 78:1072-82.
37. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood 1996; 88:3698-703.
38. Kamphuisen PW, Lensen R, Houwing-Duistermaat JJ, et al. Heritability of elevated factor VIII antigen levels in factor V Leiden families with thrombophilia. Br J Haematol 2000; 109:519-22.
39. van Hylckama Vlieg A, van der Linden IK, Bertina RM, Rosendaal FR. High levels of factor IX increase the risk of venous thrombosis. Blood 2000; 95:3678-82.
40. Meijers JC, Tekelenburg WL, Bouma BN, Bertina RM, Rosendaal FR. High levels of coagulation factor XI as a risk factor for venous thrombosis. N Engl J Med 2000; 342:696-701.
41. van Tilburg NH, Rosendaal FR, Bertina RM. Thrombin activatable fibrinolysis inhibitor and the risk for deep vein thrombosis. Blood 2000; 95:2855-9.
42. Rosendaal FR. Venous thrombosis: a multicausal disease. Lancet 1999; 353:1167-73.
43. Rosendaal FR. Thrombosis in the young: epidemiology and risk factors. A focus on venous thrombosis. Thromb Haemost 1997; 78:1-6.
44. Koeleman BP, Reitsma PH, Allaart CF, Bertina RM. Activated protein C resistance as an additional risk factor for thrombosis in protein C-deficient families. Blood 1994; 84:1031-5.
45. Kleesiek K, Schmidt M, Gotting C, et al. The 536C-->T transition in the human tissue factor pathway inhibitor (TFPI) gene is statistically associated with a higher risk for venous thrombosis. Thromb Haemost 1999; 82:1-5.
46. Lunghi B, Iacoviello L, Gemmati D, et al. Detection of new polymorphic markers in the factor V gene: association with factor V levels in plasma. Thromb Haemost 1996; 75:45-8.
47. Aiach M, Nicaud V, Alhenc-Gelas M, et al. Complex association of protein C gene promoter polymorphism with circulating protein C levels and thrombotic risk. Arterioscler Thromb Vasc Biol 1999; 19:1573-6.
48. Dahlbäck B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. Proc Natl Acad Sci U S A 1993; 90:1004-8.

49. Bertina RM, Koeleman BPC, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994; 369:64-67.
50. Greengard JS, Sun X, Xu X, Fernandez JA, Griffin JH, Evatt B. Activated protein C resistance caused by Arg506Gln mutation in factor Va. Lancet 1994; 343:1361-2.
51. Voorberg J, Roelse J, Koopman R, et al. Association of idiopathic venous thromboembolism with single point-mutation at Arg506 of factor V. Lancet 1994; 343:1535-6.
52. Zöller B, Dahlbäck B. Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis. Lancet 1994; 343:1536-8.
53. Sun X, Evatt B, Griffin JH. Blood coagulation factor Va abnormality associated with resistance to activated protein C in venous thrombophilia. Blood 1994; 83:3120-5.
54. Heeb MJ, Kojima Y, Greengard JS, Griffin JH. Activated protein C resistance: molecular mechanisms based on studies using purified Gln506-factor V. Blood 1995; 85:3405-11.
55. Kalafatis M, Haley PE, Lu DS, Bertina RM, Long GL, Mann KG. Proteolytic events that regulate factor V activity in whole plasma from normal and activated protein C (APC)-resistant individuals during clotting: An insight into the APC-resistance assay. Blood 1996; 87 (11):4695-4707.
56. Váradi K, Rosing J, Tans G, Pabinger I, Keil B, Schwarz HP. Factor V enhances the cofactor function of protein S in the APC-mediated inactivation of factor VIII: influence of the Factor VR506Q mutation. Thromb Haemostas 1996; 76(2):208-214.
57. Beauchamp NJ, Daly ME, Hampton KK, Cooper PC, Preston FE, Peake IR. High prevalence of a mutation in the factor V gene within the U.K. population: relationship to activated protein C resistance and familial thrombosis. Br J Haematol 1994; 88:219-22.
58. Nicolaes GAF, Thomassen MCLGD, Tans G, Rosing J, Hemker HC. Effect of activated protein C on thrombin generation and on the thrombin potential in plasma of normal and APC-resistant individuals. Blood Coagulation and Fibrinolysis 1997; 8:28-38.
59. Rosing J, Tans G, Nicolaes GAF, et al. Oral contraceptives and venous thrombosis: Different sensitivities to activated protein C in women using second- and third-generation oral contraceptives. British Journal of Haematology 1997; 97:233-238.
60. WHO. World Health Organization Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception. Venous thromboembolic disease and combined oral contraceptives: results of international multicentre case-control study. Lancet 1995a; 346:1575-82.
61. WHO. World Health Organization Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception. Effect of different progestagens in low oestrogen oral contraceptives on venous thromboembolic disease. World Health Organization Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception. Lancet 1995b; 346:1582-1588.
62. Jick H, Jick SS, Gurewich V, Myers MW, Vasilakis C. Risk of idiopathic cardiovascular death and nonfatal venous thromboembolism in women using oral contraceptives with differing progestagen components. Lancet 1995; 346:1589-93.
63. Meinardi JR, Henkens CMA, Heringa MP, vanderMeer J. Acquired APC resistance related to oral contraceptives and pregnancy and its possible implications for clinical practice. Blood Coagulation and Fibrinolysis 1997; 8:152-154.
64. Hellgren M, Svensson PJ, Dahlback B, et al. Resistance to activated protein C as a basis for venous thromboembolism associated with pregnancy and oral contraceptives. Am J Obstet Gynecol 1995; 173:210-3.
65. Olivieri O, Friso S, Manzato F, et al. Resistance to activated protein C in healthy women taking oral contraceptives. British Journal of Haematology 1995; 91:465-470.
66. Vandenbroucke JP, Helmerhorst FM, Bloemenkamp KWM, Rosendaal FR. Third-generation oral contraceptive and deep venous thrombosis: From epidemiologic controversy to new insight in coagulation. American Journal of Obstetrics and Gynaecology 1997; 177:887-891.
67. Koster T, Rosendaal FR, de Ronde H, Briet E, Vandenbroucke JP, Bertina RM. Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. Lancet 1993; 342:1503-6.
68. Bloemenkamp KWM, Rosendaal FR, Helmerhorst FM, B□ller HR, Vandenbroucke JP. Enhancement by factor V Leiden mutation of risk of deep- vein thrombosis associated with oral contraceptives containing third- generation progestagen. Lancet 1995; 346:1593-1596.
69. Rosendaal FR, Koster T, Vandenbroucke JP, Reitsma PH. High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). Blood 1995; 85:1504-1508.

PART I

Measurement of APC resistance

Laboratory tests for APC resistance

The discovery of APC resistance as a risk factor for venous thrombosis has led to the development of an enormous amount of assays to detect APC resistance. At first, efforts were aimed at developing assays with the highest specificity assays to detect FV_{Leiden} mutation. The most well known might be DNA-analysis of the MnI restriction site in the B-domain of FV, that is lost in carriers of the FV_{Leiden} mutation. This assay is 100% specific and sensitive to the mutation. Functional assays for APC resistance in diluted plasma also give a near 100% specificity and sensitivity to the FV_{Leiden} mutation. By diluting in V-deficient plasma, the assay becomes fully sensitive to factor V[1] without being influenced by individual variations in other plasma components (e.g. protein S[2]). Another advantage is that these assays are not influenced by anticoagulant therapy.[3] Functional analysis has the advantage over DNA analysis that is also provides insight to the thrombophilic mechanism and the *in vivo* phenotype (e.g. pseudo-homozygosity[4] or APC resistance combined with FV-deficiency[5]).

For a certain period it was thought that the panel of assays available (DNA analysis and functional dilution assays) provided sufficient insight in (pro)thrombotic status of an individual. However, in order to appraoch more closely the *in vivo* situation, measurements in undiluted plasma or even in whole blood become inevitable. Such measurements may have the disadvantage that they are less specific for the FV_{Leiden} mutation and show more individual variation. The consensus, however, is emerging that this is precisely the additional information needed in order to be able to assess whether a certain individual is at risk for the development of

thrombosis or not. With regard to APC resistance measurements this means that it is now recognised that a person may not only be at risk as a result of the defect in FV_{Leiden} but that it is the overall response to the anticoagulant action of APC (inlcuding acquired APC resistance states such as occurring during OC-use and pregnancy) that is important. This is reflected in the fact that APC resistance as such even in the absence of FV_{Leiden} has been established to be associated with the occurrence of venous thrombosis.[6-8]

Thus measurements in undiluted plasma have been firmly reestablished in the panel of laboratory testing for APC resistance. These tests can differ in trigger (e.g. kaolin (aPTT), tissue factor (PT and ETP), factor V or X activating russels viper venom (RVV-V and RVV-X respectively)) and they may be performed by either adding activated protein C or by activating the protein C in plasma (by a purified snake venom from the *Agkistrodon concortix concortix*). The assay that we chose to develop further is based on measurement of thrombin formation.[9] Rather than the more limited measurement of clotting times. The total amount of thrombin that is formed (the so-called endogenous thrombin potential or ETP) appears to be a good indicator for the pro-coagulant condition of a plasma sample.[9, 10] The total amount of thrombin formed after initiation with tissue factor, is directly proportional to the endlevel of α2-macroglobulin-thrombin (α2-M-IIa) complex after 20 minutes[11] both in the absence and presence of APC.[12] Thus, rather than determining the full course of thrombin formation both in the absence and presence of APC, APC resistance can simply be determined by quantifying the effect of APC on the endlevel of α2-M-IIa complexes formed in a certain plasma[12] (for reviews see [13, 14]). The specifics of this assay will be discussed in the next chapters.

Chapter 2

Analytic and pre-analytic variables

Based on:

Effects of (pre-)analytical variables on activated protein C resistance determined via the endogenous thrombin potential. Joyce Curvers, M. Christella L.G.D. Thomassen, Hans de Ronde, Rogier M. Bertina, Frits R. Rosendaal, Guido Tans and Jan Rosing. Submitted

Summary

The normalized activated protein C sensitivity ratio (nAPC-sr) determined with an assay that quantifies the effect of APC on thrombin formation initiated via the extrinsic coagulation pathway identifies hereditary and acquired defects of the protein C system. We investigated the influence of assay conditions (analytical variables) and plasma handling (pre-analytical variables) on nAPCsr obtained with this APC resistance test.

The effect of the analytical variables ($CaCl_2$, phospholipid and APC concentrations and the concentration and source of tissue factor) was determined in pooled normal plasma. Inhibition of thrombin formation by APC was dependent on the APC concentration and was also affected by the tissue factor, Ca^{2+} and phospholipid concentrations. Thus, strict standardization of reactant concentrations is required to obtain reproducible nAPCsr.

Three different tissue factor preparations were compared by determining nAPCsr in plasma samples obtained from 90 healthy individuals. nAPC-sr were similar for all three tissue factor preparations although, compared with the non-commercially available tissue factor used in earlier studies, values determined with commercial tissue factor preparations showed larger variation.

Pre-analytical variables, investigated in plasma of nine volunteers (3 normal individuals and 6 individuals with an APC-resistant phenotype) were: concentration of anticoagulant (3.2% vs. 3.8% trisodiumcitrate), time before processing of blood (0, 4 and 24 hours), centrifugation speed, storage temperature of plasma (-20 °C vs. -80 °C) and sample thawing. Multiple linear regression analysis showed that only the citrate concentration affected the nAPC-sr, which was higher in samples collected in 3.2% trisodiumcitrate than in samples collected in 3.8%. trisodiumcitrate.

Introduction

The identification of the A1691G mutation in the factor V gene,[15-18] resulting in a mutated FV molecule (FV_{Leiden}, FV^{R506Q}), has led to a higher number of patients with an identifiable prothrombotic disorder. Arg506 represents a predominant target for proteolytic inactivation of FVa by activated protein C (APC) and as a result of the Arg506-Gln mutation FVa_{Leiden} is more resistant to APC.[15, 19-21] Moreover, it appears that the mutation in FV_{Leiden} also results in a substantial loss of the cofactor activity of FV in APC-dependent down-regulation of FVIII(a).[22, 23]

APC-resistance, which is found in 20% of patients with venous thrombosis[24, 25] also occurs in individuals without FV_{Leiden} and recently, it was shown that APC resistance in the absence of the FV_{Leiden} mutation is a risk factor for venous thrombosis.[6, 7, 26] APC resistance without factor V_{Leiden} can be attributed to other hereditary defects of the protein C pathway or to acquired coagulation abnormalities occuring during oral contraceptive (OC) use or pregnancy.[27-30]

In 1997 we developed an assay[12, 29] in which the anticoagulant effect of APC in plasma is quantified by measuring the effect of APC on the time integral of thrombin formation (the endogenous thrombin potential, ETP[31]) initiated via the extrinsic coagulation pathway. This assay not only allows detetection of the FV_{Leiden} mutation, but is also sensitive for acquired APC resistance occurring during OC use.[29, 32, 33] In contrast, the classical aPTT-based APC resistance test is much less affected by OC use.[32]

Since APC sensitivity ratios (APCsr) determined with the ETP-based assay change parallel with the thrombotic risks reported in epidemiological studies,[34] this assay may gain the interest of other research or coagulation laboratories. In this paper we report how the quantitation of APC-resistance with the ETP-based assay is affected by analytical variables i.e. the APC, Ca^{2+} ions and phospholipid concentrations, and the source and concentration of tissue factor and by pre-analytical variables i.e. blood collection and handling and storage of plasma.

Materials and methods

Materials. Tris (Tris (hydroxymethyl amino-methane), Hepes (N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid), NaCl, EDTA (Ethylendinitrilo-

tetraacetic acid), BSA (bovine serum albumin) and ovalbumin were purchased from Sigma, St. Louis, USA. The chromogenic substrates D-Phe-(pipecolyl)-Arg-pNA (S2238) and L-pyroGlu-Pro-Arg-pNA (S2366) were supplied by Chromogenix, Mölndal, Sweden. Ancrod, obtained from the WHO International Laboratory for Biological Standards (Hertfortshire, England) was dissolved in distilled water (50 U/ml) and frozen in portions at -20 °C.

1,2-Dioleoyl-*sn*-glycero-3-phosphoserine (DOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids, Alabaster, Alabama, USA. Small unilamellar phospholipid vesicles composed of DOPS/DOPE/DOPC (20/20/60, M/M/M) were prepared by mixing appropriate quantities of phospholipid dissolved in $CHCl_3/CH_3OH$ (9/1 v/v) in a glass tube. The phospholipids were dried under a mild flow of N_2 and stored at -20 °C. Before use the dryed phospholipids were brought to room temperature, suspended in 25mM Hepes (pH 7.5), 175mM NaCl by rigorous vortexing for at least 1 minute and subsequently sonicated for 10 minutes at 4 °C with a MSE Soniprep 150 ultrasonic disintegrator set at 7.5 μm peak to peak amplitude. Phospholipid concentrations were determined by phosphate analysis.[35]

Purified human APC was from Enzyme Research Laboratories (ERL) and supplied by Kordia Laboratory Supplies, Leiden, The Netherlands. APC was diluted in 25 mM Hepes (pH 7.5 at 37 °C), 175 mM NaCl, 5 mg/ml BSA and frozen in small portions at -80°C. APC concentrations were determined with S2366 using kinetic parameters reported by Sala et al.[36]

Recomboplastin S without additives was a kind gift of Dr H. Pelzer (Dade, USA). Relipidated recombinant tissue factor Dade Innovin® was purchased from Behring. RecombiPlasTin® (Ortho) was from Instrumentation Laboratories. Tissue factor preparations were reconstituted according to the instructions of the manufacturer and stored at -80 °C. Tissue factor concentrations were determined with an antigen assay (American Diagnostics).

Plasma preparation for investigating the effect of analytical variables on the nAPCsr. Blood was obtained by clean venapuncture in the forearm, without pressure and discarding the first 2 ml of blood. Nine parts of blood were collected in

one part of 0.13 M (3.8%) trisodium citrate (pH 7.8) and the blood was centrifuged for 15 min at 3000x*g* at room temperature, followed by centrifugation for 30 min at 20000x*g* at 4°C.[29, 32] The cell free plasma was stored at -80°C until analysis. A pooled normal plasma was prepared from plasma of healthy volunteers not on medication, not using OC and non-pregnant (21 females and 44 males, mean age 35 years). The individuals who donated blood for normal pooled plasma were not screened for FV_{Leiden} or other hereditary coagulation abnormalities.

Plasma preparation for investigating the effect of pre-analytical variables on the nAPCsr. Blood was collected from nine volunteers: three factor V_{Leiden} negative individuals (2 men, 47 and 55 year; 1 woman, 51 yr), three heterozygous factor V_{Leiden} carriers (1 man, 30 yr; 1 woman, 41 yr and a 32 yr old woman who was 1 month pregnant) and three women (<30 years of age) using a monophasic oral contraceptive (OC) preparation containing 30 µg ethinyl estradiol and 150 µg desogestrel. The presence of the factor V_{Leiden} mutation was established by DNA analysis.[37]

The handling of the blood samples and the preparation and handling of plasma samples from the volunteers is schematically presented in Fig. 1. Nine parts of blood were collected in one part of 3.2% (0.109 M) or 3.8% (0.130 M) trisodium citrate and divided into three equal portions, one part of which was immediately further processed and the others were left 4 or 24 hours at room temperature before processing. Platelet poor plasma was obtained by centrifuging either 15 minutes at 2000xg at room temperature or 15 minutes at 3000x*g* at room temperature followed by 30 minutes at 20.000x*g* at 4°C. Samples were frozen in small aliquots and stored at either -20°C or -80 °C until analysis. For each variable one aliquot was thawed once and refrozen again. Taken together this resulted in 432 samples (9 individuals x 48 combinations) available for analysis. The nAPC-sr in six samples was not determined because the plasma was clotted upon thawing and 27 samples were left out of analysis because in these measurements residual thrombin formation determined in the presence of APC in normal plasma fell outside the range 6-16% (see also results section).

APC-resistance assay and determination of the nAPCsr. Plasmas were thawed and defibrinated with ancrod (1 U/ml final concentration) for 10 min at 37 °C after which the clot was removed with a plastic spatula (Sarstedt). Defibrinated plasma was used within 3 hours after defibrination. The nAPC-sr was routinely determined essentially as described before.[29, 32] Briefly, 80 µl defibrinated plasma was incubated at 37°C and thrombin formation was initiated with 45 µl starting solution (prewarmed at 37 °C) containing tissue factor, $CaCl_2$, phospholipid vesicles with or without APC in 25 mM Hepes (pH 7.5 at 37 °C), 175 mM NaCl, 5 mg/ml BSA. This resulted in final concentrations in plasma of 0.4 ng/ml tissue factor (corresponding to a final 940-fold dilution of stock solution of reconstituted tissue factor preparations), 16 mM added $CaCl_2$, 15 µM phospholipid vesicles (DOPS/DOPC/DOPE, 20/60/20, M/M/M) and if present, 5 nM APC. It should be noted that in earlier publications[12, 29, 32] the tissue factor concentration was abusively given as 0.1 ng/ml. When quantified with an antigen assay (American Diagnostics) the tissue factor concentration used in the previous studies was 0.4 ng/ml.

After 20 min an aliquot from the plasma mixture was diluted 50-fold in a 50 mM Tris buffer (pH 7.5 at 37°C) containing 175 mM NaCl, 20 mM EDTA and 0.5 mg/ml ovalbumin. The amount of α_2M-thrombin complex present in the diluted plasma mixture, which is a measure for the ETP (see below), was quantified by adding an appropriate aliquot from the diluted plasma to the well of a microtiterplate containing buffer with S2238. The well finally contained 250 µl 50 mM Tris (pH 7.5 at 37°C), 175 mM NaCl, 0.5 mg/ml ovalbumin, 20 mM EDTA and 235 µM S2238. The rate of change in absorbance was determined at 405 minus 492 nm at 37°C in a 340 ATTC microtiterplate reader (SLT Labinstruments, Salzburg, Austria) set in the kinetic mode. The amidolytic activity was corrected by blank values measured in plasma samples (80 µl) to which 45 µl 25 mM Hepes (pH 7.5 at 37 °C), 175 mM NaCl, 5 mg/ml BSA with or without APC was added and which were subjected to the same incubation and dilution procedure as described above.

In routine nAPC-sr determinations 21 subject plasmas together with 3 samples of pooled normal plasma (first, middle and last sample) were determined in duplicate to yield a total of 96 amidolytic measurements on a single microtiterplate (48 with and 48 without APC).

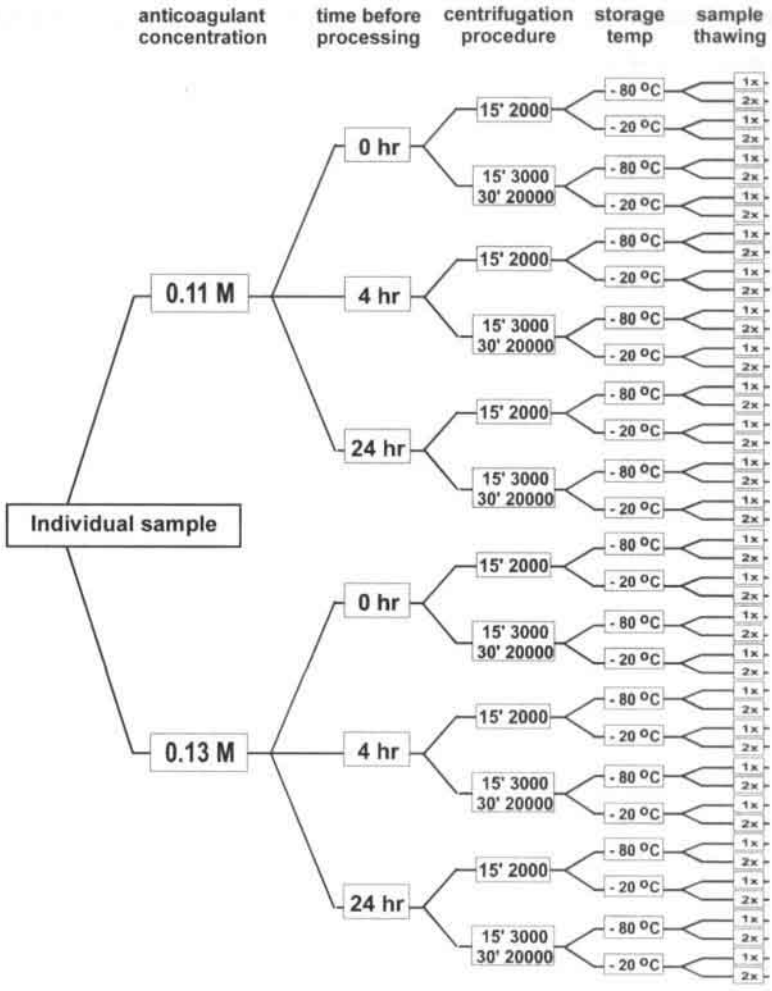


Figure 2.1 Schematic diagram of different procedures of plasma preparation and storage

Calculation of nAPCsr. Since the final level of α_2-macroglobulin-thrombin (α_2M-IIa) complexes is proportional to the ETP, the effect of APC on the ETP can be quantified by measuring the amidolytic activity of the α_2M-IIa complex generated in plasma with and without APC.[12, 38] From the α_2M-IIa amidolytic activities (+/- APC) thus obtained in subject plasma and in pooled normal plasma determined in the same microtiterplate the nAPC-sr was calculated as:

$$nAPC\text{-}sr=(\alpha 2M\text{-}IIa_{+APC}/\alpha 2M\text{-}IIa_{-APC})_{plasma\ sample}/(\alpha 2M\text{-}IIa_{+APC}/\alpha 2M\text{-}IIa_{-APC})_{pooled\ normal\ plasma}$$

Determination of the effect of variation of the analytical variables. The effect of variation of analytical variables (*i.e.* the APC, tissue factor, phospholipid and Ca^{2+} concentrations) on the efficacy by which APC inhibits thrombin formation was determined by varying the amount present in the assay mixture while keeping the others constant at the concentration at which the standard assay is performed (0.4 ng/ml tissue factor, 16 mM added $CaCl_2$, 15 μM phospholipid vesicles and 5 nM APC). The effect of the use of different tissue factor preparations in the assay was analyzed by determining the nAPC-sr in 90 normal healthy volunteers who donated blood for the normal plasma pool.

Statistics. The influence of pre-analytic variables on the nAPCsr of the plasma samples of the nine individual volunteers was assessed in a multiple linear regression model with nAPC-sr as dependent variable using indicator variables for the nine individuals. In the comparison different tissue factor preparations differences of means and 95% confidence intervals were calculated in the standard fashion.

Results

The ETP-based APC resistance test. The ETP-based APC resistance test presented in earlier reports is based on determination of the effect of APC on the time integral of thrombin generation, the ETP, initiated in plasma via the extrinsic coagulation pathway. To quantify the effect of APC on thrombin generation an APC sensitivity ratio (APCsr) was defined[12] that is equal to the ratio of the endogenous thrombin potentials determined in the presence and absence of APC. In order to improve day to day reproducibility the APCsr was normalized by division through the same ratio determined in pooled normal plasma (eq. 1).

$$\text{nAPC-sr} = (ETP_{+APC}/ETP_{-APC})_{\text{plasma sample}}/(ETP_{+APC}/ETP_{-APC})_{\text{pooled normal plasma}} \quad \text{(eq. 1}$$

which reduces to eq. 2 when the residual ETP determined in the presence of APC is expressed as percentage of the ETP determined without APC.

$$\text{nAPC-sr} = \text{Residual ETP (\%)}_{\text{plasma sample}}/\text{Residual ETP (\%)}_{\text{pooled normal plasma}} \quad \text{(eq. 2)}$$

For quantification of the effect of APC on the ETP it is not necessary to measure complete time courses of thrombin generation. Thrombin generation curves typically

reach a residual level of amidolytic activity that can be attributed to the α_2M-IIa complex [39, 40]. The α_2M-IIa level remains constant in time and is a direct indicator for the ETP in plasmas with the same α_2M levels [12, 38]. Since this is particularly the case when the same plasma is tested with and without an effector of thrombin generation, the effect of APC on the ETP can be simply quantified on the basis of single amidolytic assays of the end levels of α_2M-IIa present in plasma in which thrombin was generated in the absence and presence of APC.

Effects of tissue factor, $CaCl_2$ and phospholipid concentrations on thrombin generation in the absence and presence of APC. Fig. 2.2 shows the results of titration experiments in which the concentration of one of the reactants (tissue factor, $CaCl_2$ or phospholipid) was varied while the others were kept constant at their respective optimal concentrations. In the absence of APC (open symbols) optimal thrombin formation was observed at 0.4 ng/ml tissue factor (Fig. 2.2A), 16 mM added $CaCl_2$ (Fig. 2.2B) and 2.5 μM phospholipid vesicles in addition to the lipid present in the relipidated tissue factor preparation (Fig. 2.2C).

In the presence of 5 nM APC thrombin formation was inhibited at all reaction conditions tested (Figs. 2.2 A-C, closed symbols). However, with 5 nM APC thrombin formation increased at increasing tissue factor and $CaCl_2$ concentrations. This indicates that the efficacy by which APC inhibited thrombin formation gradually decreased at higher tissue factor (Fig. 2.2A) and $CaCl_2$ concentrations (Fig. 2.2B). In the presence of APC, thrombin formation progressively decreased when the amount of phospholipid in the assay was increased, indicating that the ability of APC to inhibit thrombin formation was enhanced at higher phospholipid concentrations (Fig. 2.2C, closed symbols).

Fig. 2.2 D-F summarizes the effect of variation of the tissue factor, $CaCl_2$ and phospholipid concentrations on residual thrombin formation in the presence of 5 nM APC (expressed as percentage of thrombin formation determined in the absence of APC, cf. Fig. 2.2 A-C). In all cases residual thrombin formation remained a function of the concentration of reactants and increased when the amounts of tissue factor (Fig. 2.2D) and $CaCl_2$ present in the assay mixture were increased (Fig. 2.2E) and decreased at increasing phospholipid concentrations (Fig. 2.2F).

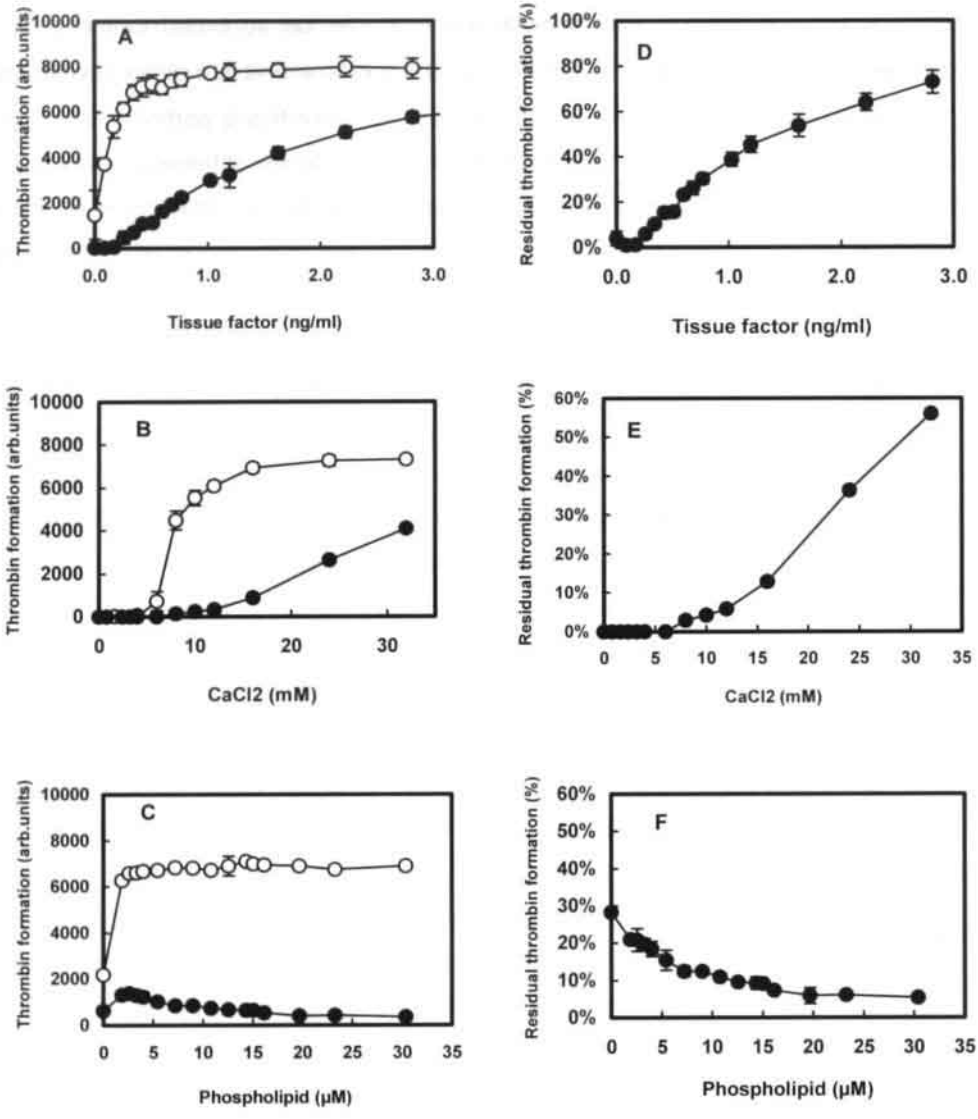


Figure 2.2 Effects of varying amounts of tissue factor, Ca^{2+} ions and phospholipid on thrombin generation in normal pooled plasma. Thrombin formation was initiated in defibrinated normal pooled plasma with a starting mixture containing varying amounts of tissue factor (Innovin), Ca^{++}-ions and phospholipid vesicles either with or without APC (A-C). Residual thrombin formation in the presence of APC, was expressed as percentage of thrombin formation determined in the absence of APC (D-F). (A, D): 16 mM Ca^{2+} ions, 15 μM phospholipid and variable amounts of tissue factor. (B, E): 0.4 ng/ml tissue factor, 15 μM phospholipid and variable amounts of Ca^{2+} ions. (C, F): 0.4 ng/ml tissue factor, 16 mM Ca^{2+} ions and variable amounts of phospholipid. The amounts of tissue factor, Ca^{++}-ions and phospholipid vesicles represent final concentrations in the assay mixture. Plotted is thrombin formation in the absence (O) or presence (•) of 5nM APC expressed in arbitrary units (final levels of α_2M- IIa complex present in plasma after 20 min). Y-error bars indicate the 95% confidence interval (n = 4).

Effects of APC on thrombin formation in normal plasma and in FV_{Leiden} plasma. The data presented in Fig. 2.2 form the basis for the reaction conditions at which the ETP-based APC resistance is performed (15μM phospholipid, 0.4 ng/ml tissue factor and 16 mM $CaCl_2$). Assay conditions were chosen such that thrombin formation in the absence of APC was near optimal, i.e. insensitive to small variations in concentrations of phospholipid, tissue factor or $CaCl_2$. The amount of phospholipid present in the assay mixture (15 μM) is higher than the concentration required for optimal thrombin formation in the absence of APC (<5 μM, Fig. 2.2C). The use of a high phospholipid concentration makes the assay insensitive to small amounts of phospholpid present in plasma. Moreover, the amount of APC required to inhibit thrombin formation is less at high phospholipid concentration (Fig 2.2F), which reduces the contribution of APC to the amidolytic activity determined to calculate the nAPCsr. The experiment presented in Fig. 2.3 shows the effect of increasing APC concentrations on thrombin generation in pooled normal plasma and in plasmas from a normal healthy male volunteer and from a male heterozygous carrier factor V_{Leiden}. In all plasmas residual thrombin formation gradually decreased at increasing APC concentrations. However, thrombin formation in the plasma from the heterozygous carrier of factor V_{Leiden} was less sensitive to APC than that in plasma from the wildtype volunteer. At all APC concentrations tested the latter plasma showed a sensitivity towards APC similar to that of pooled normal plasma.

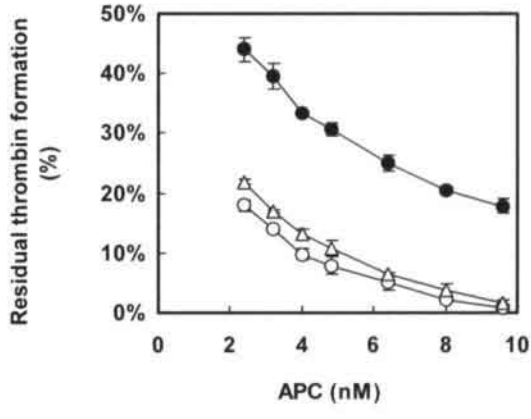


Figure 2.3 Effects of varying amounts APC on APCsr of normal pooled plasma, plasma from a normal healthy individual and factor V_{Leiden} plasma. Thrombin formation was initiated in defibrinated plasma with a mixture of tissue factor (Innovin), Ca^{2+}-ions and phospholipid vesicles containing varying amounts of APC. The final concentrations of reactants present in the assay mixture were 0.4 ng/ml tissue factor, 16 mM Ca^{2+} ions, 15 μM phospholipid and concentrations of APC indicated in the figure. Residual thrombin formation in the presence of APC, expressed as percentage of thrombin formation determined in the absence of APC in normal pooled plasma (○), plasma of a normal healthy volunteer (△) and plasma of a heterozygous carrier of factor V_{Leiden} (●). Y-error bars indicate the 95% confidence interval (n=4).

Quantification of the effect of APC on thrombin generation. In APC resistance tests the sensitivity of a particular plasma for APC is commonly determined on the basis of an APC sensitivity ratio (APCsr) which is defined as the ratio of the test result with and without APC. In the case of the ETP-based APC resistance test this yields:

$$APCsr = ETP_{+APC}/ETP_{-APC} = Residual\ ETP\ (\%)_{plasma\ sample}/100$$

in which residual ETP $(\%)_{plasma\ sample}$ is the ETP determined in the presence of APC expressed as percentage of that determined without APC. To enable comparison with normalized APC sensitivity ratios (nAPCsr, see below) the APCsr is multiplied by 10 *i.e.*:

$$APC\text{-}sr = Residual\ ETP\ (\%)_{plasma\ sample}/10$$

To minimize the effect of day-to-day variation the so-called nAPCsr was introduced[41] in which the APCsr of a plasma sample is divided by the APCsr of pooled normal plasma determined in the same experiment *i.e.*

$$nAPC\text{-}sr = Residual\ ETP\ (\%)_{plasma\ sample}/Residual\ ETP\ (\%)_{pooled\ normal\ plasma}$$

During the development of the ETP-based APC resistance test the concentration of APC added to plasma was chosen such that the residual ETP in the presence of APC was 10%.[12] This resulted in a large window for the APCsr (0-10) and in a good discrimination between normal and APC resistant individuals.[12, 29, 32] However, due to small variations in reaction conditions, the day to-day values of residual thrombin formation in normal pooled plasma may deviate from 10%. This has different effects on the APCsr and the nAPCsr of a particular plasma sample. Fig. 2.4 compares the APCsr and the nAPC-sr of plasma from the wild type volunteer (Fig. 2.4A) and from the heterozygous carrier of factor V_{Leiden} (Fig. 2.4B) when residual thrombin formation in pooled normal plasma varied between 1%-18% (data from Fig. 2.3). The APCsr of the wildtype volunteer showed a 20-fold increase at increasing levels of residual thrombin formation in pooled normal plasma, while the nAPC-sr remained virtually constant when residual thrombin formation in the pooled normal plasma varied between 5% and 18% (Fig. 2.4A). In the plasma from the factor V_{Leiden} carrier an opposite trend was observed. The nAPC-sr of factor V_{Leiden} plasma strongly depended on the percentage of residual thrombin formation in pooled normal plasma. When this was less than 5%, the nAPC-sr of the factor V_{Leiden} plasma became very high with large error margins (Fig. 2.4B). The APCsr (not-normalized)

of factor V_{Leiden} plasma was much less affected under these conditions. At levels of residual thrombin formation in normal plasma above 5% the APCsr and the nAPCsr showed a similar but opposite variation.

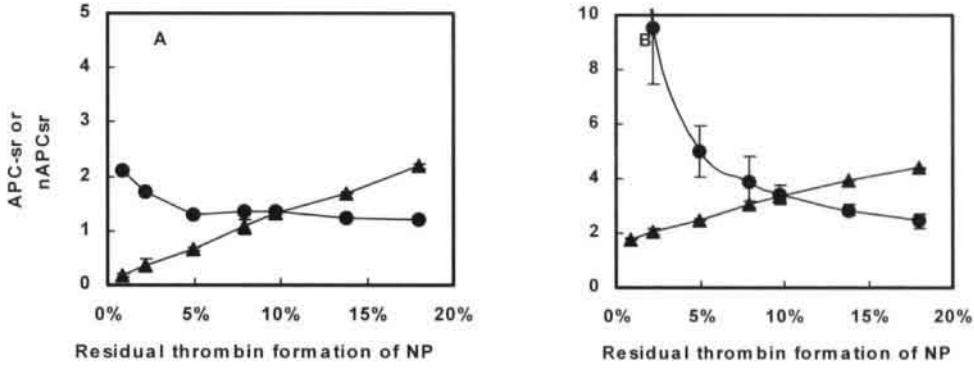


Figure 2.4 Deviations in the APC sr calculated by varying methods. APCsr determined in plasma from the normal healthy volunteer (A) and the carrier of factor V_{Leiden} (B) were calculated from the data presented in Fig. 2.3 by the three different methods described in the Results section. Plotted are deviations of the APCsr (expressed as % of the APCsr determined at 10% residual thrombin in normal pooled plasma) as function of residual thrombin in normal pooled plasma.
(●) nAPCsr = Residual ETP $(\%)_{plasma\ sample}$/Residual ETP $(\%)_{normal\ pooled\ plasma}$.
(▲) APCsr = Residual ETP $(\%)_{plasma\ sample}$/10%

Thus, normalization, which was introduced to minimize day-to-day variation of the APC resistance test may also cause deviations in the nAPCsr, especially in APC resistant plasmas. However, the data presented in Fig. 2.4 show that, irrespective of the method of calculation, reproducible (n)APCsr can be obtained when residual thrombin formation in pooled normal plasma is maintained between 6 % and 15 %. Under these circumstances normalization of the APCsr is the preferred method since the coefficients of variation of the nAPCsr were lower than those of the APCsr (see below).

Table 2.1 summarizes the in between assay variations of the (n)APCsr obtained by determining the effect of 5 nM APC on thrombin formation in pooled normal plasma, plasma from a healthy volunteer with an intermediate APCsr and a factor V_{Leiden} plasma on 18 different days. The coefficient of variation (%CV i.e. SD/mean x 100%) for residual thrombin formation of the pooled normal plasma (mean = 10.1%) was 20.8%. The in between assay variations of the (n)APCsr determined in plasma from the healthy normal volunteer were 14.8% (nAPCsr) and 23.1% (APCsr). Also in

the case of factor V_{Leiden} plasma, the in between assay variation of the nAPCsr (12.4%) was lower than that of the APCsr (17.5%).

Table 2.1 In between assay variation of the ETP-based APC resistance test.

	APCsr (mean)	In between assay variation (%CV)
Healthy individual without FVLeiden		
APCsr	1.80	23.1%
n-APCsr	1.79	14.8%
Factor V_{Leiden} plasma		
APCsr	2.75	17.5%
n-APCsr	2.76	12.4%

The effect of APC on thrombin formation was determined in normal pooled plasma and plasma from a heterozygous carrier of factor V_{Leiden} on 18 different days. Given are the mean values of the residual ETP measured in the presence of APC (expressed as percentage of the ETP determined without APC), the APCsr calculated by the three methods described in the results section and the in between assay variation (%CV).
APCsr=Residual ETP $(\%)_{plasma\ sample}/10\%$
n-APCsr=Residual ETP $(\%)_{plasma\ sample}$/Residual ETP $(\%)_{normal\ pooled\ plasma}$.

Comparison of different tissue factor preparations. In earlier studies concerning the ETP-based APC resistance test[12, 29, 32, 34] a tissue factor preparation was used that is not commercially available. To compare this preparation with commercial tissue factor preparations, we determined the nAPC-sr of 90 plasmas from normal healthy individuals (52 men, 38 women) using our earlier preparation (Recomboplastin S) and two commercially available tissue factors (Dade® Innovin® and Ortho RecombiPlasTin®) at 0.4 ng/ml tissue factor, 16 mM $CaCl_2$ and 15 □M added phospholipid vesicles. The results obtained are shown in Fig. 2.5 for men (open symbols) and women (closed symbols) separately and are summarized in Table 2.2. APC concentrations needed to attain 10% residual thrombin formation in pooled normal plasma were 4.8 nM APC for Innovin®, 5.2 nM APC for RecombiPlasTin® and 5.0 nM APC for our original tissue factor preparation (Recomboplastin S), which hints at small differences between the preparations (Table 2.2). Average nAPC-sr obtained were, however, the same for all tissue factors and with all preparations plasma of women was more resistant to APC than plasma of men (Fig. 2.5 and Table 2.2). Thus, estimation of population averages was not influenced by the choice of tissue factor preparation. However, with the commercial

tissue factor preparations a wider range of nAPC-sr values was observed, especially towards the lowest nAPC-sr values i.e. for plasmas that are more sensitive to APC than pooled normal plasma (Fig. 2.5). This was even more clear from the plots showing the correlation between the nAPCsr determined with different tissue factor preparations (Fig. 2.6).

Table 2.2 Effect of tissue factor preparations on the nAPC-sr.

Tissue factor	subjects	n (mean)	nAPC-sr	5-95% CI
Innovin	all	90	1.19	1.14-1.24
	man	52	0.95	0.91-1.01
	women	38	1.51	1.43-1.59
RecombiPlasTin	all	90	1.20	1.15-1.24
	man	52	1.02	0.97-1.07
	women	38	1.44	1.38-1.50
Recomboplastin S	all	90	1.23	1.19-1.24
	men	52	1.05	1.02-1.08
	women	38	1.46	1.42-1.52

nAPC-sr values were determined using three different tissue factor preparations in plasmas obtained from 90 healthy individuals. For more details see legend to Fig. 2.6 and Materials and Methods.With all tissue factor preparations a significant difference between the nAPCsr of men and women was observed ($p < 0.001$).

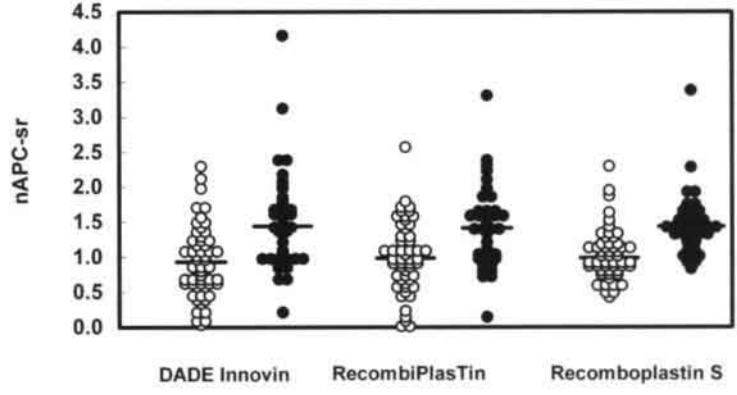


Figure 2.5 nAPC-sr determined in plasma from normal healthy individuals with three different tissue factor preparations. The nAPC-sr was determined in plasma from 90 healthy volunteers, 52 men (O) and 38 women (•), at 0.4 ng/ml tissue factor, 16 mM Ca^{2+} ions, 15 μM phospholipid and 5 nM APC (final concentrations present in the assay mixture). The following tissue factor preparations were used: Innovin, RecombiPlasTin and a not commercially available preparation (Recomboplastin S) used in earlier studies.[12, 14, 32, 34]

An excellent correlation was observed between the original non-commercial recomboplastin S and Innovin® (r = 0.92, Fig. 2.6A) and a somewhat lower correlation with RecombiPlasTin® (r = 0.79, Fig. 2.6B). However, due to a relative lack of low nAPC-sr values in the determination with the original Recomboplastin S preparation, regression lines did not cross the origin. The two commercially available tissue factor preparations showed a good correlation (r = 0.71) with a regression line which passes through the origin and which has a slope of 0.94 indicating that nAPC-sr values showed similar individual variation with these preparations (Fig. 2.6).

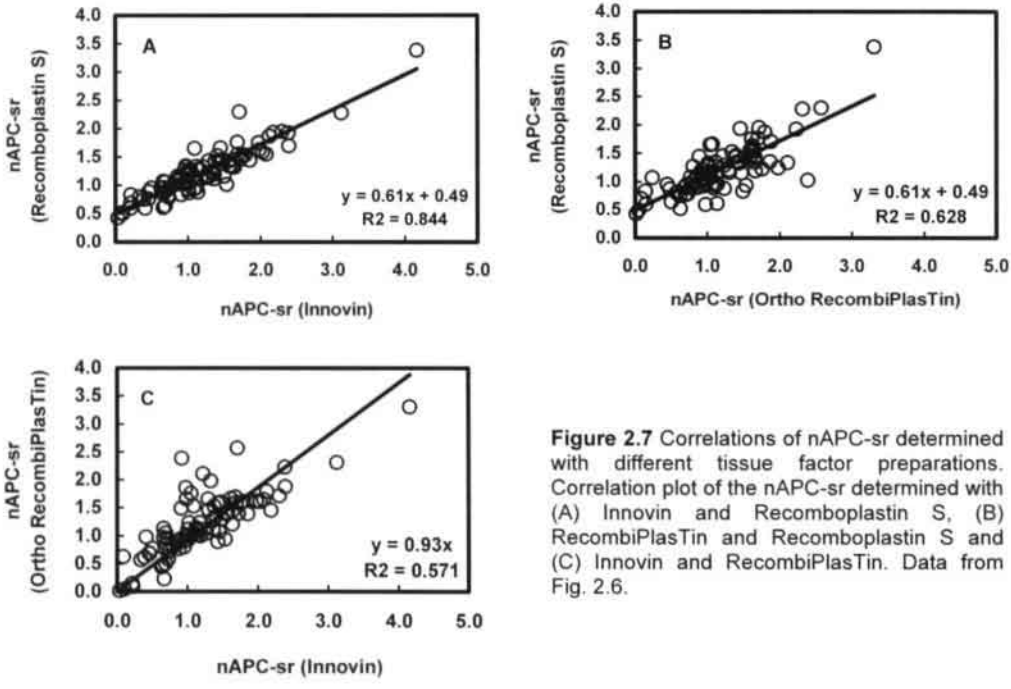


Figure 2.7 Correlations of nAPC-sr determined with different tissue factor preparations. Correlation plot of the nAPC-sr determined with (A) Innovin and Recomboplastin S, (B) RecombiPlasTin and Recomboplastin S and (C) Innovin and RecombiPlasTin. Data from Fig. 2.6.

Effect of plasma handling on the nAPC-sr of nine individual plasmas. To obtain information on the influence of plasma handling (i.e. pre-analytical variables) on the nAPC-sr, plasmas from nine individual volunteers with a large variation in the nAPC-sr were collected and subjected to different treatments as described under Materials and Methods (cf. also Figure 2.1). The pooled normal plasma used for normalisation of the APCsr was collected according to the standard procedure (nine parts of blood in one part 3.8% citrate) and processed as described in Materials and Methods.

Table 2.3 Effects of pre-analytical variables on the nAPCsr.

		Anticoagulant			
		3.2 % citrate		3.8 % citrate*	
Genotype	n	nAPC-sr	5-95% CI	nAPC-sr	5-95% CI
GG	3	1.77	1.22-2.32	0.89	0.73-1.04
AG	2	3.22	1.81-4.62	2.52	0.96-4.07
GG+O	3	4.47	3.58-5.37	3.99	3.33-4.64
AG+pregnancy		1	7.33		6.97
		Centrifugation			
		15 min 2000x*g*		15min 3000x*g*RT, 30 min 20.000x*g*4°C*	
Genotype	n	nAPC-sr	5-95% CI	nAPC-sr	5-95% CI
GG	3	1.34	1.15-1.53	1.32	0.80-1.84
AG	2	2.80	1.27-4.33	3.00	1.63-4.36
GG+OC	3	4.27	3.53-5.02	4.19	3.35-5.02
AG+pregnancy		1	7.21		7.10
		Hours at RT			
		0 hrs*		4 hrs		24 hrs	
Genotype	n	nAPC-sr	5-95% CI	nAPC-sr	5-95% CI	nAPC-sr	5-95% CI
GG	3	1.20	0.73-1.67	1.31	1.20-1.41	1.48	1.00-1.97
AG	2	2.92	1.37-4.47	2.91	1.67-4.15	2.82	1.23-4.41
GG+OC	3	4.15	3.09-5.20	4.07	3.55-4.58	4.47	3.67-5.26
AG+pregnancy		1	7.14		7.16		7.15
		Storage temperature plasma			
		-20°C		-80°C*	
Genotype	n	nAPC-sr	5-95% CI	nAPC-sr	5-95% CI
GG	3	1.32	0.99-1.66	1.34	0.96-1.72
AG	2	2.69	1.56-3.82	2.92	1.44-4.41
GG+OC	3	4.41	3.54-5.28	4.06	3.33-4.79
AG+pregnancy		1	7.23		7.08
		Thawing			
		1 x*		2 x	
Genotype	n	nAPC-sr	5-95% CI	nAPC-sr	5-95% CI
GG	3	1.33	0.92-1.74	1.33	1.03-1.63
AG	2	2.90	1.47-4.33	2.88	1.38-4.38
GG+OC	3	4.14	3.53-4.74	4.33	3.34-5.31
AG+pregnancy		1	7.34		6.97

Mean nAPC-sr values and 95% C.I. were calculated for individuals with a similar genotype/phenotype. GG = normal genotype, AG = factor V_{Leiden} heterozygous genotype, OC = oral contraceptives. * Refers to the routinely used plasma preparation procedure.

The results are summarized in Table 2.3. Under all circumstances the plasma sample from the heterozygous carrier of factor V_{Leiden}, who was 1 month pregnant, had the highest nAPCsr. The nAPC-sr determined for the heterozygous factor V_{Leiden} carriers showed values comparable to the women who did not carry the factor V_{Leiden} mutation but who were using oral contraceptives. Values close to normal plasma were observed for the non-factor V_{Leiden} men and for the non-factor V_{Leiden} woman

who was not using oral contraceptives (Table 2.3). These nAPCsr are in agreement with earlier reported values.[29, 32]

The data obtained for all possible combinations of plasma processing were analyzed in a multiple regression model which included the nine individuals as indicator variables (Table 2.4). Thereby, adjustment for individual differences (factor V_{Leiden} carriers, OC users and pregnant woman) was achieved. The model showed that the citrate concentration and the 24 hour wait at room temperature before the blood was processed were variables that clearly influenced the nAPC-sr measurement (Table 2.4). The largest effect was observed with the anticoagulant concentration. Samples collected on 3.2% citrate showed an average increase of 0.74 of the APCsr compared to plasma collected on 3.8% citrate. The difference between the APCsr of plasma collected on 3.2% and 3.8% citrate became less in samples with higher nAPCsr (Table 2.3). The other pre-analytical variations *i.e.* centrifugation procedure, 4 hour wait at room temp before processing of the blood sample, temperature during 3 month storage as well as additional sample thawing did not notably influence the nAPC-sr (Table 2.4).

Table 2.4 Regression model for the ETP-based nAPC-sr

Variables	β	95% CI
3.2% citrate vs 3.8% citrate	0.739	0.647 – 0.831
4 hrs delay vs immediate processing	-0.016	-0.130 - 0.096
24 hrs delay vs immediate processing	0.149	0.037 - 0.261
single vs double centrifugation	0.089	-0.003 - 0.181
storage at –20 vs -80	-0.023	-0.117 - 0.071
thawing twice vs once	-0.035	-0.127 - 0.057

nAPC-sr were obtained in plasmas of nine individuals processed by 48 different methods and analyzed in a multiple regression model. The nine individuals were included as indicator variables and the effect of a change in preanalytical variable was assessed against the routine method of plasma preparation (3.8% citrate as anticoagulant, immediate processing of the blood sample, double centrifugation, storage at –80 and no extra thawing).

Discussion

The data reported here concern the effects of variations in plasma handling and assay conditions on nAPCsr values determined in the ETP-based APC resistance test described in earlier publications.[12, 29, 32] It has been reported that the measurement of the nAPC-sr with this assay, apart allowing detection of the factor

V_{Leiden} mutation, is very sensitive for conditions of acquired APC-resistance such as it occurs during the use of oral contraceptives[29, 32, 33] and pregnancy.[42-44] Part of this sensitivity is likely due to the decision to use an APC concentration which diminishes thrombin formation in normal plasma by 90%. This results in a large window for the nAPC-sr (values between 1-10) of APC resistant samples. However, in order to obtain results that are comparable from study to study and between different laboratories the assay conditions need to be standardized. The assay conditions were chosen such that thrombin formation in the absence of APC is insensitive to small variations in phospholipid, $CaCl_2$ or tissue factor concentrations. The amount of phospholipid present in the assay mixture (15 μM) is higher than the concentration required for optimal thrombin formation in the absence of APC. The use of a high phospholipid concentration makes the assay insensitive to small amounts of phospholipid present in plasma and also limits the amount of APC required to inhibit thrombin formation. However, measurements obtained in the presence of APC and thus, the nAPCsr remain sensitive to variations in concentrations of reactants (cf. Figs. 2.2-2.4) and therefore, rigorous standardization is essential in order to minimize within- and between laboratory variation.

It is not surprising that the ability of APC to inhibit thrombin formation is decreased at higher tissue factor and $CaCl_2$ concentrations and increases when the amount of phospholipid present in the assay mixture is increased. At high tissue factor concentrations excessive amounts of factor Xa and factor Va will be formed, which will result in a condition at which APC is incapable to effectively inactivate factor Va and inhibit thrombin generation. The observation that in model systems containing purified proteins APC-catalyzed factor Va inactivation has a sharp Ca^{2+} optimum (3 mM) and is inhibited at high Ca^{2+} concentrations,[45] can explain the increase of residual thrombin formation at increasing amounts of Ca^{2+}. The fact that APC has a rather low affinity for negatively charged phospholipids[46] can account for the increased effectivity by which APC down-regulates thrombin formation at higher phospholipid concentrations.

In order to obtain reliable nAPCsr, it is necessary to ensure that the residual thrombin formation determined in the presence of APC in normal pooled plasma is close to 10%. A good day-to-day reproducibility is then achieved, which can be further improved upon by normalizing against the normal pooled plasma (Fig. 2.4).

When the level of residual thrombin formation in pooled normal plasma is kept within a narrow range (6%-16%) the coefficients of variation of the nAPCsr (SD/mean x 100%) were 14.8% and 12.4% for a plasma of normal healthy donor and a factor V_{Leiden} plasma, respectively (Table 2.1).

When the residual thrombin formation in pooled normal plasma is below 6%, normalization *i.e.* dividing the percentage of residual thrombin formation in the plasma sample by that determined in pooled normal plasma leads to systematic overestimation of nAPC-sr in APC resistant samples and the inter- and intra-assay variability becomes too large (Fig. 2.4). The extent of overestimation of the APCsr is then reduced by omitting the normalization procedure. There is a second reason why normalization may affect the actual value of the nAPCsr determined for an individual plasma sample. This occurs when users of oral contraceptives or carriers of factor V_{Leiden} are included in the subjects who donate blood for the pooled normal plasma. Then one obtains a pooled normal plasma that is already resistant to APC. Normalization with such a pooled normal plasma will result in lower nAPCsr for individual plasma samples than nAPCsr normalized against a pooled normal plasma from which users of oral contraceptives and carriers of factor V_{Leiden} are excluded. In order to allow comparison of nAPCsr values obtained in different studies it is recommended to use pooled normal plasma containing samples from a large number of volunteers (to minimize variations in the percentage of carriers of factor V_{Leiden}) and to exclude women who use oral contraceptives.

nAPCsr obtained with the ETP-based APC resistance test are hardly affected by the handling of plasma. The major variable to control appears to be the concentration of anticoagulant (Table 2.3, 2.4). This is not surprising considering the $CaCl_2$ dependence of the measurement in the presence of APC (cf. Fig. 2.2) and the fact that the normal plasma pool was collected at 3.8%. An influence of anticoagulant concentration has also been reported for APC resistance tests that are based on measuring the effect of APC on clotting times.[47, 48] Therefore, it is preferable to normalise APCsr against a pooled normal plasma that is collected on the same concentration of anticoagulant. The linear regression analysis further indicated a small influence of the storage time of blood before plasma is prepared and of the temperature at which plasma is stored (Table 2.4). Other variables appeared to have little or no influence.

Chapter 3

Hereditary and acquired hypercoagulable states

Based on:

Effects of hereditary and acquired risk factors of venous thrombosis on a tissue factor based APC resistance test. Joyce Curvers, M. Christella L.G.D. Thomassen, Janet Rimmer, Karly Hamulyák, Jan van der Meer, Guido Tans, F. Eric Preston and Jan Rosing. Submitted

Summary

In the past years several hereditary and acquired risk factors for venous thromboembolism (VTE) were defined that are associated with impaired down-regulation of thrombin formation via the protein C pathway. To identify individuals at risk, functional tests are needed that estimate the actual risk to develop venous thrombosis.

We determined the effects of hereditary and acquired risk factors of venous thrombosis on an activated protein C (APC) resistance test that quantifies the influence of APC on the time integral of thrombin formation (the endogenous thrombin potential, ETP) initiated in plasma via the extrinsic coagulation pathway. APC sensitivity ratios (APCsr) were determined in plasma from carriers of the factor V_{Leiden} (n=56) or the prothrombin G20210A mutation (n=18), of individuals who were deficient in antithrombin (n=9), protein C (n=7) or protein S (n=14) and of women exposed to acquired risk factors such as hormone replacement therapy (n=49), oral contraceptive use (n=126) or pregnancy (n=35). In addition, we analysed the effect of combinations of these risk factors (n=60).

The ETP-based APC resistance test was sensitive to the presence of the factor V_{Leiden} and prothrombin G20210A mutation, to protein S deficiency and to hormone replacement therapy, oral contraceptive use and pregnancy. The assay was not influenced by antithrombin- or protein C deficiency. The simultaneous presence of more than one risk factor of venous thrombosis resulted in more pronounced APC resistance. The APCsr of individuals with a single or combined risk factors of VTE correlated well with reported risk increases.

The ETP-based APC resistance test is a useful assay to identify individuals at risk for the development of venous thrombosis due to acquired risk factors and/or hereditary thrombophilic disorders that affect the protein C pathway.

Introduction

Venous thrombosis is a multifactorial disease which often results from an interaction between hereditary and acquired risk factors.[49] Mutations in genes of procoagulant proteins (factor V-R506Q also called factor V_{Leiden}[15] and prothrombin G20210A[50]), hereditary defects of anticoagulant proteins[51] (*e.g.* deficiencies of antithrombin, protein C or protein S), and phenotypic abnormalities (APC resistance[6, 7] and high levels of factor VIII,[52] factor IX[53] and factor XI[54]) predispose individuals to an increased risk of venous thrombosis.

A poor anticoagulant response to activated protein C (APC resistance) is an important risk factor for venous thrombosis.[24] APC resistance is often associated with a mutation in factor V (factor V_{Leiden}),[15] which causes the replacement of an amino acid (Arg506→Gln) at a predominant APC cleavage site in factor Va. This results in impaired inactivation of factor Va by APC[55] and in enhanced thrombin generation,[12] phenomena, which can explain the increased risk of venous thrombosis in carriers of the factor V_{Leiden} mutation.[25, 56] Later it was recognised that other mutations in factor V (R2-factor V,[57] factor $V_{Hongkong}$,[58] factor $V_{Cambridge}$[59]) and acquired coagulation disorders due to pregnancy or oral contraceptive use[30] also cause APC resistance. Recently, it was reported that APC resistance without factor V_{Leiden} is an independent risk factor of venous thrombosis.[6, 7]

We have developed a functional APC resistance test, which is based on quantification of the effect of APC on the time integral of thrombin formation (the endogenous thrombin potential, ETP) initiated in plasma via the extrinsic coagulation pathway[12]. This assay not only allowed detection of factor V_{Leiden}, but also disclosed that the use of oral contraceptives (OC)[29, 34] is associated with acquired APC resistance. A good correlation was noted between APC sensitivity ratio's (APCsr) determined with this test and the risk of venous thrombosis in carriers of factor V_{Leiden} and OC users.[29, 60] This indicates that APCsr determined with this test may predict the risk of venous thrombosis, both in the absence and presence of factor V_{Leiden}.

In the present study we evaluated the effect of hereditary and acquired risk factors of venous thrombosis on the ETP-based APC resistance test and correlated the APCsr obtained with reported risk increases of thrombosis. This study involved carriers of factor V_{Leiden} or the prothrombin G20210A mutation, individuals deficient in protein S, protein C or antithrombin (AT), OC users, women on hormone replacement

therapy (HRT) and pregnant women as well as individuals who were exposed to combinations of these risk factors.

Materials and Methods

Materials. The chromogenic substrates D-Phe-(pipecolyl)-Arg-pNA (S2238) and L-pyroGlu-Pro-Arg-pNA (S2366) were supplied by Chromogenix, Mölndal, Sweden. Ancrod, used to defibrinate plasma, was purchased from the WHO International Laboratory for Biological Standards, Hertfordshire, UK. Relipidated recombinant tissue factors, Recomboplastin S (a preparation not commercially available) and Innovin were obtained from Dade. Purified human APC (Enzyme Research Laboratories) was purchased from Kordia Laboratory Supplies, Leiden, the Netherlands. Phospholipids were from Avanti Polar Lipids, Alabaster, Alabama, USA. Small unilamellar phospholipid vesicles used in the APC resistance test were prepared as described earlier[12].

Collection and Handling of Plasma Samples. Nine parts of blood from consenting volunteers were either collected in one part of 0.105 M trisodium citrate (pH 7.8) or in one part of 0.13 M trisodium citrate (pH 7.8). Platelet poor plasma from blood samples collected on 0.105 M trisodium citrate was obtained by centrifuging 10 minutes at 2000x*g* at room temperature, removal of plasma and a second centrifugation for 10 minutes at 2000xg at room temperature. Blood samples collected in one part of 0.13 M trisodium citrate (pH 7.8) were centrifuged for 15 min at 3000x*g* at room temperature, followed by centrifugation for 30 min at 20000x*g* at 4°C. Plasma was frozen in small aliquots and stored at -80°C until analysis.

Subjects. Plasma samples were obtained from the Sheffield Haemostasis and Thrombosis Center, Royal Hallamshire Hospital, Sheffield, UK, the Academic Hospitals of the Universities of Groningen and Maastricht, the Netherlands and from the laboratory of Biochemistry of the Maastricht University, the Netherlands. Normal pooled plasma was obtained from 90 healthy volunteers (40 women that were not pregnant or using OC, 50 men, mean age 35 years).

Subjects with the factor V_{Leiden} (n=56), the prothrombin G20210A mutation (n=18) or who were deficient in AT (n=9), protein S (n=14) or protein C (n=7) and individuals with a combination of these risk factors (n=33) were patients with a previous episode of venous thrombosis (not on anticoagulant therapy) or their first degree family members. Controls for this group were healthy age- and sex-matched volunteers (34 men and 62 women, mean age 42 years) who were screened for factor V_{Leiden} but not for the prothrombin G20210A mutation or AT-, protein S- and protein C-deficiency.

The effect of hormone replacement therapy (HRT) on the APCsr was studied in plasma from 49 women (37-72 years, mean age 52 years) and 47 age- and sex-matched controls not on HRT (36-71 years, mean age 51 years). From 44 women data were available on the HRT preparation used. These were classified as estrogen only (n=22) or combined estrogen/progestagen preparations (n=22). HRT was administered orally (n=23) or via transdermal patches (n=21).

To study the influence of pregnancy, APCsr were determined in plasma from 35 healthy pregnant women (mean age 31 years and average gestational age 26 weeks) with uncomplicated pregnancies. Data on the effects of OC on the APCsr are from an earlier investigation.[32] Controls were healthy non-pregnant women without the factor V_{Leiden} mutation who were not using any hormonal preparation (n=62, age range 17-41 years, mean age 31 years). Women participating in the study on the effects of HRT, pregnancy and OC use on the APCsr and the corresponding controls (cf. Fig. 3.2) were screened for the factor V_{Leiden} mutation (carriers were excluded) but not for other coagulation abnormalities.

Diagnosis of coagulation abnormalities and APC resistance. Heterozygosity or homozygosity for the factor V_{Leiden} mutation was determined by DNA analysis or by determination of the sensitivity of diluted plasma factor Va for APC.[61] Prothrombin G20210 was established by DNA analysis. Protein S (total and free antigen) was determined by ELISA and protein S deficiency was defined by antigen levels of free protein S (< 0.64 IU/ml) or total protein S (<0.71 IU/ml).[62] Protein C was determined by ELISA (Diagnostica Stago) or after activation with Protac (Pentapharm, Basel, Switzerland) by measurement of amidolytic activity against S2366 (Chromogenix, Mölndal, Sweden). AT was determined by ELISA or by chromogenic activity (Coamatic antithrombin, supplied by Chromogenix). Subjects

were considered to be protein C deficient when they had plasma protein C levels <0.7 IU/ml and AT deficient at plasma AT levels <0.84 IU/ml.
APC sensitivity ratios (APCsr) were determined with the ETP-based APC resistance test[12] under conditions described in a later publication.[34]

Statistics. P-values for differences between the groups of individuals with a single acquired or hereditary risk factor for venous thrombosis were obtained using Student's t-test. Differences in APCsr between the groups with combined risk factors were compared using the Mann-Whitney test for independent samples. The association between the APC-sr and risk of venous thrombosis was determined by linear regression (for odd's ratios <20) and by Spearman rank correlation.

Results

Effect of hereditary risk factors of venous thrombosis on the APCsr. The APCsr of individuals with a single hereditary risk factor of venous thrombosis are summarised in figure 3.1 and table 3.1. Individuals who were either protein C or AT deficient had APCsr values that did not significantly differ from controls. Carriers of the prothrombin G20210A mutation exhibited a moderate though significant higher resistance to APC (APCsr = 1.87, 95%CI 1.53-2.22) than controls (APCsr = 1.31, 95%CI 1.20-1.41). Protein S deficient subjects had APCsr values (APCsr = 3.35, 95%CI 2.77-3.94), which were similar to those observed in heterozygous carriers of the factor V_{Leiden} mutation (APCsr = 3.75, 95%CI 3.52-3.98). The highest APCsr values were found in homozygous carriers of the factor V_{Leiden} mutation (APCsr = 4.76, 95%CI 4.34-5.18).

Both in controls and in carriers of hereditary coagulation abnormalities, men (closed symbols, figure 3.1) generally had lower APCsr *i.e.* were more sensitive to APC than women (open symbols, figure 3.1).

Effect of acquired risk factors of venous thrombosis on the APCsr. Table 3.1 and figure 3.2 show that women on hormone replacement therapy had significantly higher APCsr (1.70, 95% CI 1.55-1. 85) than age-matched controls not using HRT (APCsr = 1.44, 95% CI 1.31-1.56). Comparison of estrogen-only

preparations with preparations containing estrogen and progesterone did not reveal significant differences. However, a small difference was noted between oral HRT (APCsr = 1.86, 95% CI 1.63-2.08) and HRT administered via transdermal patches (APCsr = 1.59, 95% CI 1.34-1.84), a trend which reached borderline significance (p = 0.054).

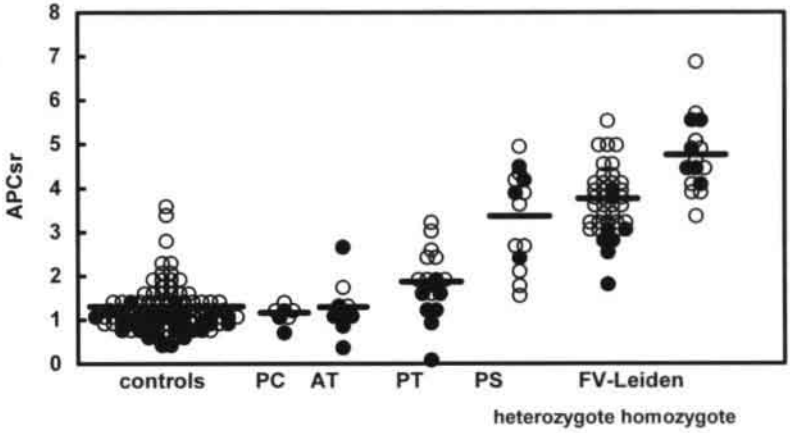


Figure 3.1 APCsr values of individuals with hereditary risk factors of VTE. APCsr values in male (●) and female (○) controls, individuals deficient in protein C (PC), antithrombin (AT) or protein S (PS), heterozygous carriers of the prothrombin G20210A mutation (PT) and heterozygous or homozygous carriers of factor V_{Leiden} were determined as described under Methods. Solid lines indicate the mean APCsr. Further details are given in Table 3.1.

Pregnancy was also associated with APC resistance (Table 3.1, figure 3.2). In the first trimester (5-13 weeks) the APCsr was significantly elevated (APCsr = 2.46, 95% CI 1.78-3.14) and further increased during the second and third trimester (Table 3.1). There appeared to be a significant correlation (r=0.52 , p=0.001) between gestational age and the APCsr.

For comparison we have presented in Table 3.1 and figure 3.2 APCsr values of OC users reported in an earlier publication [32]. It appears that APC resistance during pregnancy, particularly in the second and third trimester, was more pronounced than during third generation OC use (p<0.001) and that APCsr values of women on HRT were lower than those of second generation OC users (p<0.001).

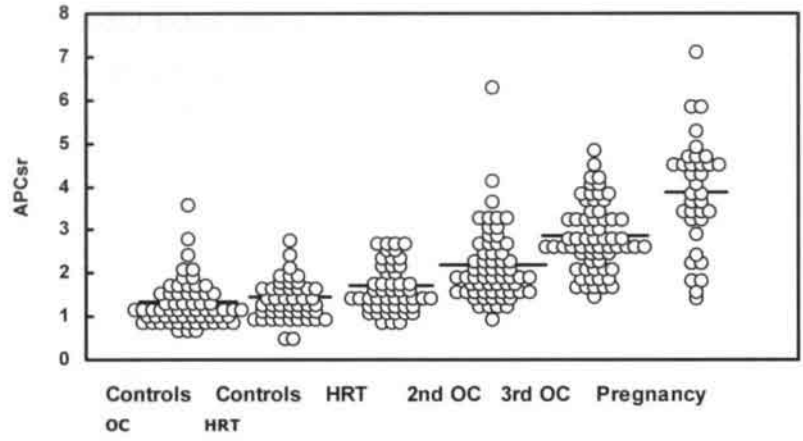


Figure 3.2 APCsr values of women with acquired risk factors of VTE. APCsr values of controls, women on hormone replacement therapy (HRT), second generation oral contraceptive users (2nd OC), third generation OC users (3rd OC) and pregnant women (gestational age ≥5 weeks) were determined as described under Methods. Solid lines indicate the mean APCsr. Further details are given in Table 3.1.

Table 3.1 APCsr values obtained with the ETP-based APC resistance test in individuals with hereditary and acquired risk factors of VTE

Risk factor	n (m/f)	mean age	mean APCsr	95% CI
Hereditary				
Controls (62 men/34 women)	96 (62/34)	42	1.31	1.20-1.41
Protein C deficient	7 (3/4)	31	1.16	0.99-1.33
Antithrombin deficient	9 (7/2)	51	1.29	0.88-1.71
Prothrombin G20210A	18 (8/10)	40	1.87	1.53-2.22
Protein S deficient	14 (4/10)	47	3.35	2.77-3.94
FV_{Leiden} heterozygous	40 (8/32)	42	3.75	3.52-3.98
FV_{Leiden} homozygous	16 (6/10)	40	4.76	4.34-5.18
Acquired				
Controls HRT	47	51	1.44	1.31-1.56
HRT	49	52	1.70	1.55-1.85
oral HRT	23	54	1.86	1.63-2.08
transdermal HRT	21	51	1.59	1.34-1.84
Controls pregnancy/OC	62	31	1.35	1.23-1.48
Pregnant women (all)	35	30	3.87	3.45-4.30
Pregnant women (5-13 weeks)	7	32	2.46	1.78-3.14
Pregnant women (14-26 weeks)	15	31	4.03	3.56-4.51
Pregnant women (27-40 weeks)	13	30	4.45	3.74-5.15
Second generation OC	62	29	2.21	2.00-2.42
Third generation OC	64	28	2.87	2.68-3.06

Data on OC users are from an earlier publication of Curvers et al.[32]

Table 3.2 APCsr values obtained with the ETP-based APC resistance test in individuals with more than one risk factor of VTE

Risk factor	n (m/f)	age	mean APCsr	95% CI
Protein C deficient + AT deficient	1 (1/0)	17	1.58	
AT deficient + Pregnant	1 (0/1)	29	3.76	
Protein C deficient + Pregnant	1 (0/1)	32	2.86	
Prothrombin G20210A + OC	1 (0/1)	22	4.04	
Prothrombin G20210A + pregnant	1 (0/1)	23	4.18	
Heterozygous FV_{Leiden} + Protein C deficient	4 (3/1)	42	3.11	1.62-4.60
Heterozygous FV_{Leiden} +Prothrombin G20210A	18 (6/12)	41	3.77	3.23-4.30
Heterozygous FV_{Leiden} + HRT	5 (0/5)	51	4.04	3.72-4.35
Heterozygous FV_{Leiden} + OC	12 (0/12)	27	6.02	5.58-6.45
Heterozygous FV_{Leiden} + Pregnancy	4 (0/4)	31	6.50	5.58-7.41
Heterozygous FV_{Leiden} + Protein S deficient	6 (3/3)	37	6.52	5.86-7.18
Heterozygous FV_{Leiden} + AT deficient + Pregnant	1 (0/1)	36	4.91	
Heterozygous FV_{Leiden} + AT deficient + Protein S deficient	1 (1/0)	35	6.11	
Heterozygous FV_{Leiden} + ProthrombinG20210A + OC	1 (0/1)	26	6.44	
Homozygous FV_{Leiden} + Pregnant	1 (0/1)	30	7.66	
Homozygous FV_{Leiden} + homozygous Prothrombin G20210	1 (1/0)	35	4.16	

AT = antithrombin; OC = oral contraceptive use; HRT = hormone replacement therapy

Effect of combinations of risk factors of venous thrombosis on the APCsr. The majority of individuals with more than one risk factor for VTE were carriers of the factor V_{Leiden} mutation who were exposed to either another hereditary thrombophilic disorder (the prothrombin G20210A mutation, protein S or AT deficiency) or to an acquired risk factor (HRT, OC, pregnancy) for venous thrombosis. Subjects with more than one risk factor generally exhibited responses to APC that reflected a combination of the effects of the individual risk factors on the APCsr (figure 3.3, table 3.2).

In line with the observation that AT deficiency and protein C deficiency were not associated with APC resistance (cf. Table 3.1, figure 3.1) no further increase of the APCsr was observed in individuals carrying this risk factor in combination with a risk factor that did affect the APCsr (figure 3.3, Table 3.2). The presence of the prothrombin G20210A mutation or HRT had virtually no additional effect on the APCsr in factor V_{Leiden} carriers (figure 3.3).

Heterozygous carriers of factor V_{Leiden} who were also proteins S deficient, using OC or who were pregnant had APCsr that were significantly higher than those of

heterozygous carriers of factor V_{Leiden} without an additional risk factor ($p<0.001$). The mean APCsr determined for these groups were even higher than the value determined for homozygous carriers of factor V_{Leiden} ($p<0.02$). The highest APCsr (7.90) was observed in a heterozygous carrier of factor V_{Leiden} who was also protein S deficient (figure 3.3).

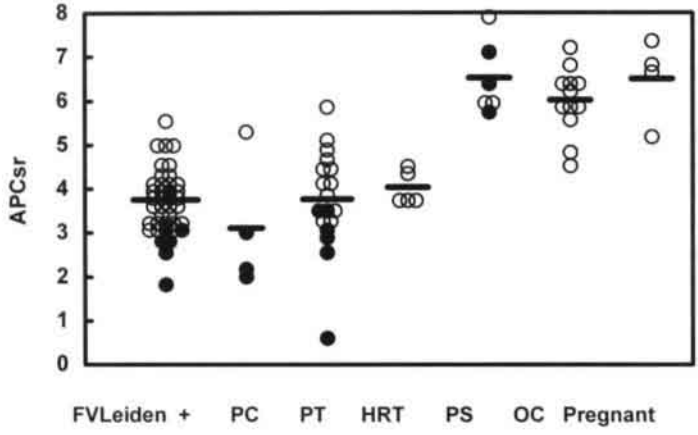


Fig 3.3 APCsr values of heterozygous carriers of factor V_{Leiden} mutation with an additional risk factor of VTE. FV_{Leiden} = heterozygous carrier of factor V_{Leiden}; PC = protein C deficiency; PT = heterozygous for the prothrombin G20210A mutation; HRT = hormone replacement therapy; PS = protein S deficiency; OC = users of oral contraceptives. Solid lines indicate the mean APCsr. Further details in Table 3.2.

Association of APC-sr with reported risks for venous thrombosis. Table 3.3 summarises the APCsr for the various subgroups together with the thrombotic risks reported in epidemiological studies. Risk factors for which only one plasma sample was available or for which no odds ratios are known were omitted from this analysis. With the exception of AT- and protein C-deficiency a general trend was observed in which increases in risk of venous thromboembolism are reflected by increased APC-resistance in the ETP-based assay. The strong association between the APC-sr and the reported risk increases for odds ratios <20 is illustrated in figure 3.4 ($r = 0.95$, $p<0.00001$). For odds ratios higher than 20 the curve deviates from linearity. This can be explained by the fact that the maximum APCsr is 10, a value obtained in a plasma that is fully (100%) resistant to APC. Spearman rank correlation (with the inclusion of all risk factors, Table 3.3) still yielded a clear correlation between the APCsr and the odds ratio ($r = 0.893$, $p < 0.00001$).

Table 3.3 APC sensitivity ratios and previously published risk for venous thromboembolism

Risk factor	n =	nAPCsr	95% C.I.	relative risk	95% C.I.	Ref
Hereditary						
Controls		96	1.31	1.20-1.41	1	
Prothrombin G20210A	18	1.87	1.53-2.22	2.8	1.4-5.6	50
Protein S deficient	14	3.35	2.77-3.94	5.0	1.5-16.8	62
FV_{Leiden} heterozygous	40	3.75	3.52-3.98	6.8	3.6-12.0	25
FV_{Leiden} homozygous	16	4.76	4.34-5.18	79.4	22.0-289	56
Acquired						
Controls HRT	47	1.44	1.31-1.56	1		
HRT	49	1.70	1.55-1.85	3.6	1.6-7.8	63
Second generation OC	62	2.21	2.00-2.42	3.6	2.5-5.1	64
Third generation OC	64	2.87	2.68-3.06	7.4	4.2-12.9	65
Pregnant women (all)	35	4.15	3.45-4.30	5		64
Combined						
Heterozygous FV_{Leiden} +						
+ Prothrombin G20210A	18	3.77	3.23-4.30	58.6	22.1-155	26
+ HRT	5	4.04	3.72-4.35	13.3	4.3-41	66
+ Protein S deficient	6	6.52	5.86-7.18	41.3	12.1-142	a
+ OC	12	6.02	5.58-6.45	34.7	7.8-154	67
+ Pregnancy	4	6.50	5.58-7.41	18.3	2.7-432	68

Corrected for age 49; 26; 66; a calculated from data of refs 69; 67; 68. In other publications 70-75 relative risk enhancements are reported that fall within the 95% conficence interval presented in this table

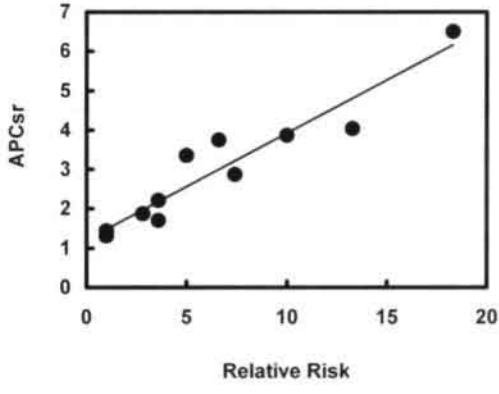


Figure 3.4 Correlation between APC-sr and relative risks of VTE. The average APC-sr determined in the various subgroups in the ETP-based APC-resistance assay were plotted against odds ratios published in literature. See Table 3.3 for further details.

Discussion

In this paper we report the effects of hereditary and acquired risk factors of venous thrombosis on APCsr determined with the ETP-based APC resistance test. It is shown that this assay is not only sensitive to the factor V_{Leiden} mutation, but that individuals with the prothrombin G20210A mutation or with protein S deficiency or women who are exposed to acquired risk factors for venous thrombosis such as HRT, OC and pregnancy also exhibited increased resistance to APC. The APCsr of individuals who were protein C- or AT-deficient fell within the range of normal healthy individuals, indicating that these coagulation abnormalities do not influence the APC resistance test.

The moderate increase of the APCsr in carriers of the prothrombin G20210A mutation is in agreement with a recent report concerning the association of elevated plasma prothrombin levels with increased APC resistance as determined in the classical aPTT-based assay.[76] The fact that prothrombin can inhibit APC-dependent factor Va inactivation[77] may provide a mechanistic explanation for these observations.

Protein S deficient subjects had APCsr similar to heterozygous carriers of factor V_{Leiden}. Since protein S is a cofactor of APC in the inactivation of factor Va,[21] it is not surprising that a decrease in plasma protein S results in higher thrombin formation in the presence of APC and hence in an increased APCsr. It is interesting to note that the aPTT-based APC resistance test, which probes the effect of APC on the intrinsic coagulation pathway, is hardly affected by plasma protein S levels.[69] This suggests that the regulation of the extrinsic coagulation pathway by the protein C system is more sensitive to variation of the plasma protein S level than the intrinsic pathway.

Individuals with protein C or AT deficiency had normal APCsr. In the case of protein C deficiency this can be explained by the fact that APC resistance tests quantify the effect of APC added to the plasma, thus by-passing the contribution of plasma protein C to the down-regulation of thrombin formation. Although it has been reported that the thrombin formation is increased in AT deficiency[9] this does not come to expression in the APC resistance test. This is likely caused by the fact that changes of the plasma levels of AT similarly influence thrombin formation in the absence and in the presence of APC, which reduces the effect on the APCsr (i.e. the ratio of thrombin formation with and without APC).

We also report increased APC resistance in plasma from pregnant women and from women on HRT. With respect to HRT, the increase in APCsr is moderate compared to that observed during OC use. This is likely caused by the fact that women on HRT are exposed to lower dosages and less potent hormone derivatives than OC users. It is interesting to note that orally administered hormones induced more pronounced APC resistance than transdermal therapy. This suggests that APC resistance is affected by route of administering HRT, a phenomenon that was also observed for other coagulation parameters.[78] Pregnancy was associated with a gestation-dependent increase of the APCsr, which, particularly in the third trimester of pregnancy, became higher than the APCsr observed in heterozygous carriers of the factor V_{Leiden} mutation and in women using OC.

The decreased sensitivity to APC observed during HRT, third generation OC use or pregnancy can, at least partially, be explained by a decrease in protein S concentration under these conditions.[79] The influence of protein S on the APCsr values may also explain the further increased resistance to APC of carriers of the FV_{Leiden} mutation who were also protein S deficient, pregnant or who used OC. Individuals exposed to a combination of these risks factors had APCsr values that were in the range of homozygous carriers of the factor V_{Leiden} mutation. The highest APCsr was observed for a woman who was a heterozygous carrier of factor V_{Leiden} and protein S deficient; APCsr = 7.90 (cf. figure 3.3) indicating that her plasma was almost fully resistant to APC.

One of the advantages of the ETP-based APC resistance measurement is that the APCsr obtained with this test correlate remarkably well with the risk of venous thromboembolism reported in epidemiological studies (cf. figure 3.4). This makes the assay potentially suitable for a more overall evaluation of individual thrombotic risks. The large variation of the APCsr within the various sub-groups indicates that a number of variables that modulate the response to APC in the APC-resistant phenotype remain to be identified.

In the present study, we did not discriminate between individuals with and without a history of venous thrombosis. A preliminary analysis of the plasma collection of the Leiden Thrombophilia Study (LETS) showed, however, that the APCsr determined with the ETP based APC resistance test predicts the risk of venous thrombosis in both patients with and without factor V_{Leiden} (G. Tans et al. unpublished observations).

Taken together, the observations reported in the present paper indicate that the ETP-based APC resistance test is a useful assay to assess the risk of venous thrombosis and to study the pathophysiology of venous thrombosis.

Chapter 4

Compared to the classical clotting assay

Based on:

Acquired APC resistance and oral contraceptives: Differences between two functional tests. Joyce Curvers, M. Christella L.G.D. Thomassen, Gerry A.F. Nicolaes, Rene van Oerle, Karly Hamulyák, H. Coenraad Hemker, Guido Tans and Jan Rosing. The British Journal of Haematology 1999, 105, 88-94

Summary

Resistance to activated protein C (APC) is often associated with a mutation in factor V (factor V_{Leiden}). Individuals without factor V_{Leiden} who exhibit a response in functional APC resistant tests similar to that of carriers of factor V_{Leiden} are considered to be acquired APC resistant. This phenomenon is particularly observed in women using oral contraceptives (OC).

In the present study we compared the response to APC in plasma samples from normal individuals, carriers of factor V_{Leiden} and women who use OC using functional tests that either quantify the effect of APC on the endogenous thrombin potential (ETP) or on the activated partial thromboplastin time (aPTT).

Both tests discriminate equally well between individuals with and without factor V_{Leiden} who were not using OC. In contrast to the aPTT-based test, the ETP-based assay yielded significant differences in sensitivity to APC between non-OC users and OC users and between users of second and third generation OC. Since there is no correlation between APC-sensitivity determined with both assays in non-carriers of factor V_{Leiden} and in women who use OC and a rather poor correlation in carriers of factor V_{Leiden}, we propose that other plasma components differentially modulate the response to APC in the aPTT- and ETP-based APC-resistance tests and that OC change the level of plasma protein(s) that modulate the effect of APC on thrombin formation initiated via the extrinsic coagulation pathway.

Introduction

Prolongation of the activated partial thromboplastin time (aPTT) by activated protein C (APC) was reported to be considerably less in a large group of patients with venous thrombosis than in a control group of healthy individuals.[24] This defect, called APC resistance, appears to be a common hereditary risk factor for venous thrombosis,[25, 56, 80, 81] which in the majority of the cases is associated with a single point mutation in factor V[15-18]. This mutation, often referred to as factor V_{Leiden}, causes the replacement of an amino acid (Arg506→Gln) at a predominant APC cleavage site which renders the activated form of factor V, factor Va, less susceptible to proteolysis by APC.[19, 20, 82, 83]

On the basis of the original observations of Dahlbäck et al.[24] several functional tests were developed for the diagnosis of APC resistance. However, screening methods that are based on measurement of the effect of APC on the aPTT of undiluted plasma do not fully discriminate between carriers and non-carriers of the factor V_{Leiden} mutation.[84-86] In particular, women who are using oral contraceptives[27-29] or who are pregnant[30] appear to be less sensitive to the anticoagulant action of APC and hence are considered to have acquired APC resistance.

Recently, we developed an APC resistance test[12] that is based on measurement of the effect of APC on the endogenous thrombin potential (ETP), i.e. the time integral of thrombin generated in plasma in which coagulation is initiated via the extrinsic pathway.[31] This so-called ETP-based assay not only enabled detection of an abnormal anticoagulant response of plasma to APC (e.g. the presence of factor V_{Leiden}), but it also appeared that the plasma of women using oral contraceptives (OC) exhibited considerable resistance to APC when analysed by this procedure.[29]

The observation that women who were using so-called third-generation oral contraceptives were more resistant to APC than women using second-generation oral contraceptives attracted much attention[87-89] in the discussion about the different risks for venous thrombosis between second and third generation OC users.[65, 71, 90] However, the impact of our observations for "pill thrombosis" was also questioned[91-94] Amongst other things it was argued that the APC resistance test developed in our laboratory has not yet been clinically validated[92-94] and that the different effects of OC in our APC resistance test might have been affected by the selection of patients or may be due to an *ex-vivo* effect.[91]

In order to gain more insight in possible differences between the aPTT- and ETP-based APC resistance tests we present in this article a comparison of APC sensitivity ratios of a large plasma collection of normal individuals, women who use OC and carriers of FV_{Leiden} determined with both tests.

Materials and Methods

Materials. The aPTT-based APC resistance test (Coatest ® APC™) and D-Phe-(pipecolyl)-Arg-pNA (S2238) and L-pyroGlu-Pro-Arg-pNA (S2366) were supplied by Chromogenix, Mölndal, Sweden. Ancrod was purchased from the W.H.O. International Laboratory for Biological Standards, Hertfordshire, U.K., and Arwin® was from Knoll, Germany. Relipidated recombinant tissue factor was obtained from Dade, U.S.A. The APC used in the ETP-based APC resistance test was purified human APC (a kind gift of Immuno A.G., Vienna, Austria) or human APC (Enzyme Research Laboratories) purchased from Kordia Laboratory Supplies, Leiden, The Netherlands. APC was quantitated as described by Sala et al.[36] Immuno-depleted factor V-deficient plasma was obtained from Organon Technika. Phospholipids were from Avanti Polar Lipids, Alabaster, Alabama, U.S.A. Small unilamellar phospholipid vesicles, composed of a mixture of 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) (20/20/60, M/M/M), were prepared by mixing appropriate quantities of phospholipids dissolved in $CHCl_3/CH_3OH$ (9/1,v/v) in a glass tube. After drying under a mild flow of N_2, the phospholipids were suspended in 2 ml buffer (25 mM Hepes, 175 mM NaCl, pH 7.5) and vigorously vortexed for 1 min. The phospholipid suspension was subsequently sonicated for 10 min at 0°C using a MSE Soniprep 150 ultrasonic disintegrator set at 7.5 μm peak to peak amplitude. Phospholipid concentrations were determined by phosphate analysis.[95]

Collection and Handling of Plasma Samples. This study was performed according to a protocol approved by our institutional ethics committee. Nine parts of blood from consenting volunteers were collected in one part of 0.13 M trisodium citrate (pH 7.8). Platelet-poor plasma was obtained by centrifugation for 25 minutes

at 3000 *g* at room temperature followed by centrifugation for 25 min at 20000 *g* at 4°C and was snapfrozen in liquid nitrogen in small aliquots and stored at -80°C until analysis.

Normal pooled plasma from healthy volunteers (40 females not using OC, 50 males, mean age 35 years) was the same as used before.[29]

Some of the plasma samples used in this study were also used in an earlier paper in which we reported the effect of OC use on the ETP-based normalized APC sensitivity ratio (nAPC-sr)[29]. Plasmas no longer available for determination of the aPTT-based nAPCsr were excluded from current analysis. Plasmas of women using triphasic OC, treated as a separate group earlier,[29] were reclassified as second (n=23) or third generation OC (n=6) depending on the kind of progestagen. When sufficient plasma was available, the ETP-based nAPC-sr was redetermined and the average of the original and newly determined value was used. When insufficient plasma was available to redetermine the ETP-based nAPC-sr, the original value[29] was used. Compared to the earlier study the plasma collection was extended with 101 new samples from individuals recruited within our own institute.

The final plasma collection contained plasma samples of: male volunteers (n = 46, 18-48 years old, mean age 30 years, new n=21), women not using OC (n = 62, 17-41 years, mean age 31 years, new n=15), women using second-generation contraceptives containing levonorgestrel or lynestrenol (n=62, 20-41 years, mean age 29 years, duration OC use 0.3-20 year, average duration OC use 9 years, new n=14), women using third-generation contraceptives containing desogestrel or gestodene (n = 64, 18-46 year; mean age 28 year, duration OC use 0.3-20 years, average duration OC use 6 years, new n=24), men heterozygous for the factor V_{Leiden} mutation (n =21, 14-78 years, mean age 40 years, new n=13) of which four with previous venous thrombolic embolism (VTE), 14 were asymptomatic relatives of a propositus with VTE and three were obtained by random sampling, women heterozygous for the factor V_{Leiden} mutation not using OC (n =26, 17-80 years, mean age 42 years, new n=12) of which nine had a histrory of previous VTE, 13 asymptomatic relatives and three obtained by random sampling. Women heterozygous for the factor V_{Leiden} mutation using second or third generation OC (n =7, 20-35 years, mean age 29 years, duration OC use 3-15 years, average duration OC use 10 years, new n=2) of which non with previous VTE, two asymptomatic

relatives and five obtained by random sampling. Compared to our previous study,[29] the group specifics, i.e. range and average of age and duration of OC use, had not changed significantly.

Women who had ceased OC therapy for >6 months were considered non-users. The OC user group consisted of women that were using the same OC for at least 3 months. We further excluded pregnant women and individuals with known hereditary risk factors for venous thrombosis other than factor V_{Leiden}, with a previous episode of venous thrombosis, a chronic or intercurrent acute disease or with medication known to interfere with blood coagulation.

Diagnosis of the presence of the factor V_{Leiden} mutation. The occurrence of heterozygosity for factor V_{Leiden} was established by determination of the sensitivity of plasma factor Va for APC[61] or by DNA analysis[37].

Determination of nAPCsr with the ETP-based and the aPTT-based APC resistance tests. Normalized APC sensitivity ratios (nAPCsr) were determined with the ETP-based APC resistance as described before[29]. The normalized APC sensitivity ratio (nAPCsr) was defined as the ratio of α2M-IIa determined in the presence and absence of APC divided by the ratio determined in the normal plasma pool (cf.[41]):

$$nAPCsr = (\alpha2M\text{-}IIa_{+APC}/\alpha2M\text{-}IIa_{-APC})_{plasma\ sample}/(\alpha2M\text{-}IIa_{+APC}/\alpha2M\text{-}IIa_{-APC})_{normal\ plasma}$$

The aPTT-based APC resistance test was performed in undiluted plasma as described by the supplier (Chromogenix, Mölndal, Sweden). Clotting times were determined on an ACL 300R coagulation analyser (Instrumentation Laboratory, Milan, Italy). The normalized APC sensitivity ratio (nAPCsr) was defined as the ratio of the clotting times (aPTT) determined in the presence and absence of APC normalized by division through the same ratio determined in the normal plasma pool[41]:

$$nAPCsr = (aPTT_{+APC}/aPTT_{-APC})_{plasma\ sample}/(aPTT_{+APC}/aPTT_{-APC})_{normal\ plasma}$$

Statistics. Statistical analysis was performed after logarithmic transformation of the data which resulted in normally distributed nAPCsr values. 95% confidence

intervals of the mean are given in Tables 4.1 and 4.2. *P* values shown in the figures were obtained by comparison of the groups using Student's *t*-test. The association between both tests (Pearson's correlation and *P* values) were obtained by regression analysis using SPSS.

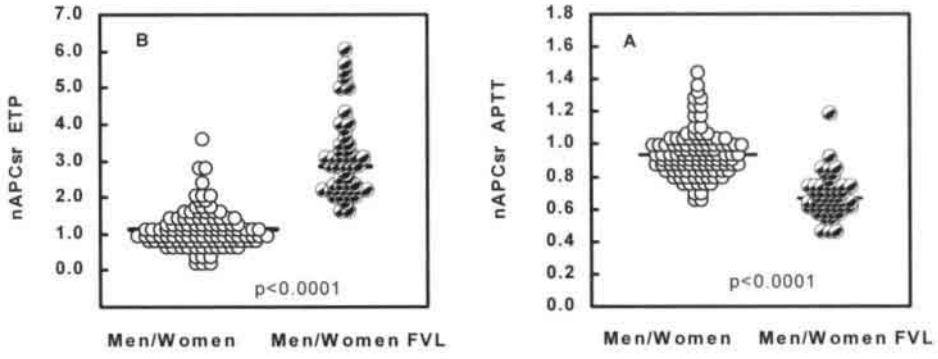


Figure 4.1 Comparison of nAPCsr of carriers and non-carriers of FV_{Leiden} determined with the (A) aPTT- and (B) ETP-based APC resistance tests.APC sensitivity ratios were determined in the same plasma collection of 109 non-carriers (46 men and 62 women) and 47 carriers of the FV_{Leiden} mutation (21 men and 26 women) with the aPTT- and ETP-based APC resistance test as described under Methods.

Results

Comparison of APC sensitivity ratios of normal individuals and carriers of factor V_{Leiden} in the aPTT- and ETP-based APC resistance tests. Normalised APC sensitivity ratios (nAPCsr) of healthy individuals (men and women not using oral contraceptives) and heterozygous carriers of the factor V_{Leiden} mutation were evaluated in the aPTT- and ETP-based APC resistance test in undiluted plasma (Fig 4.1). Due to the calculation procedure, APC resistant plasmas will give a decrease of the nAPCsr in the aPTT-based (A) and an increase in the ETP-based assay (B). At cut-off ratio's of 0.76 for the aPTT-based APC resistance test and 2.08 for the ETP-based APC resistance test (95% specificity) the sensitivity of the tests were 83% and 89%, respectively. Table 4.1 summarizes the data together with the results for men and women, separately. In both tests women appeared slightly more resistant to APC than men, confirming earlier reports,[25, 28, 29, 96] but only in the ETP-based assay did gender difference reach statistical significance ($P< 0.05$, Table 4.1).

Table 4.1 nAPCsr of men and women determined in the aPTT- and ETP-based APC resistance tests.

		aPTT-based test			ETP-based test		
	n	median	mean	95% CI	median	mean	95% CI
Men and women	108	0.94	0.95	0.93-0.98	1.13	1.20	1.10-1.30
Men and women FV-$_{Leiden}$	47	0.67	0.69	0.65-0.72	2.97	3.09	2.77-3.41
Men	46	0.95	0.97	0.93-1.02	0.98	1.00	0.86-1.13
Men FV-Leiden	21	0.71	0.72	0.68-0.76	2.33	2.67	2.30-3.05
Women							
No OC	62	0.92	0.94	0.91-0.97	1.21	1.35	1.23-1.48
Second geneartion OC	62	0.86	0.88	0.85-0.91	1.97	2.21	2.00-2.43
Third geneartion OC	64	0.86	0.86	0.83-0.89	2.75	2.87	2.68-3.07
Women FV-Leiden							
No OC	26	0.64	0.66	0.60-0.71	3.11	3.43	2.98-3.88
Using OC	7	0.56	0.58	0.53-0.62	5.99	5.74	5.14-6.35

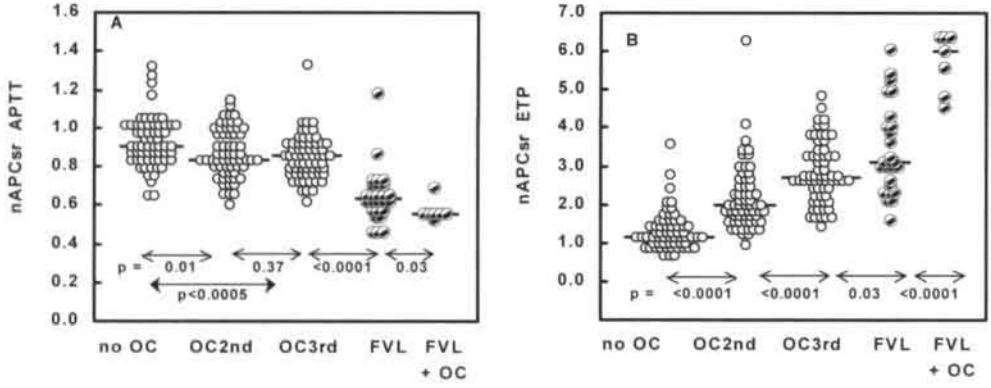


Figure 4.2 Effect of OC use on nAPCsr determined with (A) aPTT- and (B) ETP based APC resistance tests.APC sensitivity ratios were determined with the aPTT and ETP-based APC resistance tests as described under Methods in the same plasma collection of women not using OC (n=62), women using second generation OC (n=62) and women using third generation OC (n=64) who were not carrying the FV-Leiden mutation and of heterozygousfemale carriers of FV-Leiden not using OC (n=26) or using OC (n=7)

Effect of OC use on nAPCsr determined in aPTT- and ETP-based APC resistance tests. Fig 4.2 and Table 4.2 summarize the effects of OC use on nAPCsr obtained in both tests for plasmas of normal healthy women who do not carry the factor V_{Leiden} mutation and of women heterozygous for the factor V_{Leiden} mutation. Increased resistance to APC in OC users was observed in both tests, but the differences between non-users and OC users in the aPTT-based test appeared

rather small. The effects of OC use on nAPCsr determined in the ETP-based were much more pronounced and significant differences were observed between non-users and pill users independent of the kind of OC used (P<0.0001) and between users of second and third generation OC (P<0.0001). Women who used third generation OC had nAPCsr in the range of heterozygous female carriers of factor V_{Leiden} who were not using OC (Fig 4.2B). The effect of OC and the factor V_{Leiden} mutation on the nAPCsr appear to amplify each other, since female heterozygous carriers of factor V_{Leiden} who used OC were considerably more resistant to APC than carriers of the mutation who did not use OC (p<0.0001), a phenomenon that was not observed with the aPTT-based APC resistance test (Fig 4.2A).

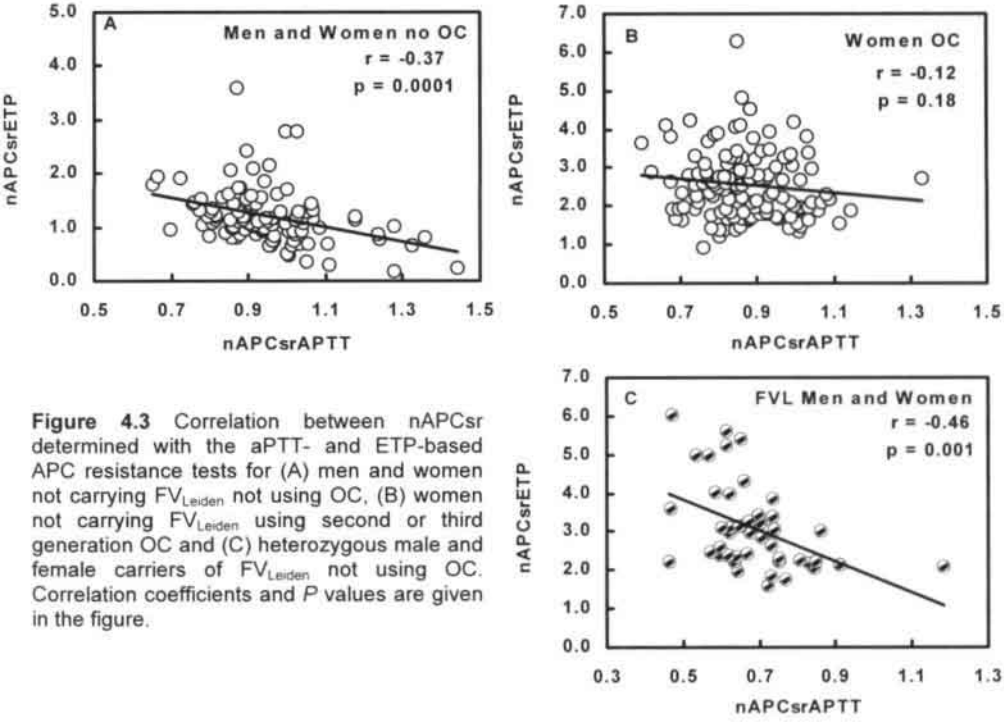


Figure 4.3 Correlation between nAPCsr determined with the aPTT- and ETP-based APC resistance tests for (A) men and women not carrying FV_{Leiden} not using OC, (B) women not carrying FV_{Leiden} using second or third generation OC and (C) heterozygous male and female carriers of FV_{Leiden} not using OC. Correlation coefficients and *P* values are given in the figure.

Correlation between nAPCsr determined in the aPTT- and ETP based APC resistant tests. Fig 4.3 shows a correlation plot of nAPCsr determined with the aPTT-based and ETP-based APC resistance tests. There appears to be a rather poor correlation between the nAPCsr determined with the two tests in the case of non-carriers of factor V_{Leiden} (r = -0.37, Fig 4.3A) and in women using oral

contraceptives (r = -0.12, Fig 4.3B). The correlations were also low (data not shown) within the populations of men (r = -0.36), women not using OC (r = -0.34), women using second generation OC (r = -0.13) and women using third generation OC (r =-0.06).

A slightly higher correlation (r = -0.46) was observed for heterozygous carriers of factor V_{Leiden}. This means that, despite the fact that both assays have approximately equal sensitivity and specificity for the diagnosis of factor V_{Leiden}, the correlation between the nAPCsr obtained in both tests is rather low. These data, taken together with the fact that OC use shows differential effects in both tests, indicates that the nAPCsr is subject to differences in modulation by other plasma components in the two APC-resistance tests. This is illustrated by the experiment in Fig 4.4 which shows the effect of the factor V_{Leiden} mutation *per se* on values of the nAPCsr of plasma in which the levels of other coagulation factors are the same. In this experiment factor V-deficient plasma was reconstituted with varying amounts of purified factor V and factor (homozygous) V_{Leiden} at a fixed total factor V concentration (20 nM). In these plasmas there appeared to be an excellent correlation (r = -0.99) between nAPCsr determined in the aPTT- and ETP based assays.

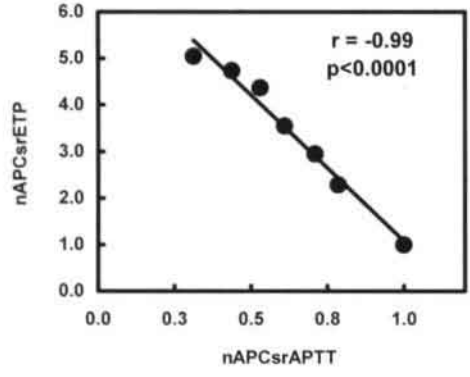


Figure 4.4 Effect of FV-Leiden on nAPCsr determined with aPTT- and ETP-based APC resistance tests. FV-deficient plasma was supplemented with varying amounts of purified normal FV and FV-Leiden at a constant level of 20nM total factor V. In the ETP-based APC resistance test ~50nM was required to reduce thrombin formation to 10% in FV-deficient plasma reconstituted with 20 nM normal FV. The nAPCsr of plasmas with varying amounts of FV and FV-Leiden was normalized against plasma with 100% normal FV.

Discussion

An impaired response to APC in functional clotting tests is associated with an increased risk for venous thrombosis.[25, 56, 80, 81] The defect, called APC resistance, is often caused by a mutation[15-18] in factor V (factor V_{Leiden}) at a predominant APC cleavage site ($Arg^{506} \rightarrow Gln$). APC resistance associated with factor V_{Leiden} is a hereditary defect that increases the risk for venous thrombosis some 7-fold in the case of heterozygosity[25, 56] and 80-fold in the case of homozygosity.[56]

In contrast to assays performed in factor V-deficient plasma,[97] commercially available aPTT-based APC-resistance tests on undiluted plasma[84-86] are generally not 100% specific and 100% sensitive for the diagnosis of the factor V_{Leiden}. Depending on the test used, there appears to be a variable number of individuals who do not carry the mutation but who show an impaired response to APC that is comparable to that observed for heterozygous carriers of factor V_{Leiden}. When no other reason for this impaired response to APC is known, these individuals are considered to be acquired APC resistant.

Acquired APC resistance particularly occurs in plasma of women who use oral contraceptives[27-29] or who are pregnant[30] and is particularly observed in a functional test in which the ability of APC to down-regulate the coagulation pathway is quantitated by measuring its effect on thrombin generation initiated in plasma via the extrinsic pathway.[29]

Since the effects of oral contraceptives on APC sensitivity ratios determined with other APC-resistance tests appeared to be marginal,[27, 28, 91] it was argued[91] that our earlier observations[29] in the ETP-based assay may have been caused by the selection of OC users or by an *ex vivo* effect on the plasma collection. The data presented here, however, show that our plasma collection yields results that are in agreement with earlier publications in which rather small effects of OC use on the aPTT-based APCsr were reported.[27, 28, 91]

Therefore we propose that the different observations in the two APC resistance tests are due to the fact that levels of other plasma proteins differentially modulate the response to APC in the ETP-based test (effect of APC on thrombin generation initiated via the extrinsic pathway) and in the aPTT-based test (effect of APC on clotting times after initiation of the intrinsic pathway).

Differences in modulation of the nAPCsr determined in the two tests by other plasma proteins becomes evident after correlation of nAPCsr determined with the aPTT- and ETP-based assays in a population of heterozygous carriers of the factor V_{Leiden} mutation. Although the effect of the mutation *per se* (variation of relative amounts of normal factor V and factor V_{Leiden} in a single plasma) shows an excellent correlation between nAPCsr determined with the two assays (Fig 4.4), the correlation between the nAPCsr of a population of heterozygous carriers of the factor V_{Leiden} mutation appears to be considerably less (Fig 4.3C). In our opinion these observations support the hypothesis that the actual value of the nAPCsr of an individual, especially in OC-users, is subject to differential modulation by other plasma proteins in the ETP- and aPTT-based APC resistance tests.

With respect to the different effects of oral contraceptives on nAPCsr determined in the aPTT- and ETP-based assays, this would imply that OC change the level plasma protein(s) that particularly affect the response to APC probed by measuring its effect on thrombin generation initiated via the extrinsic coagulation pathway. At present we have no information about possible proteins that might be responsible for such a phenomenon.

Another major question is the clinical significance of acquired APC resistance. There are as yet no reports that directly associate acquired APC resistance with an increased risk for venous thrombosis. To investigate this possibility we are presently comparing nAPCsr of populations of patients with a previous unexplained venous thrombosis and proper controls.

References

1. Le DT, Griffin JH, Greengard JS, Mujumdar V, Rapaport SI. Use of a generally applicable tissue factor--dependent factor V assay to detect activated protein C-resistant factor Va in patients receiving warfarin and in patients with a lupus anticoagulant. Blood 1995; 85:1704-11.
2. Halbmayer WM, Haushofer A, Schon R, Fischer M. The prevalence of poor anticoagulant response to activated protein C (APC resistance) among patients suffering from stroke or venous thrombosis and among healthy subjects [see comments]. Blood Coagul Fibrinolysis 1994; 5:51-7.
3. Cadroy Y, Aiach M. evaluation of apc resistance in the plasma of patients with q506 mutation of factor V (factor V Leiden) and treated by oral anticoagulants. Thromb and Haemost 1995; 38.
4. Castaman G, Lunghi B, Missiaglia E, Bernardi F, Rodeghiero F. Phenotypic homozygous activated protein C resistance associated with compound heterozygosity for Arg506Gln (factor V leiden) and His1299Arg substitutions in factor V. British Journal of Haematology 1997; 99 (2):257-261.
5. Delahousse B, Iochmann S, Pouplard C, Fimbel B, Charbonnier B, Gruel Y. Pseudo-homozygous activated protein C resistance due to coinheritance of heterozygous factor V Leiden mutation and type I factor V deficiency. Variable expression when analyzed by different activated protein C resistance functional assays. Blood Coagulation and Fibrinolysis 1997; 8 (8):503-509.
6. Rodeghiero F, Tosetto A. Activated protein C resistance and factor V Leiden mutation are independent risk factors for venous thromboembolism. Ann Intern Med 1999; 130:643-50.
7. de Visser MC, Rosendaal FR, Bertina RM. A reduced sensitivity for activated protein C in the absence of factor V Leiden increases the risk of venous thrombosis. Blood 1999; 93:1271-6.
8. Tans G, Rosendaal FR, Curvers J, Thomassen MCLGD, Bertina RM, Rosing J. APC Resistance Determined With the Endogenous Thrombin Potential Is Associated With Venous Thrombosis: A Blinded Clinical Evaluation. Thromb Haemost 1999; Suppl:202-203.
9. Wielders S, Mukherjee M, Michiels J, et al. The routine determination of the endogenous thrombin potential, first results in different forms of hyper- and hypocoagulability. Thromb Haemost 1997; 77:629-36.
10. Rotteveel RC, Roozendaal KJ, Eijsman L, Hemker HC. The influence of oral contraceptives on the time-integral of thrombin generation (thrombin potential). Thromb Haemost 1993; 70:959-62.
11. Hemker HC, Wielders S, Kessels H, Beguin S. Continuous registration of thrombin generation in plasma, its use for the determination of the thrombin potential. Thromb Haemost 1993; 70:617-24.
12. Nicolaes GAF, Thomassen MCLGD, Tans G, Rosing J, Hemker HC. Effect of activated protein C on thrombin generation and on the thrombin potential in plasma of normal and APC-resistant individuals. Blood Coagulation and Fibrinolysis 1997; 8:28-38.
13. Tans G, Nicolaes GA, Rosing J. Regulation of thrombin formation by activated protein C: effect of the factor V Leiden mutation. Semin Hematol 1997; 34:244-55.
14. Rosing J, Hemker HC, Tans G. Oral contraceptives and venous thromboembolism: Acquired APC resistance? British Journal of Haematology 1997; 98:491-492.
15. Bertina RM, Koeleman BPC, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994; 369:64-67.
16. Greengard JS, Sun X, Xu X, Fernandez JA, Griffin JH, Evatt B. Activated protein C resistance caused by Arg506Gln mutation in factor Va. Lancet 1994; 343:1361-2.
17. Voorberg J, Roelse J, Koopman R, et al. Association of idiopathic venous thromboembolism with single point-mutation at Arg506 of factor V. Lancet 1994; 343:1535-6.
18. Zöller B, Dahlbäck B. Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis. Lancet 1994; 343:1536-8.
19. Heeb MJ, Kojima Y, Greengard JS, Griffin JH. Activated protein C resistance: molecular mechanisms based on studies using purified Gln506-factor V. Blood 1995; 85:3405-11.
20. Kalafatis M, Bertina RM, Rand MD, Mann KG. Characterization of the molecular defect in factor VR506Q. Journal of Biological Chemistry 1995; 270:4053-7.

21. Rosing J, Hoekema L, Nicolaes GAF, et al. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor VaR506Q by activated protein C. J Biol Chem 1995; 270:27852-8.
22. Váradi K, Rosing J, Tans G, Pabinger I, Keil B, Schwarz HP. Factor V enhances the cofactor function of protein S in the APC-mediated inactivation of factor VIII: influence of the Factor VR506Q mutation. Thromb Haemostas 1996; 76(2):208-214.
23. Thorelli E, Kaufman RJ, Dahlback B. The C-terminal region of the factor V B-domain is crucial for the anticoagulant activity of factor V. J Biol Chem 1998; 273:16140-5.
24. Dahlbäck B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. Proc Natl Acad Sci U S A 1993; 90:1004-8.
25. Koster T, Rosendaal FR, de Ronde H, Briet E, Vandenbroucke JP, Bertina RM. Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. Lancet 1993; 342:1503-6.
26. Salomon O, Steinberg DM, Zivelin A, et al. Single and combined prothrombotic factors in patients with idiopathic venous thromboembolism: prevalence and risk assessment. Arterioscler Thromb Vasc Biol 1999; 19:511-8.
27. Olivieri O, Friso S, Manzato F, et al. Resistance to activated protein C in healthy women taking oral contraceptives. British Journal of Haematology 1995; 91:465-470.
28. Henkens CM, Bom VJ, Seinen AJ, van der Meer J. Sensitivity to activated protein C; influence of oral contraceptives and sex. Thrombosis and Haemostasis 1995; 73:402-4.
29. Rosing J, Tans G, Nicolaes GAF, et al. Oral contraceptives and venous thrombosis: Different sensitivities to activated protein C in women using second- and third-generation oral contraceptives. British Journal of Haematology 1997; 97:233-238.
30. Meinardi JR, Henkens CMA, Heringa MP, vanderMeer J. Acquired APC resistance related to oral contraceptives and pregnancy and its possible implications for clinical practice. Blood Coagulation and Fibrinolysis 1997; 8:152-154.
31. Hemker HC, Beguin S. Thrombin generation in plasma: its assessment via the endogenous thrombin potential. Thrombosis and Haemostasis 1995; 74:134-8.
32. Curvers J, Thomassen MCLGD, Nicolaes GAF, et al. Acquired APC resistance and oral contraceptives: differences between two functional tests. British Journal of Haematology 1999; 105:88-94.
33. Rosing J, Middeldorp S, Curvers J, et al. Low-dose oral contraceptives and acquired resistance to activated protein C: a randomised cross-over study. Lancet 1999; 354:2036-40.
34. Rosing J, Tans G. Effects of oral contraceptives on hemostasis and thrombosis. American Journal of Obstetrics and Gynecology 1999; 180:S375-82.
35. Rouser G, Fkeischer S, Yamamoto A. Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 1970; 5:494-6.
36. Sala N, Owen WG, Collen D. A functional assay of protein C in human plasma. Blood 1984; 63:671-675.
37. Beauchamp NJ, Daly ME, Cooper PC, Preston FE, Peake IR. Rapid two-stage PCR for detecting factor V G1691A mutation. Lancet 1994; 344:694-695.
38. Duchemin J, Pittet JL, Tartary M, et al. A new assay based on thrombin generation inhibition to detect both protein C and protein S deficiencies in plasma. Thromb Haemost 1994; 71:331-8.
39. Fischer AM, Tapon-Bretaudiere J, Bros A, Josso F. Respective roles of antithrombin III and alpha 2 macroglobulin in thrombin inactivation. Thromb Haemost 1981; 45:51-4.
40. Hemker HC, Willems GM, Beguin S. A computer assisted method to obtain the prothrombin activation velocity in whole plasma independent of thrombin decay processes. Thromb Haemost 1986; 56:9-17.
41. de Ronde H, Bertina RM. Laboratory diagnosis of APC-resistance: a critical evaluation of the test and the development of diagnostic criteria. Thrombosis and Haemostasis 1994; 72:880-886.
42. Sugimura M, Kobayashi T, Kanayama N, Terao T. Detection of decreased response to activated protein C during pregnancy by an endogenous thrombin potential-based assay. Semin Thromb Hemostasis 1999; 25:497-502.

43. Sugimura M, Kobayashi T, Kanayama N, Terao T. Detection of marked reduction of sensitivity to activated protein C prior to the onset of thrombosis during puerperium as detected by endogenous thrombin potential-based assay. Thromb Haemost 1999; 82:1364-1365.
44. Thomassen MCLGD, Curvers J, Rimmer JE, et al. Influence of hormone replacement therapy, oral contraceptives and pregnancy on APC resistance. Thrombosis and Haemostasis 1999; 82 (Suppl):770-771.
45. Bakker HM, Tans G, Janssen Claessen T, et al. The effect of phospholipids, calcium ions and protein S on rate constants of human factor Va inactivation by activated human protein C. Eur J Biochem 1992; 208:171-8.
46. Nelsestuen GL, Kisiel W, Di Scipio RG. Interaction of vitamin K dependent proteins with membranes. Biochemistry 1978; 17:2134-8.
47. Duncan EM, Casey CR, Duncan BM, Lloyd JV. Effect of concentration of trisodium citrate anticoagulant on calculation of the international normalised ratio and the international sensitivity index of thromboplastin. Thrombosis and Haemostasis 1994; 72 (1):84-88.
48. Chantarangkul V, Tripodi A, Clerici M, Negri B, Mannucci PM. Assessment of the influence of citrate concentration on the international normalized ratio (INR) determined with twelve reagent-instrument combinations. Thrombosis and Haemostasis 1998; 80:258-262.
49. Rosendaal FR. Venous thrombosis: a multicausal disease. Lancet 1999; 353:1167-73.
50. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood 1996; 88:3698-703.
51. Bertina RM. Molecular risk factors for thrombosis. Thromb Haemost 1999; 82:601-609.
52. Kraaijenhagen RA, in't Anker PS, Koopman MM, et al. High plasma concentration of factor VIIIc is a major risk factor for venous thromboembolism [see comments]. Thromb Haemost 2000; 83:5-9.
53. van Hylckama Vlieg A, van der Linden IK, Bertina RM, Rosendaal FR. High levels of factor IX increase the risk of venous thrombosis. Blood 2000; 95:3678-82.
54. Meijers JC, Tekelenburg WL, Bouma BN, Bertina RM, Rosendaal FR. High levels of coagulation factor XI as a risk factor for venous thrombosis. N Engl J Med 2000; 342:696-701.
55. Rosing J, Tans G. Coagulation factor V: an old star shines again. Thromb Haemost 1997; 78:427-33.
56. Rosendaal FR, Koster T, Vandenbroucke JP, Reitsma PH. High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). Blood 1995; 85:1504-1508.
57. Lunghi B, Iacoviello L, Gemmati D, et al. Detection of new polymorphic markers in the factor V gene: association with factor V levels in plasma. Thromb Haemost 1996; 75:45-8.
58. Chan WP, Lee CK, Kwong YL, Lam CK, Liang R. A novel mutation of Arg306 of factor V gene in Hong Kong Chinese. Blood 1998; 91:1135-9.
59. Williamson D, Brown K, Luddington R, Baglin C, Baglin T. Factor V Cambridge: A new mutation (Arg(306)->Thr) associated with resistance to activated protein C. BLOOD 1998; 91 (4):1140-1144.
60. Vandenbroucke JP, Rosendaal FR. End of the line for "third-generation-pill" controversy? Lancet 1997; 349:1113-1114.
61. Nicolaes GAF, Thomassen MCLGD, vanOerle R, et al. A prothrombinase-based assay for detection of resistance to activated protein C. Thombosis and Haemostasis 1996; 76:404-410.
62. Makris M, Leach M, Beauchamp NJ, et al. Genetic analysis, phenotypic diagnosis, and risk of venous thrombosis in families with inherited deficiencies of protein S. Blood 2000; 95:1935-41.
63. Jick H, Derby LE, Myers MW, Vasilakis C, Newton KM. Risk of hospital admission for idiopathic venous thromboembolism among users of postmenopausal oestrogens [see comments]. Lancet 1996; 348:981-3.
64. Prevention of venous thrombosis and pulmonary embolism. NIH Consensus Development. JAMA 1986; 256:744-9.
65. WHO. World Health Organization Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception. Venous thromboembolic disease and combined oral contraceptives: results of international multicentre case-control study. Lancet 1995a; 346:1575-82.
66. Lowe G, Woodward M, Vessey M, Rumley A, Gough P, Daly E. Thrombotic variables and risk of idiopathic venous thromboembolism in women aged 45-64 years. Relationships to hormone replacement therapy. Thromb Haemost 2000; 83:530-5.

67. Vandenbroucke JP, Koster T, Briet E, Reitsma PH, Bertina RM, Rosendaal FR. Increased risk of venous thrombosis in oral-contraceptive users who are carriers of factor V Leiden mutation [see comments]. Lancet 1994; 344:1453-7.
68. Dilley A, Austin H, El-Jamil M, et al. Genetic factors associated with thrombosis in pregnancy in a united states population [In Process Citation]. Am J Obstet Gynecol 2000; 183:1271-7.
69. Zöller B, Berntsdotter A, Garcia de Frutos P, Dahlbäck B. Resistance to activated protein C as an additional genetic risk factor in hereditary deficiency of protein S. Blood 1995; 85:3518-23.
70. Daly E, Vessey MP, Hawkins MM, Carson JL, Gough P, Marsh S. Risk of venous thromboembolism in users of hormone replacement therapy. Lancet 1996; 348:977-80.
71. Jick H, Jick SS, Gurewich V, Myers MW, Vasilakis C. Risk of idiopathic cardiovascular death and nonfatal venous thromboembolism in women using oral contraceptives with differing progestagen components. Lancet 1995; 346:1589-93.
72. Bloemenkamp KWM, Rosendaal FR, Helmerhorst FM, B□ller HR, Vandenbroucke JP. Enhancement by factor V Leiden mutation of risk of deep- vein thrombosis associated with oral contraceptives containing third- generation progestagen. Lancet 1995; 346:1593-1596.
73. Grandone E, Margaglione M, Colaizzo D, et al. Genetic susceptibility to pregnancy-related venous thromboembolism: roles of factor V Leiden, prothrombin G20210A, and methylenetetrahydrofolate reductase C677T mutations [see comments]. Am J Obstet Gynecol 1998; 179:1324-8.
74. Aznar J, Vaya A, Estelles A, et al. Risk of venous thrombosis in carriers of the prothrombin G20210A variant and factor V Leiden and their interaction with oral contraceptives. Haematologica 2000; 85:1271-6.
75. Gerhardt A, Scharf RE, Beckmann MW, et al. Prothrombin and factor V mutations in women with a history of thrombosis during pregnancy and the puerperium [see comments]. N Engl J Med 2000; 342:374-80.
76. Tripodi A, Chantarangkul V, Mannucci PM. Hyperprothrombinemia may result in acquired activated protein C resistance. Blood 2000; 96:3295-6.
77. *Smirnov MD, Safa O, Esmon NL, Esmon CT. Inhibition of activated protein C anticoagulant* activity by prothrombin. Blood 1999; 94:3839-3846.
78. Koh KK, Horne MK, 3rd, Cannon RO, 3rd. Effects of hormone replacement therapy on coagulation, fibrinolysis, and thrombosis risk in postmenopausal women. Thromb Haemost 1999; 82:626-33.
79. van der Meer FJ, Koster T, Vandenbroucke JP, Briet E, Rosendaal FR. The Leiden Thrombophilia Study (LETS). Thromb Haemost 1997; 78:631-5.
80. Griffin JH, Evatt B, Wideman C, Fernandez JA. Anticoagulant protein C pathway defective in majority of thrombophilic patients. Blood 1993; 82:1989-93.
81. Svensson PJ, Dahlback B. Resistance to activated protein C as a basis for venous thrombosis. New England Journal of Medicine 1994; 330:517-22.
82. Nicolaes GAF, Tans G, Thomassen MCLGD, et al. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor VaR506Q by activated protein C. Journal of Biological Chemistry 1995; 270:21158-66.
83. Aparicio C, Dahlbäck B. Molecular mechanisms of activated protein C resistance. Properties of factor V isolated from an individual with homozygosity for the Arg506 to Gln mutation in the factor V gene. Biochemical Journal 1996; 313:467-72.
84. Zöller B, Svensson PJ, He X, Dahlbäck B. Identification of the same factor V gene mutation in 47 out of 50 thrombosis-prone families with inherited resistance to activated protein C. J Clin Invest 1994; 94:2521-4.
85. Engel H, Zwang L, Van Vliet HHDM, Michiels JJ, Stibbe J, Lindemans J. Phenotyping and genotyping of coagulation factor V Leiden. Thrombosis and Haemostasis 1996; 75:267-269.
86. Rosen SB, Sturk A. Activated protein C resistance--a major risk factor for thrombosis. European Journal of Clinical Chemistry and Clinical Biochemistry 1997; 35:501-16.
87. Vandenbroucke JP, Helmerhorst FM, Bloemenkamp KWM, Rosendaal FR. Third-generation oral contraceptive and deep venous thrombosis: From epidemiologic controversy to new insight in coagulation. American Journal of Obstetrics and Gynaecology 1997; 177:887-891.
88. Lidegaard O, Milsom I. Oral Contraceptives and Thrombotic Diseases: Impact of new epidemiological studies. Contraception 1996; 53:135-139.
89. Alexandre JM, Strandberg K. EMEA and third-generation oral contraceptives. Lancet 1997; 350:290.

90. WHO. World Health Organization Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception. Effect of different progestagens in low oestrogen oral contraceptives on venous thromboembolic disease. World Health Organization Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception. Lancet 1995b; 346:1582-1588.
91. Schramm W, Heinemann LAJ. Oral contraceptives and venous thromboembolism: Acquired APC resistance? British Journal of Haematology 1997; 98:491-491.
92. Balasch J. The pill scare two years later. European Journal of Contraception and Reproductive Health Care 1997; 2:149-159.
93. Winkler UH. Blood Coagulation and Oral Contraceptives. A Critical Review. Contraception 1998; 57:203-209.
94. Spitzer WO. The 1995 pill scare revisited: anatomy of a non-epidemic. Human Reproduction 1997; 12:2347-57.
95. Böttcher CJF, van Gent CM, Pries C. A Rapid and Sensitive Sub-micro Phosphorus Determination. Analitica Chimica Acta 1961; 24:203-207.
96. De Stefano V, Paciaroni K, Mastrangelo S, Rutella S, Bizzi B, Leone G. Instrument effect on the activated protein C resistance plasma assay performed by a commercial kit. Thrombosis and Haemostasis 1996; 75:752-756.
97. Sun X, Evatt B, Griffin JH. Blood coagulation factor Va abnormality associated with resistance to activated protein C in venous thrombophilia. Blood 1994; 83:3120-5.

PART II

APC resistance during hormonal changes: sex steroids and APC resistance

Introduction*

Risk factors for venous thrombosis include transient states of high hormonal levels as oral contraceptive (OC) use, pregnancy and the use of so-called hormone replacement therapy (HRT) in postmenopausal women. Female sex hormones (estrogens) have always been associated with a risk for venous thrombosis. The triad of Virchow (1859) represents three factors involved in thrombosis: changes in the vessel wall, reduction in blood flow and changes in blood composition. Since the latter component plays a predominant role in thrombus formation in the veins, venous thrombosis can be regarded as the clinical symptom of hypercoagulable plasma. Many investigators have therefore, put effort in studying changes of haemostatic parameters in individuals at risk of venous thrombosis in an attempt to understand and explain the causes of this disease.

In numerous studies the effects of oral contraceptive use, pregnancy and hormone replacement therapy on haemostatic variables have been investigated. In the next sections of this introduction an overview will be given on the thrombotic risks and changes of the haemostatic variables associated with the use of oral contraceptives, with pregnancy and with hormone replacement therapy.

Oral contraceptives

Soon after the marketing of oral contraceptive (OC) preparations in the early 60's it was reported that women who use OC are exposed to an increased risk of

* This introduction is based on: J.Curvers, G.Tans and J. Rosing. Sex steroids and blood coagulation

venous thromboembolism. The incidence of venous thrombosis decreased when the estrogen dose in oral contraceptive pills was lowered to 50µg ethinylestradiol or less[1]. In the mid-eighties so-called third generation OC pills were introduced which instead of levonorgestrel, lynestrenol or norethisterone contained a less androgenic progestagen (desogestrel or gestodene). These third generation oral contraceptives were developed to reduce the risk of cardiovascular disease. However, in 1995 it was reported that the risk of venous thrombosis in third generation OC users was increased 2- to 3-fold compared to users of the older second generation preparations containing levonorgestrel.[2-5] Until the present day, reports are appearing in literature that either deny or confirm this risk difference.[6, 7]

The effects of oral contraceptives on the haemostatic system have been studied extensively during the past years (for reviews see references[8-10]). In women who use oral contraceptives the plasma level of almost every protein that participates in blood coagulation changes. The concentration of many coagulation factors (prothrombin, VII, VIII, IX, X, XI) is increased. Together with the decrease of the anticoagulant proteins antithrombin and protein S this is indicative for a net prothrombotic effect. It has been argued, however, that these procoagulant changes may be counterbalanced by antithrombotic effects of the pill, such as elevated levels of protein C, α_1-antitrypsin, plasminogen, a decrease of plasminogen activator inhibitor-1 (PAI-1) and by an increased fibrinolytic activity.

Recently, a cross-over study was published, of which part will be presented in Chapter 6[11, 12], in which a direct comparison was made between a second generation OC (containing 30µg ethinylestradiol and 150µg levonorgestrel) and a third generation OC (containing 30µg ethinylestradiol and 150µg desogestrel).

The OC-induced changes of the plasma levels of proteins involved in the procoagulant, anticoagulant and fibrinolytic pathways are moderate and in most OC users the values of the haemostatic parameters stay within the normal range. However, nowadays it is known that even relatively small increases in levels of coagulation factors are associated with an increased risk of venous thrombosis[13-17]. This suggests that the modest changes of plasma levels of coagulation factors and the differential effects of second and third generation OC thereon cannot be ignored in the discussion about the thrombotic effects of OC.

In 1997 observations were published that are indicative for another prothrombotic effect of OC. It was reported that plasma of users of OC is resistant to APC and that there was a significant difference in APC resistance between users of second and third generation OC.[18] The criticism on the design of this study was taken away in the randomised cross-over study mentioned above which established that women who use OC pills with desogestrel are more resistant to APC than users of levonorgestrel-containing OC.[11] It is interesting to note that women who were using OC and who were also heterozygous carriers of factor V_{Leiden} had a degree of APC resistance that is normally observed for homozygous carriers of the factor V_{Leiden} mutation (Chapter 4 and [18]). The amplifying effects of OC use and factor V_{Leiden} in the APC resistance test may explain the high risk of venous thrombosis of women with factor V_{Leiden} who use OC.[4]

Pregnancy

It is generally accepted that there is a state of hypercoagulability during pregnancy and puerperium. This hypercoagulable state may prevent excessive blood loss during delivery,[19] but it also accounts for thrombotic complications that may occur during pregnancy. During pregnancy the incidence of venous thromboembolism is 0.3-1.0 per 1000 pregnant-women years[20, 21] of which 75% occurs antepartum.[22, 23] Thus, the risk of venous thrombosis during pregnancy rises some three- to sevenfold. However, the post-partum period can be regarded as the most dangerous period, since the majority of cases of pulmonary embolism, occur post-partum[23]. Correction of the epidemiological data for the shorter duration of the post-partum period yields a twenty fold risk increase during the puerperium.[21, 24, 25] Although a number of additional factors can be designated as risk factor for post-partum thrombosis (>35 years of age, multiparity, caesarean section, hereditary thrombophilia), it is likely that changes in the haemostatic system also play a major role in the thrombotic risk during this period.

A detailed report on the effect of normal pregnancy on coagulation parameters was published in 1984 by Stirling et al.[26] They showed that the plasma levels of a number of coagulation factors consistently rise during pregnancy: factor VII (to 170%), factor VIII (to 200%, an increase that sustained after delivery), factor X (to 130%) and fibrinogen (to 4.2 g/L). Prothrombin and factor V initially also increased,

but after 20-30 weeks decreased again, a phenomenon that may be due to haemodilution. The levels of the protease inhibitors α_1-antitrypsin and α_2-macroglobulin, which probably do not play an important role in the *in vivo* regulation of coagulation, considerably increased during pregnancy. However, the plasma level of the major physiological inhibitor antithrombin hardly changed. Furthermore, the clot lysis time decreased during pregnancy but increased enormously after delivery indicating a vast drop in fibrinolytic capacity in the post partum period. On the basis of these observations Stirling and co-workers[26] concluded that the initial increase of the level of coagulation factors and fibrinogen was mediated by estradiol and that this was a mechanism to ensure integrity of the endometrium and placenta.

With respect to markers of coagulation van Wersch et al.[27] showed significant positive correlations between gestational age and thrombin-antithrombin (TAT) complexes and fibrin monomers. In several other studies increased levels of TAT complexes,[28-30] soluble fibrin monomers[28, 31] and F1+2[29-32] were observed. F1+2 is formed when prothrombin is converted to thrombin by factor Xa and like TAT complexes and fibrin monomers is a marker for *in vivo* thrombin formation. F1+2 formation increases with gestational age (correlation = 0.69), especially after 20 weeks of pregnancy.[31, 33, 34] Since increased plasma levels of TAT complexes, F1+2 and D-dimer are indicative for thrombin formation and for fibrinolytic activity, it appears that in pregnant women both the coagulation and fibrinolytic system are in an activated state.

Protein S, the cofactor of APC in the anticoagulant protein C pathway, circulates in plasma in a free form and in complex with C4B binding protein. The total plasma protein S levels decrease during pregnancy to 70% of normal.[35] Later it was shown that not only total protein S decreases, but that free protein S is also decreased.[28, 31, 32, 36] Low levels of free protein S are already observed after 10 weeks of gestation[31] and in some cases free protein S dropped to 40% of normal. This low level sustains until after delivery (even until 8 weeks postpartum). In contrast, changes of protein C during pregnancy are much less pronounced or absent.[28, 36] Since free protein S acts as a cofactor of APC in the down regulation of coagulation, the impairment of the protein C pathway due to low levels of free protein S may contribute to the increased risk of venous thrombosis during pregnancy.

Soon after the discovery of APC resistance and factor V_{Leiden} it was reported that pregnant women who do not carry the factor V_{Leiden} mutation become resistant to APC,[34, 37-40] a phenomenon that was called acquired APC resistance. Since increased resistance to APC was measured with an APC resistance assay that is based on a clotting test and clotting times are influenced by factor VIII and protein S levels, it is possible that increased APC resistance is due to the high levels of FVIII and/or the low levels of protein S during pregnancy. Although a number of studies suggested that the changes in APC resistance correlated with the changes in factor VIII[32, 38, 39] and protein S,[32] Kjellberg and co-workers[31] did not find such correlations. This indicates that other plasma components may also contribute to APC resistance.

Although significant, the extent of APC resistance determined with classical APC resistant assays is in general less pronounced than that of carriers of the factor V_{Leiden} mutation. In 1997 we described an APC resistance test that is based on measurement of the effect of APC on thrombin formation in full plasma.[41] We found that this assay is strongly affected by pregnancy and we have shown that APC resistance determined with this test increases with gestational age (Chaper 4 and [42]). Using a similar assay, Sugimura et al.[43] reported an enormous increase in APC resistance in one woman who developed thrombosis during delivery demonstrating that APC resistance that develops during pregnancy may contribute to the risk for venous thrombosis.

Hormone Replacement Therapy

Although early epidemiological studies did not show that HRT is associated with an increased risk for VTE,[44-46] recent case-control studies established that compared to non-users, current users of hormone replacement therapy have some 3-fold higher risk of VTE.[47, 48]

With respect to the effect of HRT on haemostatic variables there are many conflicting reports. This is likely caused by the fact that the changes of coagulation parameters during HRT are less pronounced than those occurring during OC use or pregnancy. Also the amount and source of estrogen, the route of administration (oral or transdermal) and the absence or presence of opposing progestagens may have different impacts on the coagulation system. Orally administered preparations seem to have a more pronounced effect than transdermally applied preparations.

Only limited information is available on the effect of HRT on the coagulation factors II, V, VIII, IX, X and XI. Several studies focussed on the effects of HRT on factor VII and fibrinogen. The interest in these coagulation factors, which substantially increase during OC use and pregnancy, is presumably caused by the fact that they have been implicated with an increased risk of arterial thrombosis[49, 50]. Oral estrogen-only therapy appears to be associated with increased levels of factor VII[51-54], whereas combined HRT or transdermal estrogens have no effect or even lower the plasma level of factor VII.[52-55] In contrast to oral contraceptives and pregnancy, HRT with both estrogen-only and combined preparations causes a reduction of fibrinogen.[51, 56-60] Since high factor VII and fibrinogen levels are associated with an increased risk of cardiovascular disease[49, 50] the lowering of the plasma levels of these proteins during HRT may have a beneficial effect on the risk. These beneficial effects may, however, be counteracted by decreases of the plasma levels of the anticoagulant proteins antithrombin,[59, 61-66] protein C[65] and protein S.[66-68] We would like to mention, however, that there are also studies in which increases of protein C[51, 66, 69] and protein S[70] were reported. Like OC users and pregnant women, users of HRT develop acquired APC resistance (Chapter 4 and [42, 71]), the extent of which is, however, much less pronounced than during OC use and pregnancy.

Markers of ongoing coagulation such as F1+2,[66, 68, 72] fibrinopeptide A[66] and soluble fibrin[72] are increased during HRT. Despite modest effects of HRT on the levels of individual coagulation factors this is indicative for activation of the coagulation system. Increased D-dimer[72] and plasmin-antiplasmin[54] levels show that not only the coagulation system but that fibrinolysis is also enhanced during HRT. However, there are also studies in which no changes of F1+2,[54] TAT-complexes[68] and D-dimer[54] were observed. In the cases in which increased coagulant and fibrinolytic activities were observed there appeared to be no correlation between the elevation of markers of coagulation and fibrinolysis.[73, 74] This suggests that the potentiation of fibrinolysis during HRT is not a response to enhanced activation of coagulation.

Chapter 5

Acquired APC resistance during OC use

Based on:

Low dose oral contraceptives and acquired resistance to activated protein C: a randomised cross-over study. Jan Rosing, Saskia Middeldorp, Joyce Curvers, M.Christella, L.G.D. Thomassen, Gerry A.F. Nicolaes, Joost C.M. Meijers, Bonno N. Bouma, Harry R. Buller, Martin H. Prins, Guido Tans. Lancet, 1999, 354, 2036-2040

A randomised cross-over study on the effects of levonorgestrel- and desogestrel-containing oral contraceptives on the anticoagulant pathways.

Guido Tans, Joyce Curvers, Saskia Middeldorp, M.Christella L.G.D. Thomassen, Joost C.M. Meijers, Martin H. Prins, Bonno N. Bouma, Harry R. Buller, Jan Rosing. Thrombosis and Haemostasis, 2000, 84, 15-21

Summary

The use of oral contraceptives (OC) causes disturbances of the procoagulant, anticoagulant and fibrinolytic pathways of blood coagulation which may contribute to the increased risk for venous thrombosis associated with OC therapy. We have reported previously that, compared with use of second generation oral contraceptives, the use of third generation OC is associated with increased resistance to the anticoagulant action of activated protein C (APC). Owing to the cross-sectional design of that study, these obsevations may have been subject to unknown bias or uncontrolled effects of the menstrual cycle. We aimed to overcome these sources of bias by doing a cycle-controlled randomised cvross-over trial. A number of anticoagulant parameters and the response to APC in plasma was assessed in 33 women, who were randomly prescribed either a second generation (150 μg levonorgestrel/30 μg ethinylestradiol) or a third generation OC (150 μg desogestrel/30 μg ethinylestradiol) and who switched preparations after two pill-free cycles. The anticoagulant parameters determined were: antithrombin (AT), α_2-macroglobulin (α_2-M), α_1-antitrypsin, protein C inhibitor (PCI), protein C, total and free protein S and activated protein C sensitivity ratios (APC-sr) measured with two functional APC resistance tests which quantify the effect of APC on either the activated partial thromboplastin time (aPTT) or on the endogenous thrombin potential (ETP). Of the 33 women, five were excluded because not all required plasma samples were available. During the use of desogestrel-containing OC the plasma levels of α_2-M, α_1-antitrypsin, PCI and protein C significantly increased, whereas AT and protein S significantly decreased. Similar trends were observed with levonorgestrel-containing OC, although on this kind of OC the changes in AT, PCI and protein S (which was even slightly increased) did not reach significance. Compared with levonorgestrel, desogestrel-containing OC caused a significant decrease of total ($p<0.005$) as well as free protein S ($p<0.0001$) and more pronounced APC resistance in the aPTT-based APC resistance tests ($p=0.02$). The additional increase (0.51, 95% CI 0.37-0.66) in APCsr in the ETP-based APC resistance tests was highly singificant ($p<0.0001$). Normalised APC sensitivity ratios during OC treatment correlated with values before OC use.

These observations indicate that the activity of the anticoagulant pathways in plasma from users of desogestrel-containing OC is more extensively impaired than in plasma from users of levonorgestrel-containing OC.

Introduction

Since the early 1960s, the use of oral contraceptives has been associated with an increased risk of venous thromboembolism. After the introduction of low-dose pills containing 50µg ethinyl estradiol or less the thrombotic risk gradually decreased[75]; thus the oestrogen component of oral contraceptives was generally accepted as being the cause of the increased risk for venous thromboembolism (VTE). However recent epidemiological reports suggest that the type of progestagen in oral contraceptives may also have a role in the increased risk on VTE. In 1995 and 1996 it was reported that women who used third generation OC (containing desogestrel or gestodene) had a thrombotic risk two to three times that of women using second generation OC containing levonorgestrel.[3-5, 76] The outcome of these studies was, however, questioned in later publications.[77, 78] This controversy resulted in several critical reviews in which the effects of progestagen on the occurrence of VTE were discussed.[79-82]

So far, there is no good biological explanation for the thrombotic effect of oral contraceptives. These preparations affect the concentrations of many proteins involved in blood coagulation,[10, 83] but since changes are small and because concentrations of coagulation factors usually stay within normal range during oral contraceptive use, most investigators agreed that the effect of such preparations on haemostatic variables could not explain the incresaed risk of venous thromboembolism.

Earlier we have reported that oral contraceptive use is associated with acquired resistance to activated protein C (APC), and that the plasma of women who use third generation OC is more resistant to the anticoagulant action of APC than the plasma of users of second generation OC.[18] Since hereditary APC resistance,[84] which is due to a mutation in factor V (factor V_{Leiden}) at an APC cleavage site,[85] is associated with an increased risk of venous thrombosis,[86] several investigators[18, 80, 87] have postulated that acquired APC resistance (measured as the effect of APC on thrombin generation) may explain the thrombotic effect of oral contraceptives and the further risk associated with the use of third generation preparations.

Since our previous study was cross sectional and did not control for menstrual-cycle effects, selection-bias or uncontrolled effects may have contributed to the observed differences between the different kinds of oral contraceptive.[79, 83, 88-90] We now report

the effects of a second generation OC containing levonorgestrel and a third generation OC containing desogestrel on the anticoagulant action of APC and other proteins involved in the anticoagulant pathways in the plasma of women who took part in a cycle controlled randomised cross-over study.

Design

Thirty three healthy female volunteers between the ages of 18 and 40 years, with no history of venous thromboembolism, no previous pregnancy, and who had not used oral contraceptives for at least 12 months before the start of the study, were assigned in random order two oral contraceptive preparations: a levonorgestrel-containing monophasic oral contraceptive preparation (30µg ethinylestradiol and 150µg levonorgestrel) and a desogestrel-containing monophasic oral contraceptive preparation (30µg ethinylestradiol and 150µg desogestrel). Randomisation was done in blocks of ten, by use of a central facility (Academic Medical Center).

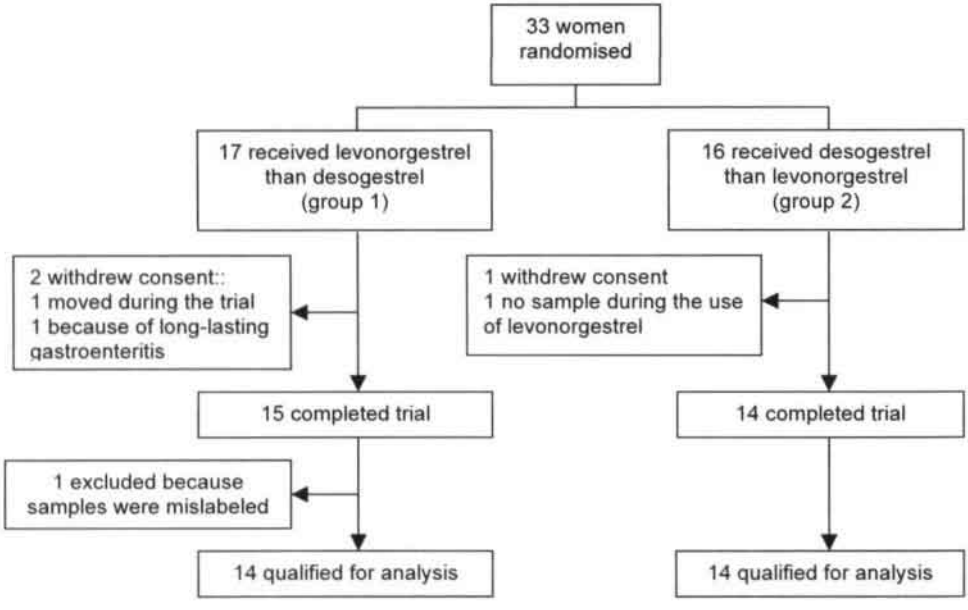


Figure 5.1 Trial profile

The first OC was used for two consecutive cycles and after a washout of two menstrual periods the volunteer was switched to the other preparation for two more cycles. Blood samples were obtained between day 18 and 21 of six menstrual cycles.

That is at baseline (before starting on the OC), during the two cycles of the first period, in the second menstrual cycle of the washout period and during the two cycles of the second period (see Figure 5.1). All volunteers gave written informed consent. The study was approved by the Institutional Review Board of the Academic Medical Center, Amsterdam, The Netherlands.

All blood samples (nine parts blood in one part 0.13M sodium citrate, pH 7.8) were drawn under standard conditions, i.e. at 9.00 a.m., after a period of 15 minutes rest, and after abstinence of caffeine, alcohol or nicotine for 12h. Cell free, citrated plasma was prepared and centrally stored at -70°C. Control normal plasma was from 90 healthy volunteers (40 women not using OC and 50 men, mean age 35 years). Plasma samples were analyzed after all volunteers had completed the study protocol. Measurements were performed in duplicate and without knowledge of the OC preparations used.

Laboratory methods

The chromogenic substrates D-Phe-(pipecolyl)-Arg-pNA (S2238) and L-pyroGlu-Pro-Arg-pNA (S2366) were supplied by Chromogenix, Mölndal, Sweden. Phospholipids were from Avanti Polar Lipids, Alabaster, Alabama, USA. Small unilamellar phospholipid vesicles, composed of a mixture of 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) and/or 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) (20/20/60, M/M/M), were prepared as described before [41, 91]. Phospholipid concentrations were determined by phosphate analysis [92].

Quantitation of plasma protein levels The plasma levels of the proteins determined (antigen or activity) were expressed as percentage of that present in normal pooled plasma determined in the same experiment.

All commercially available assays were carried out according the manufacturer's instructions. α_2-Macroglubulin and α_1-antitrypsin were determined via their ability to inhibit trypsin added to diluted plasma samples using the Coaset α_2-macroglubulin/α_1-antitrypsin kit from Chromogenix (Mölndal, Sweden). Antithrombin activity was determined with the Coamatic antithrombin kit (Chromogenix). Antigen levels of protein C inhibitor (PCI) were determined by ELISA using a monoclonal

antibody against PCI (API-93) as capturing antibody and rabbit polyclonal anti-PCI serum as secondary antibody [93]. This assay will be described in detail elsewhere. Protein C was determined using the kits from Chromogenix (Coamatic protein C activity) and from Boehringer/Stago (protein C antigen). Protein S (total and free antigen) was determined using the protein S antigen kit from Reaads Medical Products Inc, USA. At the time of measurement of protein S, some plasmas were no longer available for analysis. This resulted in incomplete sets (samples from start, first phase, washout and second phase) and in smaller groups (total protein S antigen: n = 13 starting with levonorgestrel- and n = 8 starting with desogestrel-containing OC; free protein S antigen: n = 13 starting with levonorgestrel- and n = 10 starting with desogestrel-containing OC). Coefficients of variation (%CV = 100*SD/mean) were estimated from repeated measurements (on various days) of reference samples and were similar to those given by the manufacturer for the commercially available assays. The %CV for the PCI antigen determination and for the nAPC-sr determined with the ETP-based assay (see below) were 11.6 and 6.9%, respectively.

APC-resistance assays Normalized APC-sensitivity ratios (nAPCsr) in the ETP-based assay were determined by comparing the effect of 5 nM human APC (Enzyme Research Laboratories purchased from Kordia Laboratory Supplies, Leiden, The Netherlands) on thrombin generation initiated in subject plasma and in normal pooled plasma with 0.4 µg/L tissue factor (Dade, USA), 15 µM phospholipid and 16 mM added $CaCl_2$ (final concentrations in plasma) as described previously [18]. APC was quantitated as described by Sala et al. [94] and tissue factor was determined with an antigen assay (American Diagnostics). To 80 µl defibrinated plasma incubated at 37°C was added 45 µl starting solution containing tissue factor, $CaCl_2$ and phospholipid vesicles, with or without APC to result in the abovementioned final concentrations. After 20 min incubation at 37°C amidolytic activity of α_2-macroglobulin-thrombin (α_2M-IIa) complexes was determined in diluted aliquots using S2238 by measurement of the rate of change in absorbance determined at 405 minus 492 nm at 37°C in a microtiter plate reader (SLT Labinstruments, Salzburg, Austria) set in the kinetic mode The normalized APC sensitivity ratio (nAPCsr) was

defined as the ratio of the amounts of α_2-macroglobulin-thrombin complex (α_2M-IIa) determined in the presence and absence of APC divided by the ratio determined in the normal plasma pool (cf. De Ronde [95]):

$$nAPCsr = (\alpha_2M\text{-}IIa_{+APC}/\alpha_2M\text{-}IIa_{-APC})_{plasma\ sample}/(\alpha_2M\text{-}IIa_{+APC}/\alpha_2M\text{-}IIa_{-APC})_{normal\ plasma}$$

The aPTT-based APC resistance test (Coatest ® APC™) was performed in undiluted plasma as described by the supplier (Chromogenix, Mölndal, Sweden). Clotting times were determined on an ACL 300R coagulation analyser (Instrumentation Laboratory, Milan, Italy). The normalized APC sensitivity ratio (nAPCsr) was defined as the ratio of the clotting times (aPTT) determined in the presence and absence of APC normalized by division through the same ratio determined in the normal plasma pool:

$$nAPCsr = (aPTT_{+APC}/aPTT_{-APC})_{plasma\ sample}/(aPTT_{+APC}/aPTT_{-APC})_{normal\ plasma}$$

The presence of the factor V_{Leiden} mutation was determined by DNA analysis.

Statistical analysis Analysis was limited to subjects, of whom both baseline samples and at least one sample during each OC exposure were available. For each parameter determined, all samples of an individual were assayed in duplicate in a single experiment and compared to normal plasma determined in the same experiment. Values obtained during the two cycles on a given OC preparation were averaged and the change in parameter was calculated relative to the value determined immediately prior to the start of OC use (*i.e.* relative to baseline in phase 1 or washout in phase 2). Graphs of this treatment effect were inspected for interaction. In the absence of interaction (protein C antigen, protein C inhibitor antigen, total and free protein S antigen, antithrombin, α_1-antitrypsin and nAPC-sr in the aPTT-based assay) the changes in parameter in the first and second phase of treatment were combined for each oral contraceptive and the statistical significance of the change relative to baseline/washout was assessed with a paired t-test. The difference between the values of the parameters during the use of levonorgestrel and desogestrel was also assessed using a paired t-test.

Since there was an indication for a modest treatment by phase interaction for α_2-macroglobulin, protein C activity and the ETP-based APC-resistance assay the phase difference (phase 1 minus phase 2) as a result of changing OC was calculated for the individuals in each separate group[96]. The statistical significance of the phase

difference between the two treatment groups (desogestrel → levonorgestrel and levonorgestrel → desogestrel) was assessed with a t-test assuming non-equal variances[96]. It was calculated that the inclusion of 30 volunteers would allow detection of differences of changes between desogestrel and levonorgestrel use which are 60% or more of the standard deviation.

Associations between parameters were searched for by calculation of the Pearson correlation coefficient before and during OC use and for the change occurring as a result of OC use (relative to baseline or washout).

Results

Five of the 33 eligible volunteers were excluded from analysis. One because blood samples were only obtained during use of one OC type, one due to mislabelling of samples and three because they withdrew consent. None of the volunteers developed clinical signs or symptoms of venous thromboembolism. Thus,

Table 5.1 Effect of oral contraceptives on anticoagulant plasma parameters

	Levonorgestrel			*Desogestrel*		
	before OC mean (SD)	during OC mean (SD)	increase* mean (SD)	before OC mean (SD)	during OC mean (SD)	increase* mean (SD)
Individual plasma components (expressed as % of normal pooled plasma)						
Antithrombin	111 (9)	110 (8)	-1.3 (8.0)^{NS}	112 (11)	109 (10)	-3.1 (7.8)
α2-macroglobulin	119 (32)	128 (34)	8.9 (14.9)	114 (31)	128 (31)	14.1 (14.6)
α1-antitrypsin	94 (22)	125 (29)	31.8 (13.8)	92 (20)	128 (27)	36.1 (11.0)
PC antigen	84 (14)	93 (19)	9.1 (13.2)	85 (17)	95 (1)	9.8 (12.1)
PC activity	89 (15)	97 (18)	7.6 (10.3)	89 (16)	100 (18)	11.0 (9.6)
PC Inhibitor antigen	83 (18)	84 (17)	0. (11.4)^{NS}	82 (13)	89 (18)	6.6 (12.0)
PS total antigen (n=21)	99 (13)	104 (16)	5.0 (20.4)^{NS}	99 (10)	88 (13)	-11.4 (9.7)‡
PS free antigen (n=23)	94 (14)	99 (15)	4.3 (17.9)^{NS}	94 (12)	82 (12)	-12.1 (9.3)‡
APC resistance assays						
nAPCsr (ETP-based)	1.39 (0.49)	2.17 (0.58)	0.77 (0.33)	1.40 (0.55)	2.69 (0.76)	1.29 (0.44)¥
nAPCsr (aPTT-based)	0.83 (0.11)	0.84 (0.12)	0.00 (0.10)^{NS}	0.83 (0.11)	0.80 (0.09)	-0.04 (0.08)‡

PC= protein C, PS=protein S, nAPCsr=normalized APC sensitivity ratioDifferences before and during OC use were statistically significant (P<0.05) unless indicated with NS (NS=non-significant) using a paired t-test. ‡ Significant difference between levonorgestrel and desogestrel OC preparations assessed with a paired t-test. ¥ Significant difference between OC preparations assessed with a t-test (assuming non-equal variances)

plasma samples of 28 subjects were available for analysis, group 1 starting with levonorgestrel (n=14) and group 2 starting with desogestrel (n=14).

Effects of OC on the plasma levels of anticoagulant proteins. Table 5.1 summarizes the plasma levels of protein C, protein S, antithrombin, α_2-macroglobulin, α_1-antitrypsin and protein C inhibitor determined at the start of each OC regime (baseline or washout), during OC treatment and the calculated change observed as a result of OC use. One woman was found to have a combined heterozygosity for the factor V_{Leiden} mutation and protein C deficiency (antigen and activity levels 40% of normal). With the exception of this woman, plasma levels of the various anticoagulant proteins at the start of the study were similar for both treatment groups.

During treatment with levonorgestrel-containing OC, the plasma levels of protein C (both antigen and activity), α_2-macroglobulin and α_1-antitrypsin significantly increased, while the changes in antithrombin, protein C inhibitor and total and free protein S did not reach statistical significance. The use of desogestrel-containing OC had a more pronounced effect on the levels of the anticoagulant proteins and resulted in statistically significant increases of α_2-macroglobulin, α_1-antitrypsin, protein C inhibitor and protein C and in significant decreases in antithrombin and total and free protein S. (Table 5.1). Differences between the two OC preparations, however, only reached significance for the decrease in both the total and free antigen levels of protein S.

The individual values determined for protein S (total and free antigen) are shown in Fig. 5.2. Both total protein S and free protein S antigen showed a small increase (4–5%, $p>0.25$) with levonorgestrel, whereas with desogestrel-containing OC a significant decrease was observed (11-12%, $p<0.005$). The overall difference between desogestrel- and levonorgestrel-containing OC was estimated to be approximately 16% for both total protein S ($p<0.005$) and free protein S ($p<0.0001$, Table 5.1). The individual with the factor V_{Leiden} mutation, who was also deficient in protein C had relatively high protein S levels (Fig. 5.2, open circles), but the changes in protein S during OC use were similar as for the other volunteers.

APC-resistance measurements

Two functional assays were used to evaluate the effect of OC's on the ability of APC to down-regulate in vitro coagulation *i.e.* the classical aPTT-based APC resistance test, which quantifies the effect of APC on the clotting time of plasma in which coagulation is initiated via the intrinsic pathway and an assay which determines the effect of APC on the time integral of thrombin formation (the endogenous thrombin potential, ETP) initiated in plasma via the extrinsic coagulation pathway. It should be noted that due to the calculation procedure APC-sensitivity ratios (APCsr) show opposite trends in the two assays *i.e.* compared with normal plasma, APC resistant plasmas will give a lower APC-sr in the aPTT-based assay and higher APC-sr in the ETP-based assay (see also Fig. 5.3).

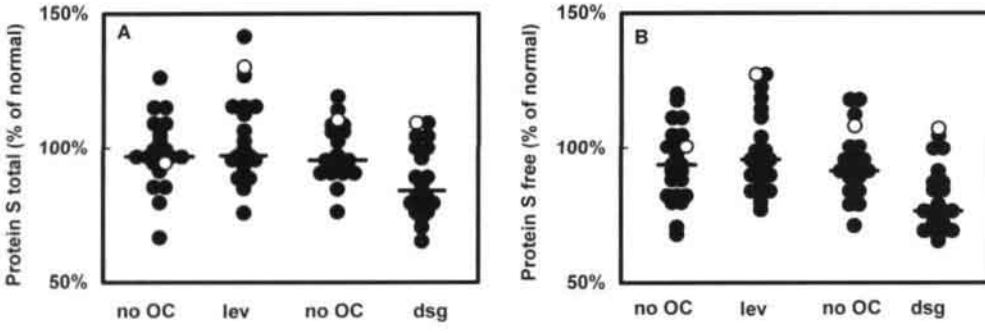


Figure 5.2 Protein S antigen levels at different time intervals in the cross-over. (A) Total protein S antigen. (B) Free protein S antigen. Lev= levonorgestrel containing OC. Dsg= desogestrel containing OC. The open symbol represents the individual combined heterozygous for FV-Leiden and protein C deficient.

Normalised APC sensitivity ratios were calculated for each treatment group at each sampling time during the study (Fig 5.3). With the exception of one women with a factor V_{Leiden} mutation (baseline ratio 3.13), the APCsr before OC use were similar in the two groups and within the normal range. Fig. 5.3A shows the results obtained with the aPTT-based APC resistance test. Levonorgestrel-containing OC caused a minimal increase of the nAPC-sr whereas the use of desogestrel-containing OC resulted in a slight decrease of the nAPC-sr (Fig. 5.3A, Table 5.1).

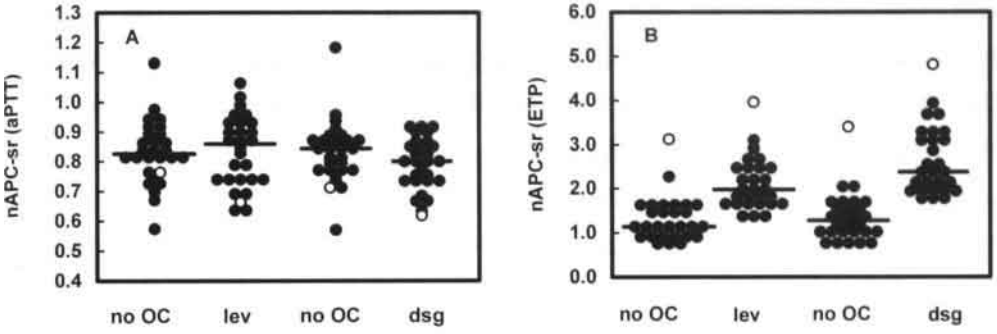


Figure 5.3 nAPCsr values determined in the (A) classical undiluted aPTT-based assay and the (B) ETP-based assay. The open symbol represents the individual combined heterozygous for FV-Leiden and protein C deficient.

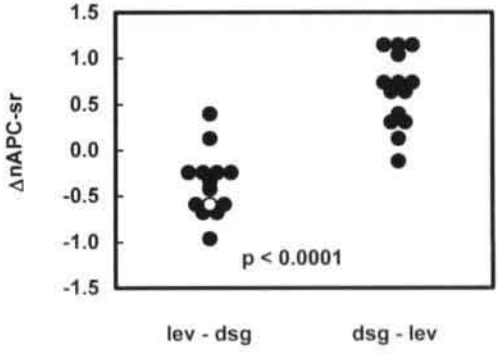


Figure 5.4 Change in normalised APC sensitivity ratio during cross-over. Difference between average increases in ratios in phase 1 and phase 2, relative to baseline or wash-out, respectively. Open symbol represents heterozygous factor V_{Leiden} carrier.

Thus, compared to levonorgestrel, exposure to desogestrel-containing OC was associated with an increased resistance to APC in the aPTT-based APC-resistance assay ($p = 0.02$, Table 5.1).

In both groups (including the FV_{Leiden} individual), the APCsr's increased substantially during OC treatment (Fig. 5.3B). Also, the difference between the two OC preparations was more pronounced in the ETP-based test (Fig 5.4) than observed in the aPTT based APC resistance test. The use of desogestrel-containing OC caused significantly higher resistance to APC than the use of levonorgestrel-containing OC ($p<0.0001$, Table 5.1, Fig. 5.2), the mean difference being 0.51 (95% CI 0.37-0.66, Fig 5.4).

The average nAPCsr for the two cycles of either contraceptive (irrespective of the treatment period) were plotted against the values calculated before the start of the respective treatments (Fig 5.4). For both regimens, a highly significant correlation

was observed between the ratio before and during OC therapy (r=0.81 (0.64-0.91) for levonorgestrel and 0.83 (0.66-0.92) for desogestrel). The correlation remained when the FV-Leiden individual was omitted from the regression analysis (0.69 (0.42-0.84) and 0.75 (0.52-0.88) respectively).

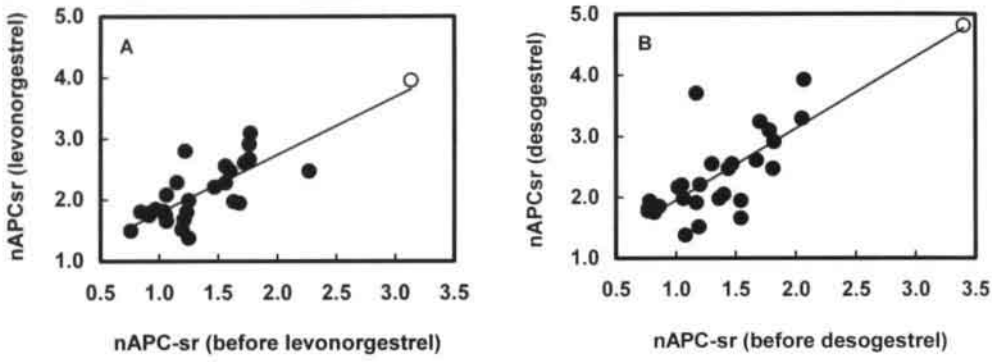


Figure 5.5 Correlation plots of normalised APC sensitivity ratios before and during OC use. The open symbol represents the FV-Leiden carrier.

Table 5.2 illustrates that the APCsr obtained with both APC-resistance assays showed a rather weak correlation both before using OC and during levonorgestrel treatment and a somewhat stronger association during the use of desogestrel-containing OC. However, no association was observed between the changes of the APCsr determined with both tests occurring as a result of OC-use.

Table 5.2 Correlations between ETP-based APC resistance mesurements and the aPTT-based APC resistance measurements, total protein S and free protein S.

	Correlation aPTT nAPCsr	p-value	Correlation Total Protein S	p-value	Correlation Free Protein S	p-value
Before OC use	-0.43	0.02	-0.32	0.16	-0.54	0.01
During OC use						
Levonorgestrel	-0.49	0.01	-0.52	0.01	-0.49	0.02
Change with levonorgestrel	-0.31	0.11	-0.53	0.02	-0.60	0.003
Desogestrel	-0.61	0.001	-0.51	0.02	-0.66	0.001
Change with desogestrel	-0.23	0.25	-0.45	0.05	-0.44	0.04

Pearson correlations (excluding the combined heterozygous FV-Leiden en Protein C deficient individual) for the association between nAPC-sr determined in the ETP-based assay and the nAPCsr determined in the aPTT and plasma protein S levels before and during OC use and for the change in parameters as a result of OC treatment (relative to baseline or washout).

APC-resistance and protein S levels. Since protein S is a cofactor for APC in the inactivation of factors V(a) and VIII(a) and hence may modulate the response to APC in APC resistance tests, the data obtained with both assays were analysed with respect to a possible association with protein S levels. In this analysis the individual who was combined factor V_{Leiden} positive and protein C deficient was excluded. The aPTT-based nAPC-sr values did not show an association with the protein S levels determined (not shown). Table 5.2 shows the Pearson correlations observed for the ETP-based assay. The nAPC-sr obtained with the ETP-based APC resistance test showed association with protein S levels and also the change of the nAPC-sr observed during treatment with both OC preparations correlated with the change in protein S levels (Table 5.2). Free protein S values showed somewhat stronger correlations than total protein S (Table 5.2).

Discussion

In order to obtain more information on possible differential effects of second and third generation OC on the anticoagulant pathways we have performed a randomized cross-over study in which we compared the effects of levonorgestrel- and desogestrel-containing OC on anticoagulant parameters known to be associated with an increased risk for venous thromboembolic disease. These include protein C, protein S and antithrombin as well as two APC-resistance assays, which assess the ability of added APC to down-regulate coagulation. In addition, we determined the plasma levels of protease inhibitors that may be involved in the regulation of the activity of APC *i.e.* protein C inhibitor, α_2-macroglobulin and α_1-antitrypsin.

With regard to the trends of the changes of the anticoagulant proteins induced by OC, the data presented here are in good agreement with earlier reports (for recent reviews see.[10, 83] Both OC preparations caused some 10% increase in protein C (both antigen and activity) and α_2-macroglobulin and a marked increase (30-35%) in α_1-antitrypsin. The latter inhibitors have been implicated in the control of APC *in vitro* [97-99] and *in vivo* [99] and their increase may, therefore, attenuate the effect of the raised protein C level. With levonorgestrel, changes in the other proteins were not significant but the use of desogestrel-containing OC resulted in significant changes of the plasma levels of antithrombin, protein C inhibitor and protein S (cf. Table 5.1).

A highly significant difference between the two OC preparations was observed with respect to the effect of OC on protein S levels (cf. Table 5.1, Fig. 5.1). In agreement with earlier reports we observed that total protein S [100-105] as well as free protein S levels [101-103] decreased during the use of desogestrel-containing OC. In contrast, treatment with levonorgestrel-containing OC resulted in a slight, but not significant increase of the protein S level. This finding differs from an early report in which lower protein S levels were found to be associated with the use of a levonorgestrel-containing OC,[106] but it is in agreement with a later study in which 11 women were followed during the first cycle of levonorgestrel-containing OC use[107] and in which a decrease in total protein S was observed during the second week of hormone administration but no significant change was observed at the end of the pill cycle. Thus, the increased protein C levels observed with both OC preparations will be counteracted by decreased protein S levels during the use of desogestrel-containing OC but not during the use of levonorgestrel-containing OC. Hence, the anticoagulant potential of plasma of desogestrel-users will likely be less than that of levonorgestrel users.

This assumption was corroborated by the overall APC-resistance measurements. In agreement with others[88, 108-111] we observed that the nAPC-sr as determined with the aPTT-based APC resistance test was marginally influenced by OC use (Table 5.1, Fig. 5.2). APTT-based nAPC-sr values were not affected by levonorgestrel-containing OC whereas treatment with desogestrel-containing OC caused a small increase in APC-resistance ($p<0.05$, Table 1). Compared with second generation OC, the use of third generation OC resulted in a small overall increased resistance to added APC in the aPTT-based assay ($p=0.02$). Resistance to APC as determined in the ETP-based assay was strongly increased on both OC, with desogestrel-containing OC causing more pronounced APC resistance than levonorgestrel-containing OC ($p<0.001$, Table 5.1, cf. also Fig. 5.2), the latter result is in concordance with previous publications.

The mechanistic basis of the occurrence of acquired APC resistance during OC use and of the different extents by which second and third generation OC affect the anticoagulant action of APC remains to be established. Since neither the protein C antigen and activity assays, nor the level of protein C inhibitor did show a significant change it is tempting to speculate that other plasma components that regulate the protein C pathway are involved in the increased resistance to APC. Rotteveel et al.[112]

reported that the time integral of thrombin formation (ETP) in plasma of OC users increased some 20%. Consistent with this, we observed that the ETP in the absence of added APC (as reflected in the measurement of α_2-macroglobulin-thrombin complexes, α_2M-IIa) increased about 24% with levonorgestrel and 30% with desogestrel-containing OC (data not shown). This increase is well explained by the increased levels of prothrombin [113] (13% and 16%, respectively) and α_2-macroglobulin and the small decrease in antithrombin (Table 5.1). Since the ETP_{-APC} (α_2M-IIa_{-APC}) is in the denominator of the APCsr, a sole increase of thrombin generation in the absence of APC during OC use would result in a decrease of the APCsr rather than in the observed increase. Thus, the increase of the APCsr during OC therapy observed with the ETP-based APC resistance test must be caused by a profound increase of thrombin generation in the presence of APC. Since the anticoagulant action of APC quantified in the aPTT-based assay is hardly affected by OC, this indicates that the use of OC impairs the efficacy by which thrombin formation, initiated via the extrinsic coagulation pathway is down-regulated by the protein C system.

Although both APC-resistance assays are equally sensitive for the presence of the factor V_{Leiden} mutation,[108] the correlation between APCsr determined with both assays is surprisingly weak [108] (Table 5.2). On the basis of these observations we proposed that both assays are differentially modulated by other plasma components [108]. One such a component appears to be protein S. The classical aPTT-based APC-sr is rather insensitive to variations in protein S [114, 115]. The results obtained in the present study confirm that protein S levels (both total and free antigen) do not correlate with the aPTT-based nAPC-sr. The ETP-based assay, however, appears to be influenced to some extent by protein S levels, particularly by free protein S (Table 5.2). It should be noted that the observed changes in protein S levels cannot fully account for the increased nAPC-sr values during OC use since in the period of treatment with levonorgestrel-containing OC the average protein S levels did not significantly change, while the nAPC-sr determined with the ETP-based assay significantly increased (Table 5.1). It appears, therefore, that the overall increase in nAPC-sr observed in the ETP-based assay during OC use is modulated by changes in protein S, but that these changes can only partially explain the OC-induced increase of the

APCsr. However, the data presented here clearly point to an important role for protein S in determining the APC-resistant plasma phenotype observed with the ETP-based assay and in the different extents of acquired APC resistance occurring during the use of levonorgestrel- and desogestrel-containing OC preparations.

Hereditary APC resistance caused by the FV-Leiden mutation is associated with an increased risk for venous thrombosis. Moreover, there seems to be a correlation between normalised APC sensitivity ratios calculated by quantification of the effect of APC on thrombin generation and the risk increase of venous thrombosis in OC users and in carriers of the FV-Leiden mutation (PART I, Chapter 4).Taken together these findings suggest that acquired APC resistance may explain the increased thrombotic risk for OC users, and the suggested further risk reported for users of desogestrel-containing preparations. One report, however, concluded that acquired APC resistance, established via a tissue-factor-initiated test, had no association with venous thromboembolism. Analysis of the plasma collection of the Leiden Thrombophilia Study showed that APC sensitivity ratios calculated with the same APC resistance test as that used in our study predict the risk for venous thrombosis in both carriers and non-carriers of FV-Leiden. In addition, there are several other indications that acquired APC resistance may have clinical implications and that APC resistance in the absence of the FV-Leiden mutation is a risk factor for venous thrombosis.

Chapter 6

Changes in APC Resistance during IVF-treatment

Based on:

Effects of In Vitro Fertilization Treatment and subsequent pregnancy on the Protein C pathway. J. Curvers, A. W. Nap, M.C.L.G.D. Thomassen, S. J. Nienhuis, K. Hamulyák, J. L. H. Evers, G. Tans and J. Rosing. Accepted for publication

Summary

Subfertile women who were planned for an In Vitro Fertilization (IVF) cycle donated blood at four time points during treatment; at baseline and after down regulation, hyperstimulation and luteal support. Levels of progesterone, 17ß-estradiol and indicators of the protein C pathway, *i.e.* APC sensitivity ratios (APCsr), protein C, protein C inhibitor and protein S, were measured.

Compared to baseline, estradiol decreased 2-fold at down-regulation and increased 40-fold at hyperstimulation. Progesterone was elevated 2.5 fold at hyperstimulation and 40-fold at luteal support. The APCsr slightly increased at down regulation, significantly increased during hyperstimulation and remained high during luteal support. The plasma levels of the anticoagulant proteins did not or moderately change during treatment. During down regulation, progesterone correlated negatively with APCsr ($r = -0.398$, $p=0.024$). At hyperstimulation estradiol correlated with the APCsr ($r=0.615$, $p<0.0005$). Moreover, there was a significant correlation ($r=0.599$, $p<0.0005$) between the difference in baseline and hyperstimulation values of estradiol ($\Delta E2 = 6.6$ nmol/L) and the APCsr ($\Delta APCsr = 0.30$). Six women who participated in this study became pregnant. Compared to baseline the APCsr was increased 1.9-fold ($\Delta APCsr = 1.48$) and free protein S free level decreased 30% at 7 weeks of pregnancy.

This study demonstrates that despite the considerable changes of endogenous estradiol and progesterone during an IVF cycle, changes in plasma levels of anticoagulant proteins are moderate. The significant increase of the APCsr during hyperstimulation indicates that acquired APC resistance observed during sex steroid hormone changes in women is at least partially caused by high estrogen levels. Our findings demonstrate that IVF treatment is accompanied by the development of a mild prothrombotic condition.

Introduction

Hormonal changes in women occurring during pregnancy, the use of oral contraceptives (OC) or hormone replacement therapy (HRT), have been associated with an increased risk for venous thromboembolism (VTE)[2, 47, 76, 116] Many investigators have studied the effect of hormonal changes on haemostatic parameters in an attempt to explain the increased risk of VTE. Both during pregnancy[26, 31, 32] and OC use[10, 12, 113, 117, 118] marked changes in the proteins involved in coagulation, anticoagulation and fibrinolysis were observed. The use of OC is associated with changes in the procoagulant (increased prothrombin, factor VII and factor X) and anticoagulant pathways (decreased protein S and increased APC resistance) that result in a prothrombotic state which might be responsible for the increase in VTE. Particularly the occurrence of APC resistance, which is a risk factor for VTE both in the presence[86, 119] and absence of the factor V_{Leiden} mutation,[120, 121] was suggested to contribute to the thrombotic risk during OC use[18, 80, 122] and pregnancy.[43]

The mechanistic basis for the hormone-induced changes of haemostatic parameters is still unclear. In vitro fertilisation (IVF) treatment is a unique model to study effects of hormones on the coagulation system since during the IVF cycle endogenous estradiol and progesterone levels change considerably within a short period of time. Yet, thrombosis as a consequence of IVF treatment is a rare event[123, 124] which mainly occurs during ovarian hyperstimulation syndrome.[124] The fact that changes of coagulation parameters during IVF treatment are modest[125-128] is in line with the low risk for venous thrombosis. Recently, however, two case reports were published of APC resistant women who suffered a thrombotic event during IVF treatment.[129, 130] According to the literature IVF treatment itself does not cause APC resistance[127, 131] but in these studies an APC resistance test was used which quantifies the effect of APC on the intrinsic coagulation pathway and which is much less sensitive to changes of sex steroids than the tissue factor-based APC resistance test used in investigations of OC.[108]

To obtain insight in the biological basis and the extent of hormone-induced changes of coagulation parameters, especially of APC resistance determined via the extrinsic coagulation pathway, we have investigated the effect of estrogen and progesterone

changes on the protein C system in women undergoing IVF treatment and in the subgroup of women who became pregnant during the IVF cycle.

Materials and methods

Design This study was approved by the Medical Ethics Committee of the Academic Hospital in Maastricht. At intake for IVF treatment, women were asked to participate in this study and were included after written informed consent was given. The women who agreed to participate donated blood at four time points (cf. Fig. 1) during IVF treatment: (1) at baseline on menstrual cycle day 2, 3 or 4 before starting with an OC preparation containing 30µg ethinylestradiol and 150µg levonorgestrel (Microgynon 30, Schering), (2) at maximal down regulation after approximately 14 days of treatment with nasal spray containing the gonadotropin releasing hormone (GnRH-) analogon, Synarel (Nafarelin, Searle), (3) at maximal stimulation after treatment (average 15 days) with follicle stimulating hormone (FSH, Puregon, 100 to 250 IU follitropin, Organon, Oss, The Netherlands) and before a human chorionic gonadotropin (hCG) injection (5000 IU Pregnyl, Organon) was given and (4) one week after the embryo transfer during the luteal support (3x200 µg Progestan, Organon) period. From the women who became pregnant additional blood samples were taken 2 and 4 weeks after embryo transfer which means at 5 and 7 weeks pregnancy.

Collection and handling of blood samples Women were asked to abstain from food overnight. The next morning, after 15 minutes rest, they were punctured in the anticubical vein of the fore-arm. Blood was collected in EDTA (Vacutainer, Becton Dickinson), in thrombin-coated tubes (Vacutainer) and in 0.129 M sodium citrate (Greiner, Germany). Blood collected in citrate was centrifuged 5 min at 3,000xg at room temperature. The cell free plasma was subsequently centrifuged for 10 min. at 11,000xg at 4°C. Plasma was frozen at -80°C in small aliquots until use.

Determination of blood and plasma variables. Haemoglobin, haematocrit, platelet count, mean platelet volume and mean cell volume were determined using a Coulter® Gen S system in whole blood collected in EDTA that was left for 2 hours at

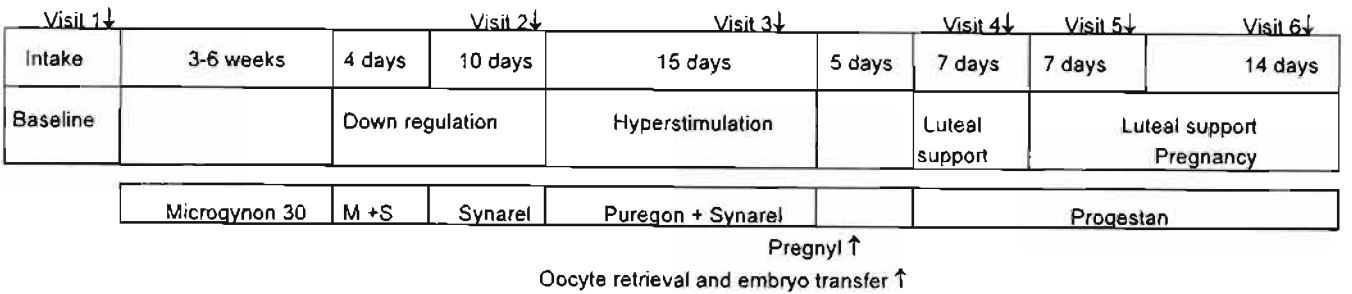


Figure 6.1 Design of the study. Arrows indicate the time of blood sampling. M+S = Microgynon 30 + Synarel.

room temperature. Hormone levels of progesterone and 17β-estradiol were determined in serum by a fluorescence-enzyme immunoassay (AutoDELFIA TM, Wallac Oy, Turku, Finland) at the Department of Clinical Chemistry of the Academic Hospital Maastricht.

The ETP (endogenous thrombin potential)-based APC resistance assay, the principle of which was described before [18, 122], quantifies the effect of 5 nM human APC (Enzyme Research Laboratories, Kordia Laboratory Supplies, Leiden, The Netherlands) on thrombin generation initiated in plasma with 0.4 μg/L recombinant tissue factor (DADE®Innovin®), 16 mM $CaCl_2$ and 15 μM phospholipid vesicles containing 20 mole% 1,2-dioleoyl-sn-glycero-3-phosphoserine, 20 mole% 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine and 60 mole% 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (Avanti Polar Lipids, Alabaster, Alabama, USA). The concentrations given are final concentrations present in plasma during thrombin generation. The amount of thrombin captured by α_2-macroglobulin, *i.e.* the amidolytic activity of the α_2-macroglobulin-thrombin complex (α_2M-IIa) was used as an end-point marker to quantify thrombin generation [41]. The APC sensitivity ratio (APCsr) was defined as the ratio of the amounts α_2M-IIa determined in the presence and absence of APC: $APCsr = (\alpha_2M\text{-}IIa_{+APC}/\alpha_2M\text{-}IIa_{-APC})_{plasma\ sample}$ which was normalised through division by the same ratio determined in the normal plasma pool (cf. [95]). Since APC inhibits thrombin generation and prolongs the activated partial thromboplastin time (aPTT), APC-sr determined with the ETP-based assay exhibit trends that are the reverse of APC-sr determined with aPTT-based APC resistance tests ($APC\text{-}sr = aPTT_{+APC}/aPTT_{-APC}$) [95]. Compared to normal pooled plasma, samples of APC resistant individuals yield higher APC-sr in the ETP-based assay and lower APC-sr in aPTT-based assays.

The presence of the factor V_{Leiden} phenotype was determined with a functional assay that measures the sensitivity for APC of factor Va present in a highly diluted plasma [91]. Since this assay is performed with more than 1000-fold diluted plasma it is 100% sensitive for the factor V_{Leiden} mutation. Thus far no individuals were found who were APC resistant in this assay and who were not carrying the factor V_{Leiden} mutation. Total and free protein S were measured by enzyme-linked immunosorbent assay (ELISA) using a polyclonal antibody against total protein S or a monoclonal

antibody against free protein S, respectively (REAADS purchased from Nodia, Amsterdam, The Netherlands). Protein C activity was determined after activation of protein C in diluted plasma to which 0.05 U/ml Protac (Kordia Laboratory Supplies, Leiden, The Netherlands) was added. Activated protein C was quantified with S2366 (Chromogenix, Mölndahl, Sweden). Protein C antigen was measured with an ELISA using a polyclonal anti-human protein C as capturing antibody (DAKO) and a horseradish peroxidase conjugated rabbit-anti-human protein C (DAKO) as secondary antibody. Protein C inhibitor (PCI) levels were also determined by ELISA, using a monoclonal against PCI (API-93) as capturing antibody and a horseradish peroxidase conjugated polyclonal rabbit anti-PCI as secondary antibody [118].

Values determined for total and free protein S, activity and antigen levels of protein C and protein C inhibitor are expressed as percentage of that determined in pooled normal plasma prepared from plasma of 49 men and 25 women (pregnant women and OC users were excluded) with a mean age of 37 years.

Statistics All samples of one woman were determined in duplicate in a single experiment and compared to normal pooled plasma determined in the same run. Differences between visit 1 (baseline) and the following visits were compared using a paired t-test. Correlations between variables were calculated on the basis of non-parametric Spearman rank correlation coefficients.

Results

Characteristics of participants

The schedule of IVF treatment and the time points at which blood samples were taken are indicated in Fig 6.1. Baseline characteristics of the women who participated in this study are summarised in Table 6.1. Of the 41 women who had given informed consent and who started IVF treatment, 33 completed the cycle. One woman was excluded since embryo transfer was not possible due to unsuccessful fertilisation of the oocytes. Two women, responded poorly to FSH and treatment was stopped. Five women discontinued treatment because of signs of threatening of ovarian hyperstimulation syndrome. The coagulation parameters of these women at visit 1 and 2 did not significantly differ from those of the women who completed the IVF

Table 6.1 Baseline characteristics of the women who were included in the study and completed the IVF treatment

Variable		number of women (%)
Indication for assisted reproduction	tuba obstruction	1 (3%)
	unexplained	12 (37%)
	male factor	20 (59%)
IVF-cycle	1	15 (46%))
	2	11 (33%)
	3	5 (15%)
	>3	2 (6%)
Amount of Puregon (FSH)	100	1 (3%)
	150	2 (6%)
	200	13 (39%)
	250	17 (52%)
Nr of oocytes retrieved	<5	10 (30%)
	5-10	12 (37%)
	>10	11 (33%)
Nr of fertilised oocytes (2PN)*	<5	16 (49%)
	5-10	15 (46%)
	>10	2 (6%)

* Of one women the oocytes could not succesfully be fertilized.

cycle. From the women with threatening ovarian hyperstimulation syndrome no blood samples of visit 3 and 4 were available. The average age of the 33 women who completed IVF treatment was 33 years. The mean body mass index was 24.2 (95% CI 23.8-25.1) and six women were smokers who on the average smoked 10 cigarettes per day.

None of the volunteers experienced clinical symptoms of venous thrombosis during treatment. In 33 women oocytes were successfully retrieved and fertilised. One woman exhibited the factor V_{Leiden} phenotype. She is indicated by the half filled circles in Fig 2 and her APCsr values during IVF treatment are separately given in Table II. Six women became pregnant and donated additional blood samples at 5 weeks and 7 weeks of pregnancy *i.e.* 2 and 4 weeks after embryo transfer.

Effects of IVF treatment on whole blood measurements

Of the whole blood measurements (Table 6.2), haemoglobin, the haematocrit and the mean cell volume significantly increased from baseline values after down-regulation ($p<0.05$). At hyperstimulation these variables returned to base-line and at luteal they dropped to values below base-line. The changes of haematocrit during

IVF treatment were relatively minor. Hence, the effects of haemoconcentration or haemodilution on the haemostatic variables reported below were negligible.

Effects of IVF treatment on hormone levels

Progesterone and 17β-estradiol changed as expected (Table 6.2). Compared to the baseline level (0.15 nmol/L), 17β-estradiol was significantly decreased in the down-regulated phase (0.08 nmol/L) and rose during hyperstimulation to 6.76 nmol/L (95% CI 5.10-8.42). During luteal support 17β-estradiol levels decreased to 0.38 nmol/L, which was still higher than the baseline level. Progesterone did not significantly change during down-regulation, but became significantly elevated during hyperstimulation (from 1.16 to 2.86 nmol/L) and increased considerably during luteal support (46.2 nmol/L, 95% CI 39.6-52.8 nmol/L).

Table 6.2 Effects of IVF treatment on hormones, erythrocytes, platelets and the protein C system in 33 women who completed IVF treatment

Variable	Baseline (visit 1) mean (95% C.I.)	Downregulation (visit 2) mean (95% C.I.)	Hyperstimulation (visit 3) mean (95% C.I.)	Luteal support (visit 4) mean (95% C.I.)
17-β-Estradiol	0.15 (0.13-0.17)	0.08‡ (0.06-0.10)	6.76‡ (5.10-8.42)	0.38‡ (0.23-0.53)
Progesterone	1.16 (1.00-1.32)	1.13 (0.88-1.38)	2.86‡ (2.44-3.29)	46.2‡ (39.6-52.8)
Haemoglobin	8.3 (8.1-8.4)	8.5‡ (8.4-8.7)	8.2 (8.0-8.3)	8.1# (8.0-8.3)
Haematocrit	0.40 (0.39-0.41)	0.41‡ (0.41-0.42)	0.40 (0.39-0.40)	0.39‡ (0.38-0.40)
Mean cell volume	91.1 (90.2-92.0)	91.6# (90.7-92.5)	91.2 (90.2-92.1)	90.9 (90.0-91.8)
Platelet count (x10^9/L)	271 (250-291)	276 (255-296)	247‡ (230-264)	276 (255-298)
Mean platelet volume	8.2 (8.0-8.4)	8.3 (8.0-8.5)	8.4 (8.1-8.6)	8.4# (8.1-8.6)
Protein S total (%)	91.5 (85.2-97.8)	95.4# (89.1-101.8)	88.4# (82.0-94.9)	90.3 (84.5-96.2)
Protein S free (%)	87.7 (80.5-94.9)	92.8# (84.9-100.7)	87.1 (81.0-93.2)	87.8 (81.9-93.6)
Protein C activity (%)	101.4 (95.9-106.9)	105.7# (99.1-112.3)	104.2 (98.0-110.4)	102.3 (96.0-108.6)
Protein C antigen (%)	105.3 (99.6-111.1)	105.1 (98.2-112.0)	105.6 (99.2-112.0)	104.8 (97.4-112.2)
Protein C inhibitor (%)	83.5 (78.4-88.6)	84.0 (79.2-88.7)	84.7 (80.0-89.5)	82.2 (77.0-87.5)
APCsr*	1.73 (1.49-1.97)	1.79 (1.54-2.04)	2.03‡ (1.72-2.35)	1.93‡ (1.71-2.15)
APCsr of FV_{Leiden}	4.41	5.50	6.37	5.29

* mean and 95% CI are given excluding the women with heterozygous factor V_{Leiden} phenotype
significantly different from baseline #($p<0.05$) or ‡($p<0.005$)

Effect of IVF treatment on anticoagulant parameters

Table 6.2 also summarizes the effects of IVF treatment on the anticoagulant parameters. Compared to the baseline value (91.5%), the total protein S levels increased slightly at down-regulation (95.4%), decreased during hyperstimulation (88.4%) and did not differ from baseline levels during luteal support (90.3%). Free protein S only increased at down-regulation (92.8%) and returned to baseline values during hyperstimulation (87.1%) and during luteal support (87.8%). Protein C antigen did not substantially change during IVF treatment. A modest increase of the protein C activity was observed at down-regulation and during the other phases of treatment (Table 6.2). Protein C activity levels correlated with the antigen measurements ($r=0.848$, $p<0.0001$). Protein C inhibitor levels did not change during the in vitro fertilisation cycle.

Table 6.2 and Fig. 6.2 show that the APCsr values slightly increased from 1.73 at baseline to 1.79 during down-regulation. At hyperstimulation the APCsr reached a maximum (2.03) and decreased slightly during luteal support (1.93). The subject with the factor V_{Leiden} phenotype, who was excluded from the analysis of the mean APCsr values, had a baseline APCsr of 4.41. During down-regulation her APCsr increased to 5.50, increased further to 6.37 at hyperstimulation and decreased again during luteal support (APCsr = 5.29).

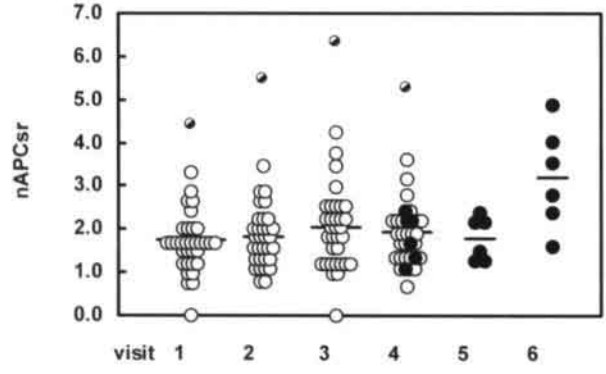


Figure 6.2 Effect of IVF treatment on the APCsr. Visit 1 refers to baseline, Visit 2 refers to downregulation after treatment with GnRH analogon, visit 3 is at maximum stimulation and visit 4 is during luteal support. Visit 5 and visit 6 refer to plasma samples taken at 5 and 7 weeks of pregnancy following IVF treatment. The half filled circle represents a woman with heterozygous FV_{Leiden} phenotype. The closed circles at visit 4, 5 and 6 represent the six women who became pregnant after OVF treatment. APC sensitivity ratios were determined as described in the materials and methods section. Horizontal bars represent the mean APCsr value.

Effects of early pregnancy on anticoagulant parameters

Six women who participated in this study became pregnant. The plasma levels of anticoagulant proteins (Table 6.3) and the APCsr of these women (Fig 6.2, Table 6.3) did not differ from the women who did not become pregnant. Analysis of additional blood samples showed that protein C antigen and activity did not change during the first weeks of pregnancy (Table 6.3). Protein C inhibitor levels increased in 5 out of 6 women at week 5 and returned to normal levels after 7 weeks of pregnancy. The plasma protein S level and APC resistance measurements revealed no changes at week 5. However, after 7 weeks of pregnancy the APCsr was increased from 1.93 at the end of IVF treatment (luteal support) to 3.20 (mean change 1.27, 95%CI 0.54-2.01). Total protein S decreased 12% (95%CI 4-21%) and the decrease of free protein S was even more pronounced (mean change 27%, 95%CI 17-38%).

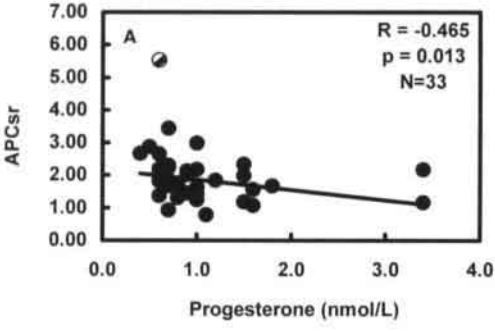
Table 6.3 Effects of IVF treatment on hormones, erythrocytes, platelets and the protein C system in 6 women who became pregnant

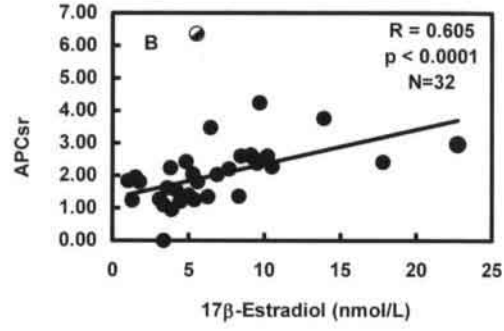
Variable	Baseline (visit 1) mean (95% C.I.)	Luteal support (visit 4) mean (95% C.I.)	5 week pregnant (visit 5) mean (95% C.I.)	7 week pregnant (visit 6) mean (95% C.I.)
17-β-Estradiol	0.17 (0.13-0.21)	0.51 (0.17-0.85)	2.31 (0.29-4.32)	6.44 (0.76-12.13)
Progesterone	0.95 (0.61-1.29)	56.0 (22.4-89.6)	258 (30.7-486)	210 (0-492)
Haemoglobin	8.1 (7.7-8.5)	8.1 (7.7-8.4)	8.2 (7.7-8.6)	7.6 (7.1-8.0)
Haematocrit	0.39 (0.37-0.41)	0.39 (0.37-0.41)	0.39 (0.37-0.42)	0.36 (0.34-0.39)
Mean cell volume	90.5 (88.7-92.4)	90.3 (87.8-92.7)	90.3 (88.2-92.3)	89.1 (85.4-92.7)
Platelet count ($x10^9$/L)	278 (228-328)	287 (255-318)	324 (288-370)	289 (274-305)
Mean platelet volume	8.1 (7.8-8.3)	8.4 (7.7-9.1)	8.3 (7.7-8.9)	8.0 (7.2-8.8)
Protein S total (%)	87 (81-93)	97 (84-111)	96 (83-108)	85 (77-93)
Protein S free (%)	88 (75-102)	87 (79-95)	81 (69-93)	60 (46-74)
Protein C activity (%)	98 (80-116)	105 (74-136)	103 (85-121)	100 (81-120)
Protein C antigen (%)	110 (86-134)	110 (75-144)	104 (81-127)	102 (77-127)
Protein C inhibitor (%)	86 (79-93)	91 (76-105)	98 (87-110)	87 (77-98)
APCsr	1.72 (1.07-2.37)	1.93 (1.42-2.43)	1.79 (1.29-2.29)	3.20(2.02-4.39)

Correlations between hormone levels and coagulation parameters

During down regulation *i.e.* at low estradiol levels the plasma progesterone concentration correlated negatively with the APCsr (r=- 0.398, p=0.024, Fig 3A).

During hyperstimulation there was a correlation between 17β-estradiol and the APCsr (r=0.615, p<0.0005, Fig 3B). Total protein S correlated slightly with 17β-estradiol at hyperstimulation (r=-0.386, p=0.029). The change in 17β-estradiol from baseline to hyperstimulation correlated with the change in APCsr, r=0.599, p<0.0005 (Fig 3C). No other significant correlations were observed between the levels or the changes of hormones and coagulation parameters.





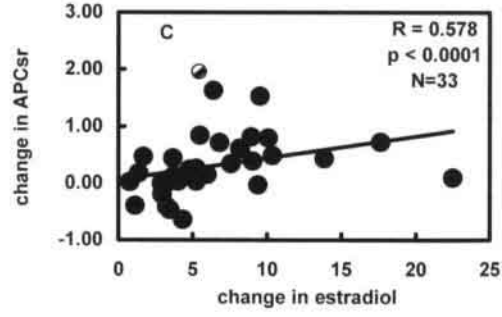


Figure 6.3 Correlation plots between progesterone, 17ß-estradiol and APCsr values. Correlations between (A) APCsr and progesterone at the down-regulated state, visit 2; (B) APCsr and 17β-estradiol at hyperstimulation, visit 3; (C) the change of the APCsr and 17β-estradiol from visit 1 to visit 3 (hyperstimulation minus baseline). The half-filled circle represent a women with a heterozygous factor V_{Leiden} phenotype (omitted from the correlation analysis). The lines given in these plots were obtained by linear regression. Correlation coefficients and p-values presented in the results section were calculated using Spearman's rho

Correlations between coagulation parameters

At baseline the APCsr correlated with total protein S levels (r= - 0.43, p=0.013), a correlation that was maintained at down-regulation (r= - 0.624, p<0.0001), hyperstimulation (r= - 0.483, p=0.005) and during luteal support (r= - 0.534, p=0.002). A similar correlation was observed between the APCsr and free protein S levels during the first three visits (r= - 0.511, p=0.003; r= - 0.483 p=0.005 and r= - 0.390, p=0.02, respectively), but not during luteal support. Although the

absolute values of the APCsr and protein S correlated with each other during the different phases of IVF treatment there appeared to be no correlation between the changes of the APCsr and protein S. However, a strong correlation was observed between the decrease of free protein S and the increase of the APCsr in the women who became pregnant after IVF treatment ($r = -0.657$, baseline minus 7 weeks pregnancy). This correlation did not reach significance ($p = 0.156$) which is presumably due to the fact that only a low number of pregnant women ($n = 6$) were available for statistical analysis.

Discussion

We have investigated the effects of the changes in endogenous estradiol and progesterone during IVF treatment and subsequent pregnancy on haemoglobin, haematocrit, platelets and on a number of haemostatic parameters that probe the activity of the protein C pathway. Despite considerable changes in the plasma concentrations of estradiol and progesterone, IVF treatment had modest effects on haemoglobin, haematocrit, platelets and on the plasma levels of the proteins involved in the protein C pathway. With respect to the anticoagulant proteins our observations confirm earlier studies which indicated that hyperstimulation induced only marginal changes of coagulation parameters.[125-127] Our study also shows that during down-regulation and luteal support the changes of the plasma levels of anticoagulant proteins are virtually negligible.

The only coagulation parameter that changed considerably during IVF treatment was the APCsr. In this study we observed that hyperstimulation *i.e.* high estrogen levels induce APC resistance and that under these conditions both the absolute values and the changes of the APCsr and the estrogen levels (hyperstimulation - baseline) correlate significantly. Previously, it was reported that high estrogen levels are not associated with APC resistance [127, 131]. However, the APC resistance test used in these studies, which is based on quantification of the effect of APC on the clotting of plasma initiated via the intrinsic coagulation pathway, is not very sensitive to changes of sex hormones.[108] In the present study an assay was used that quantifies down-regulation of extrinsic coagulation by APC and that is particularly sensitive to hormonal changes in women.[18, 42, 43, 122] The fact that

according to this test the large increase of estrogen during ovarian stimulation is accompanied by APC resistance indicates that acquired APC resistance occurring during OC use[18, 122] and pregnancy[42, 43] is at least partially due to elevated levels of estrogen. However, second and third generation OC have different effects on coagulation parameters[12, 122] while they contain the same amount of ethinylestradiol which suggests that the progestagens (levonorgestrel or desogestrel/gestodene) also influence the coagulation system. The present study shows that profound changes in natural progestagen (40-fold) have modest effects on the APCsr. Only during down-regulation of estradiol a significant negative correlation was found between the progesterone level and the APCsr. This suggests that under certain conditions progesterone can counteract the response of the coagulation system to estradiol, a phenomenon that was also observed by Kluft et al.[132]

In OC users a decrease of the plasma level of protein S partially explains acquired APC resistance.[12] However, in all phases of IVF treatment the changes in the levels of proteins involved in the protein C pathway (including protein S) were minimal and did not correlate with the changes in APCsr. Thus, it is likely that changes in plasma proteins other than protein S are responsible for the acquired APC resistance induced by hyperstimulation.

It is well established that pronounced APC resistance develops during pregnancy.[42, 43] Six women who participated in the present study became pregnant. At 7 weeks of pregnancy they had APCsr that were two times higher than the baseline value (ΔAPCsr = 1.48) and their plasma level of free protein S decreased ~30%. It is interesting note that in these women the estrogen levels at 7 weeks of pregnancy were similar to those observed after hyperstimulation. Therefore, the large increase of the APCsr and the decrease of the plasma level of free protein S in early pregnancy are likely caused by other changes occurring in the first weeks of pregnancy. These changes primarily cause a decrease in protein S which for a large part is responsible for the increase in APC resistance. This is concluded from the fact that the differences between the protein S levels at baseline and during early pregnancy correlated with the increase of the APCsr. A change of the APCsr and plasma protein S levels that early in pregnancy has not been shown before. Kjellberg et al[31] reported that protein S decreases to 40% after 10 weeks of pregnancy. The decrease in free protein S and the increase in APC resistance observed here

indicates that a hypercoagulable state already develops within the first 7 weeks of gestation.

Although estradiol and progestagen levels increase considerably during in vitro fertilization, clinical complications such as venous thrombosis are very rare. The synthetic hormones present in OC are far more potent than the natural/endogenous 17β-estradiol and progesterone.[133] This might explain why, compared to OC, high concentrations of natural hormones have only marginal effects on haemostatic parameters. The change of the APCsr caused by hyperstimulation (ΔAPCsr = 0.30) was less than that occurring during the use of second generation (ΔAPCsr = 0.77) or third generation (ΔAPCsr = 1.29) oral contraceptives[12, 122] or early pregnancy (ΔAPCsr = 1.48, this paper). It should be emphasized, however, that the effect of hyperstimulation concerns a mean change. In some women (n = 5) the increase in APCsr was > 0.80. When such a change occurs in women with a hereditary risk factor of venous thrombosis this results in a temporary aggravation of an existing prothrombotic condition and in a further increased risk of venous thrombosis.[129, 130]

Chapter 7

The effects of progestagen only therapy on the anticoagulant pathway

Based on:

Effect of second and third generation oral contraceptives on the protein C system in the absence or presence of the factor V_{Leiden} mutation.Jeanet M. Kemmeren, Ale Algra, Joost C.M. Meijers, Guido Tans, Bonno N. Bouma, Joyce Curvers, Jan Rosing, Diederik E. Grobbee. Submitted

Summary

Compared with second generation oral contraceptives, the use of third generation oral contraceptives has been associated with a higher risk of venous thrombosis, particularly in women with the factor V_{Leiden} mutation. To find an explanation for these risk differences we investigated the effects of second and third generation oral contraceptives and of the progestagens used in these pills on the anticoagulant pathway in women with and without factor V_{Leiden}.

In a single center, double blind trial, 51 women without and 35 women with factor V_{Leiden} were randomized to either a second generation (30μg ethinylestradiol / 150μg levonorgestrel) or a third generation (30μg ethinylestradiol / 150μg desogestrel) oral contraceptive. After two cycles of use and a wash-out of 2 cycles, the participants received the corresponding progestagen-only preparation containing 150μg levonorgestrel or 150μg desogestrel. In plasma of the participating women hemostatic variables that probe the activity of the anticoagulant protein C system were determined.

Both combined preparations induced APC resistance, increased plasma levels of protein C and protein C inhibitor and caused a decrease of protein S, C4b-binding protein and the APC independent anticoagulant activity of protein S. Compared with levonorgestrel, desogestrel-containing oral contraceptives significantly decreased protein S and increased APC resistance in both study populations. Oral contraceptives with desogestrel had more pronounced effects in carriers than in non-carriers of factor V_{Leiden}, a trend which reached statistical significance for APC resistance and C4b-binding protein. Progestagen-only preparations caused changes of anticoagulant parameters opposite to those of combined oral contraceptives and which in a number of cases were more pronounced with levonorgestrel.

Progestagens in combined oral contraceptives counteract the thrombotic effect of the estrogen component. The observation that of desogestrel is less antithrombotic than levonorgestrel, can explain the different thrombotic risks associated with the use of second and third generation oral contraceptives, especially in women with factor V_{Leiden}.

Introduction

In 1995 and 1996 four studies reported that women who used so-called third generation oral contraceptives containing desogestrel or gestodene were at higher risk of venous thromboembolism than users of oral contraceptives with the second generation progestagen, levonorgestrel.[3-5, 76] The absolute risk of venous thrombosis associated with the use of particularly third generation oral contraceptives is further elevated among carriers of the factor V_{Leiden} mutation,[4, 134] a hereditary disorder in which activated factor V is inactivated by activated protein C (APC) at a lower rate than normal factor Va.[135-137] This hereditary defect, also called APC resistance, is a common risk factor for venous thrombosis which is associated with a three- to seven-fold increased risk in heterozygous individuals.[135-137]

A plausible biological mechanism for the thrombotic effects of second- and third-generation oral contraceptives is still lacking. Rosing et al[11, 18] reported that oral contraceptive use leads to acquired APC resistance. Women using third generation oral contraceptives were more resistant to the anticoagulant action of APC than users of second generation oral contraceptives and that the effects of oral contraceptives and factor V_{Leiden} on the APC sensitivity ratio (APC-sr) were additive.[18] A recent cross-over study indicated that second and third generation oral contraceptives not only cause differences in acquired APC resistance, but also have different effects on a large number of other procoagulant, anticoagulant and fibrinolytic parameters.[12, 113, 118] Particularly the differential changes of the proteins involved in the protein C system may contribute to the thrombotic effects of oral contraceptives.[12] The protein C system, which comprises the plasma proteins protein C and protein S, down-regulates *in vivo* blood coagulation[138] via proteolytic inactivation of coagulation factors Va and VIIIa. Protein S, which acts as cofactor of APC, also exhibits anticoagulant activity independent of APC by directly inhibiting thrombin formation.[139] The anticoagulant activity of protein S is modulated by C4b- binding protein, which binds some 60% of plasma protein S.[140, 141] The physiological importance of the protein C system is illustrated by the fact that defects in this pathway are associated with an increased risk of venous thromboembolism.[142,18,19,19,20] The thrombotic risk of women with hereditary defects in the protein C pathway is enhanced by oral contraceptive use.[134]

For a long time, the increased thrombotic risk associated with oral contraceptive use was attributed to the estrogen component. Because low dose second and third generation oral contraceptives contain the same estrogen dose, the differences may reflect a progestagen-specific effect. Although it has been proposed that progestagens have estrogen-like effects,[143] among others on hemostasis,[22] there is as yet no data on the influence of levonorgestrel or desogestrel in the absence of the estrogen component on the protein C pathway.

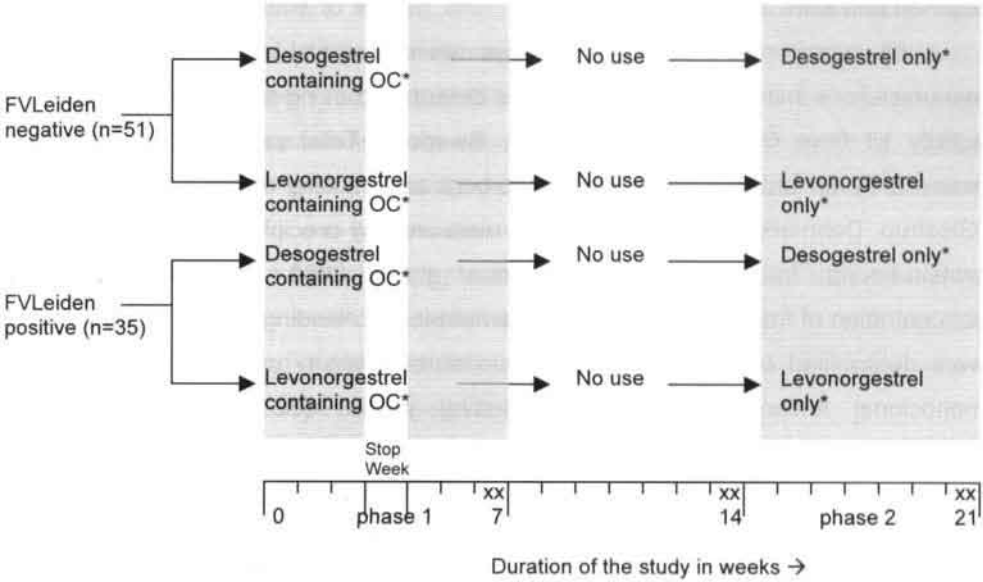
To gain more insight in the biological basis of an enhanced risk of venous thrombosis in oral contraceptive users, particularly in carriers of factor V_{Leiden}, we performed a double blind, randomized trial in which we determined the effects of second and third generation progestagens, alone or in combination with estrogens, on the protein C pathway in the absence or presence of the factor V_{Leiden} mutation.

Methods

Study design and participants Figure 1 illustrates the design of the trial in which 51 women without and 35 with factor V_{Leiden} were randomly assigned to one of two different combination pills containing either 30 μg ethinylestradiol and 150 μg levonorgestrel or 30 μg ethinylestradiol and 150 μg desogestrel. These formulations are identical to those commercially marketed. The oral contraceptives were used for a period of 2 menstruation cycles (phase 1). After a wash-out of two menstrual periods, the participants were treated with a corresponding progestagen-only preparation containing either 150 μg levonorgestrel or 150 μg desogestrel. These progestagens were used for a period of 2 menstruation cycles without a stopweek to provide full contraceptive safety (phase 2). Randomization was done in blocks of ten. Blood samples were taken at the end of phase 1 (days 47±2 and 49±2), at the end of the wash-out period (days 101±2 and 105±2), and at the end of phase 2 (days 152±2 and 154±2). For reasons of safety, ultrasound examination of the veins of both legs was performed before the start of the study, after the wash-out period and at the end of phase 2, to exclude venous thrombosis.

Exclusion criteria were a history of any malignant disorder, cardiovascular, cerebrovascular, hepatic or renal diseases, venous thrombosis, epilepsy or classical migraine, vaginal bleeding of unknown etiology, pregnancy, rheumatoid arthritis,

diabetes mellitus, psychiatric disorders, alcohol abuse or drug abuse within the last 12 months, heavy smoking, excessive obesity, chronic infectious diseases and suffering from any other serious disease. Moreover, volunteers with a contra-indication to estrogens and/or progestagens were excluded.



* Desogestrel containing OC: *30µg ethinyl estradiol + 150µg desogestrel.* Levonorgestrel containing OC: *30µg ethinyl estradiol + 150µg levonorgestrel.* Desogestrel only: *150µg desogestrel.* Levonorgestrel only: *150µg levonorgestrel.*

x Time of blood sampling

Figure 7.1 Trial profile.

To include women who carry factor V_{Leiden}, a screening was arranged among students and employees of Utrecht University. We screened 1083 women aged 18-40 years. Sixty (5.5%) were heterozygous carrier of the factor V_{Leiden} mutation; 36 agreed to participate of whom one did not meet the inclusion criteria (psychiatric disorder). From the women without factor V_{Leiden}, 111 were approached for the trial and 51 agreed to participate; all fulfilled the inclusion criteria. One participant was excluded from phase 2 because the wrong medication was supplied. Another woman withdrew consent in phase 2 because of side effects of the progestagen-only-pill (symptoms of depression). Both were factor V_{Leiden} negative.

All participants gave written informed consent. The study was approved by the Ethics Committee of the University Medical Center Utrecht.

Laboratory methods All blood samples were drawn in the morning after an overnight abstinence from intake of food, caffeine, alcohol or nicotine. Cell-free, citrated plasma was prepared and centrally stored at -80°C. Anticoagulant parameters were determined after all participants had completed the treatment regimen and were done in duplicate.

All commercially available assays were carried out according to the manufacturer's instructions. Protein C was determined using the Coamatic protein C activity kit from Chromogenix (Mölndal, Sweden). Total protein S antigen was assayed by an enzyme-linked immunosorbent assay using antibodies from DAKO (Glostrup, Denmark). Free protein S was measured by precipitating the C4b-binding protein-bound fraction with polyethylene glycol 8000 and measuring the concentration of free protein S in the supernatant. C4b-binding protein antigen levels were determined by enzyme-linked immunosorbent assay using a combination of monoclonal antibodies against C4b-binding protein (8C11 and horse-radish peroxidase-labelled 9H10). The APC independent anticoagulant activity of protein S (PS_{APCind}) was determined as described by van Wijnen et al[23] with two modifications. To avoid contamination with endogenous phospholipids all plasma samples were centrifuged for 10 min at 13,000 g at 20°C. Plasma aliquots taken from the bottom of the tube were used for the assay. Recombinant human tissue (Innovin, Dade Behring, Germany) diluted 100 fold was used to initiate coagulation. Antigen levels of protein C inhibitor (PCI) were determined by ELISA using a monoclonal antibody against PCI (API-93) as capturing antibody and rabbit polyclonal anti-PCI serum as secondary antibody[93]. APC sensitivity ratios (APC-sr) were determined by quantifying the effect of APC on thrombin generation as described before [12]. The plasma levels of the proteins determined (antigen or activity) were expressed as percentage of that present in normal pooled plasma determined in the same experiment. The presence of the factor V_{Leiden} mutation was assessed by DNA analysis [144]. All tests were performed without knowledge of the carrier status of the individual and the oral contraceptive preparation used.

Statistical analysis To increase the precision of the measurements, the results of the two visits at the end of phase 1, at the end of the wash-out period, and at the end of phase 2 were averaged. If only one sample was available, the values

obtained for that sample were used in the analysis. According to the design of the trial (Figure 1) three comparisons were made in the data analysis and represented in the corresponding tables. To assess the effect of progestagens in combined oral contraceptives, mean differences in anticoagulant parameters between the two treatment groups relative to values when no oral contraceptives were used, were tested with unpaired t-tests for non-carriers and carriers of factor V_{Leiden} separately. The effect of combined oral contraceptives (progestagens plus estrogen) versus progestagen-only preparations was calculated by testing differences in means between phase 1 and phase 2 using paired t-tests. Again, these analyses were performed separately for non-carriers and carriers of factor V_{Leiden}.

Differential effects of a hormone preparation between women with and without the factor V_{Leiden} mutation were assessed from the mean change in anticoagulant parameters in the period of oral contraceptive use relative to no use using unpaired t-tests. For all estimates 95% confidence intervals were calculated.

Results

Characteristics of the Volunteers Participating in the Study No differences in general characteristics were present between the study groups (Table 7.1). The values of the anticoagulant parameters determined at the various stages in the trial are summarized in Table 7.2. During the study none of the volunteers showed clinical signs or symptoms of venous thromboembolism or abnormalities on ultrasound examination of the leg veins.

Table 7.1. Baseline characteristics according to factor V_{Leiden} and type of oral contraceptive (OC)

	Factor V_{Leiden} negative		*Factor V_{Leiden} positive*	
	Levonorgestrel containing OC (n=24)	Desogestrel containing OC (n=27)	Levonorgestrel containing OC (n=19)	Desogestrel containing OC (n=16)
Age (years)	22.9 ± 3.4	24.1 ± 5.9	23.3 ± 2.7	22.6 ± 2.8
Body weight (kg)	67.6 ± 10.6	67.6 ± 9.5	64.0 ± 7.9	65.9 ± 10.6
Height (cm)	168.6 ± 5.5	170.9 ± 6.5	169.2 ± 6.4	171.5 ± 6.7
BMI (kg/m²)	23.4 ± 3.4	23.2 ± 3.4	22.3 ± 2.5	22.4 ± 3.5
Previous OC use (%)	19 (79)	20 (74)	15 (79)	15 (94)
Second generation	5 (26)	9 (45)	6 (40)	3 (20)
Third generation	13 (68)	5 (25)	9 (60)	5 (33)
Others	1 (5)	6 (30)	0	7 (47)

Values are means ± SD or numbers with percentage in parentheses

Table 7.2a Effects of combined oral contraceptives on anticoagulant parameters in the absence and presence of the factor V_{Leiden} mutation.

	Levonorgestrel containing OC		*Desogestrel containing OC*		
	No use	OC use	No use	OC use	Mean difference
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	in change (95% CI)
Factor V_{Leiden} negative					
Protein C	96.0 ± 18.3	108.8 ± 21.7	92.0 ± 14.0	103.7 ± 17.8	-1.1 (-7.2 - 5.0)
Protein S total	88.5 ± 13.2	83.4 ± 13.6	85.9 ± 9.9	71.7 ± 10.2	-9.1 (-13.4 - -4.7)
Protein S free	39.9 ± 7.0	43.9 ± 8.0	38.9 ± 5.8	33.8 ± 7.5	-9.2 (-12.0 - -6.4)
C4BP**	89.9 ± 20.1	77.1 ± 17.3	88.8 ± 15.8	79.1 ± 15.1	2.6 (-1.5 - 6.6)
PS_{APCind}**	1.32 ± 0.12	1.19 ± 0.08	1.34 ± 0.13	1.14 ± 0.07	-0.08 (-0.13 - -0.02)
PCI**	90.6 ± 16.6	101.0 ± 15.0	90.5 ± 13.7	99.5 ± 18.1	-1.4 (-8.4 - 5.6)
APC-sr**	1.92 ± 0.61	3.26 ± 0.72	2.04 ± 0.63	3.76 ± 0.77	0.38 (0.08 - 0.67)
Factor V_{Leiden} positive					
Protein C	92.1 ± 12.3	102.5 ± 16.3	106.6 ± 15.9	122.7 ± 22.2	5.7 (-1.8 - 13.3)
Protein S total	88.2 ± 11.4	85.9 ± 12.9	96.9 ± 10.9	77.6 ± 11.1	-16.9 (-23.8 - -10.1)
Protein S free	42.2 ± 9.4	45.9 ± 9.9	42.1 ± 8.1	35.3 ± 8.4	-10.5 (-15.0 - -5.9)
C4BP**	80.5 ± 11.2	72.3 ± 9.8	101.5 ± 12.7	85.4 ± 16.6	-8.0 (-13.9 - -2.1)
PS_{APCind}**	1.24 ± 0.10	1.16 ± 0.10	1.33 ± 0.12	1.14 ± 0.05	-0.10 (-0.15 - -0.05)
PCI**	86.0 ± 14.9	92.2 ± 16.3	92.5 ± 17.0	106.9 ± 17.6	8.2 (2.2 - 14.1)
APC-sr**	4.60 ± 1.02	5.95 ± 1.38	4.29 ± 1.00	7.06 ± 1.40	1.41 (0.76 - 2.07)

Note: the individual plasma components are expressed as % of normal pooled plasma

** For this analysis, one woman was excluded due to protocol violation in phase 2*

** PCI, Protein C inhibitor; APC-sr, activated protein C sensitivity ratio; C4BP, C4b-binding protein; PS_{APCind}, APC-independent anticoagulant activity of protein S

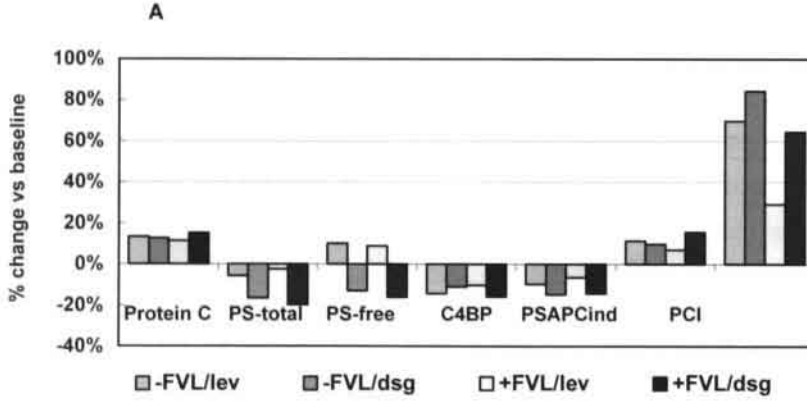


Figure 7.2a Changes of anticoagulant parameters during the use of combined oral contraceptives, according to pill type and presence of the factor V Leiden mutation.
PS = protein S, C4BP = C4b-binding protein, PS_{APCind} = APC-independent anticoagulant activity of protein S, PCI = protein C inhibitor, APC-sr = activated protein C sensitivity ratio

Table 7.2b Effects of progestagen only preparations on anticoagulant parameters in the absence and presence of the factor V_{Leiden} mutation.

	Levonorgestrel containing OC		*Desogestrel containing OC*		
	No use	OC use	No use	OC use	Mean difference
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	in change (95% CI)
Factor V_{Leiden} negative					
Protein C	96.7 ± 18.3	92.7 ± 18.6	92.0 ± 14.0	84.9 ± 11.5	-3.1 (-6.8 - 0.6)
Protein S total	88.6 ± 13.4	94.9 ± 13.4	85.9 ± 9.9	92.5 ± 13.2	0.3 (-4.2 - 4.7)
Protein S free	40.0 ± 7.1	47.9 ± 8.4	38.9 ± 5.8	42.7 ± 7.1	-4.0 (-6.4 - -1.7)
C4BP**	89.2 ± 20.3	82.0 ± 15.8	88.8 ± 15.8	87.5 ± 17.8	6.0 (0.03 - 12.0)
PS_{APCind}**	1.32 ± 0.12	1.46 ± 0.23	1.34 ± 0.13	1.35 ± 0.13	-0.13 (-0.20 - -0.06)
PCI**	88.9 ± 14.6	90.4 ± 17.7	90.5 ± 13.7	90.0 ± 14.4	-2.0 (-7.8 - 3.9)
APC-sr**	1.93 ± 0.63	1.58 ± 0.61	2.04 ± 0.63	1.80 ± 0.52	0.11 (-0.09 - 0.30)
Factor V_{Leiden} positive					
Protein C	92.1 ± 12.3	86.2 ± 11.1	106.6 ± 15.9	96.5 ± 14.9	-4.2 (-9.0 - 0.7)
Protein S total	88.2 ± 11.4	95.2 ± 10.5	96.9 ± 10.9	100.6 ± 11.2	-3.2 (-8.1 - 1.6)
Protein S free	42.2 ± 9.4	48.9 ± 7.8	42.1 ± 8.1	45.9 ± 6.8	-2.9 (-5.9 - 0.1)
C4BP**	80.5 ± 11.2	75.1 ± 9.7	101.5 ± 12.7	95.3 ± 12.1	-0.8 (-6.4 - 4.9)
PS_{APCind}**	1.24 ± 0.10	1.33 ± 0.16	1.33 ± 0.12	1.33 ± 0.10	-0.09 (-0.14 - -0.03)
PCI**	86.0 ± 14.9	85.7 ± 18.3	92.5 ± 17.0	91.0 ± 20.8	-0.6 (-8.0 - 6.8)
APC-sr**	4.60 ± 1.02	4.09 ± 0.97	4.29 ± 1.00	4.10 ± 1.04	0.32 (-0.05 - 0.68)

Note: the individual plasma components are expressed as % of normal pooled plasma
** For this analysis, one woman was excluded due to protocol violation in phase 2*
** PCI, Protein C inhibitor; APC-sr, activated protein C sensitivity ratio; C4BP, C4b-binding protein; PS_{APCind}, APC-independent anticoagulant activity of protein S

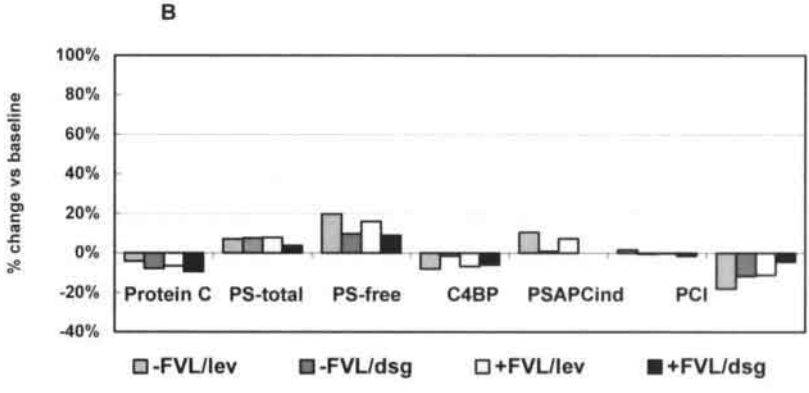


Figure 7.2b Changes of anticoagulant parameters during the use of progestagen only preparations, according to pill type and presence of the factor V Leiden mutation.
PS = protein S, C4BP = C4b-binding protein, PS_{APCind} = APC-independent anticoagulant activity of protein S, PCI = protein C inhibitor, APC-sr = activated protein C sensitivity ratio

Effect of Combined Oral Contraceptives The use of combined oral contraceptives caused considerable changes in anticoagulant parameters (Table 7.2 and Figure 7.2A). With the exception of free protein S, the direction of these changes were similar for the two kinds of contraceptives. However, in non-carriers as well as in carriers of factor V_{Leiden} clear differences were observed between the magnitude of the effects of levonorgestrel- and desogestrel-containing oral contraceptives.

Table 7.3 Differential effects of combined oral contraceptives and progestagen-only preparations on anticoagulant plasma parameters in the absence or presence of the factor V_{Leiden} mutation

	Levonorgestrel containing OC vs. Levonorgestrel-only Mean difference (95%CI)	Desogestrel-containing OC vs. Desogestrel-only Mean difference (95%CI)
Factor V_{Leiden} negative		
Protein C	17.0 (12.8 to 21.3)	18.8 (14.7 to 23.0)
Total Protein S	-11.3 (-15.2 to -7.3)	-20.8 (-26.0 to -15.6)
Free protein S	-3.7 (-5.9 to -1.5)	-8.9 (-11.6 to –6.2)
C4BP*	-5.7 (-11.2 to -0.2)	-9.0 (-12.9 to –5.2)
PS_{APCind}*	-0.27 (-0.35 to -0.19)	-0.22 (-0.26 to -0.17)
Protein C inhibitor	9.7 (5.2 to 14.2)	9.5 (3.4 to 15.6)
APC-sr*	1.66 (1.39 to 1.93)	1.96 (1.72 to 2.19)
Factor V_{Leiden} positive		
Protein C	16.3 (12.4 to 20.2)	26.2 (18.2 to 34.3)
Total Protein S	-9.3 (-14.1 to -4.5)	-23.0 (-28.9 to -17.1)
Free protein S	-3.0 (-6.3 to 0.3)	-10.6 (-13.3 to -7.8)
C4BP*	-2.8 (-6.4 to 0.8)	-10.0 (-17.6 to -2.3)
PS_{APCind}*	-0.17 (-0.23 to -0.11)	-0.19 (-0.23 to -0.15)
Protein C inhibitor	7.1 (4.2 to 10.1)	15.9 (9.6 to 22.2)
APC-sr*	1.86 (1.41 to 2.31)	2.96 (2.46 to 3.46)

Note: the individual plasma components are expressed as % of normal pooled plasma
* C4BP, C4b-binding protein; PS_{APCind}, APC-independent anticoagulant activity of protein S; APC-sr, activated protein C sensitivity ratio

Women without factor V_{Leiden} who used desogestrel-containing oral contraceptives became significantly more resistant to APC (as indicated by the increase of the APC-sr) than users of levonorgestrel-containing oral contraceptives (Figure 7.3). Total and free protein S significantly decreased in women using desogestrel-containing oral contraceptives, whereas in users of levonorgestrel-containing oral contraceptives there was a minor decrease of total protein S and even an increase of free protein S.

In addition, protein S_{APCind} was significantly more decreased on desogestrel than on levonorgestrel-containing oral contraceptives. No differential changes were observed for protein C, C4b-binding protein and protein C inhibitor.

In carriers of factor V_{Leiden}, the largest changes were observed with desogestrel-containing contraceptives (Table 7.2 and Figure 7.3).

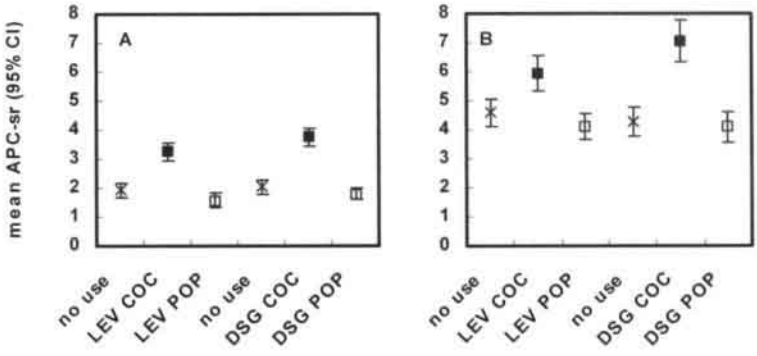


Fig 7.3 n**APC-sr levels (95% CI) on combined oral contraceptives (COC), progestagen-only** (POP) or during no oral contraceptive use according to the absence (A) or presence (B) of the factor V_{Leiden} mutation.

Effect of Progestagens-Only Preparations Relative to the period of no use, the progestagen-only preparations caused changes in anticoagulant parameters that, with the exception of C4b-binding protein, were opposite to those observed during the use of combined oral contraceptives (Table 7.2 and Figure 7.2B). The effects of progestagen-only preparations were in general more pronounced with levonorgestrel. The increase in free protein S and PS_{APCind} and the decrease in C4b-binding protein in women without factor V_{Leiden} were significantly more pronounced on levonorgestrel than on desogestrel. In carriers of factor V_{Leiden} the only significant differential effect between levonorgestrel and desogestrel was observed for PS_{APCind}.

The effects of the combination of estrogens and progestagens versus progestagen-only were estimated from the difference between the anticoagulant parameters in phase 1 and phase 2 (Table 7.3). Almost all anticoagulant parameters differed significantly between the periods that combined oral contraceptives and progestagen-only preparations were used.

Comparison of women with and without the factor V_{Leiden} mutation The magnitude of the changes induced by levonorgestrel-containing contraceptives was not materially different in carriers compared to non-carriers of the factor V_{Leiden} mutation (Table 7.4). In contrast, the changes observed during the use of desogestrel-containing oral contraceptives were more pronounced in carriers than in non-carriers of factor V_{Leiden} which was statistically significant for the increase in the APC-sr (Figure 7.3) and the decrease in the plasma level of C4b-binding protein.

Table 7.4 Differences between the effect of combined oral contraceptives and progestagen only preparations on anticoagulant plasma parameters in the absence or presence of the factor V_{Leiden} mutation

	Factor V_{Leiden} positive *vs.* factor V_{Leiden} negative Mean difference (95%CI)	
	Levonorgestrel containing OC	*Levonorgestrel-only*
Protein C	-2.4 (-8.7 to 3.9)	-1.9 (-6.3 to 2.5)
Total Protein S	2.8 (-2.8 to 8.3)	0.6 (-3.5 to 4.8)
Free protein S	-0.3 (-4.0 to 3.5)	-1.1 (-3.6 to 1.4)
C4BP*	4.7 (0.5 to 8.8)	1.8 (-4.8 to 8.5)
PS_{APCind}*	0.05 (0.01 to 0.09)	-0.05 (-0.13 to 0.03)
Protein C inhibitor	-4.2 (-9.7 to 1.4)	-2.4 (-8.6 to 3.9)
APC-sr*	0.01 (-0.44 to 0.47)	-0.16 (-0.42 to 0.10)
	Desogestrel containing OC	*Desogestrel-only*
Protein C	4.4 (-2.9 to 11.8)	-3.0 (-7.0 to 1.1)
Total Protein S	-5.1 (-10.5 to 0.2)	-2.8 (-8.1 to 2.4)
Free protein S	-1.6 (-5.0 to 1.8)	0.07 (-2.7 to 2.9)
C4BP*	-5.9 (-11.4 to -0.3)	-4.9 (-10.3 to 0.4)
PS_{APCind}*	0.03 (-0.04 to 0.09)	0.00 (-0.05 to 0.05)
Protein C inhibitor	5.4 (-2.7 to 13.5)	-1.0 (-8.0 to 5.9)
APC-sr*	1.05 (0.48 to 1.62)	0.05 (-0.27 to 0.37)

Note: the individual plasma components are expressed as % of normal pooled plasma
* C4BP, C4b-binding protein; PS_{APCind}, APC-independent anticoagulant activity of protein S; APC-sr, activated protein C sensitivity ratio

Discussion

We found that third generation oral contraceptives have a stronger effect on anticoagulant parameters than preparations of the second generation. These differential effects disappear or go in an opposite direction with the progestagen components of these pills only. The contrasts are similar, but more outspoken among

women with the factor V_{Leiden} mutation than among non-carriers. Our findings suggests that, compared to levonorgestrel, desogestrel is less effective in counteracting the thrombotic effects induced by the estrogen component in combined oral contraceptives.

Oral contraceptive use has repeatedly been associated with an increased risk of venous thrombosis [75, 100, 27] Particularly, the differential changes of the proteins involved in the protein C system may contribute to the thrombotic effects of oral contraceptives. This study was designed to investigate the effect of second and third generation oral contraceptives and their respective progestagens (levonorgestrel or desogestrel) on the protein C pathway in carriers and non-carriers of the factor V_{Leiden} mutation. We showed that earlier reported changes in anticoagulant parameters induced by oral contraceptives in healthy women[12, 145, 146] also occurred in women with factor V_{Leiden}[12, 145] In carriers as well as in non-carriers of the factor V_{Leiden} mutation both oral contraceptives induced APC resistance, increased the plasma levels of protein C and protein C inhibitor and decreased the APC-independent anticoagulant activity of protein S and the plasma levels of total protein S and C4b-binding protein. In both study populations free protein S increased on levonorgestrel- and decreased on desogestrel-containing oral contraceptives. This increase of free protein S on combined pills with levonorgestrel is explained by the fact that on this contraceptive total protein S hardly changes which together with the decrease of C4b-binding protein (which complexes protein S) results in an elevation of the plasma level of free protein S. In both carriers and non-carriers of factor V_{Leiden} the changes in anticoagulant parameters were most pronounced in the women using desogestrel-containing oral contraceptives. Since hereditary defects of the protein C pathway are associated with an increased risk of venous thromboembolism,[18-20] the differential effects of levonorgestrel- and desogestrel-containing oral contraceptives particularly on the plasma levels of protein S and on the degree of acquired APC resistance may well explain the differences in thrombotic risk associated with these preparations[3, 5, 76, 147]. The pronounced increase in APC resistance in women with factor V_{Leiden} who use desogestrel-containing oral contraceptives may account for the reported elevated risk of venous thrombosis in carriers of this mutation taking third generation oral contraceptives [4].

To determine to what extent the progestagens levonorgestrel and desogestrel are responsible for the changes of anticoagulant parameters induced by combined oral contraceptives, we also investigated the effect of progestagens-only on the protein C pathway. Some years ago, Winkler et al [148] compared two progestagen-only pills containing either 30 μg levonorgestrel or 75 μg desogestrel. They reported increased protein S levels for desogestrel compared to levonorgestrel. However, the doses of progestagen used in these pills differed from those normally used in oral contraceptives. In our study we compared progestagen-only preparations that contained the same dose of progestagen as present in oral contraceptives (150 μg levonorgestrel or desogestrel). Almost all effects of combined oral contraceptives on anticoagulant parameters disappeared or even went in an opposite direction when the participants used progestagen-only preparations. Our observations are supported by reports indicating that the changes of several other hemostatic parameters on progestagen-only pills and implants are the inverse of those induced by combined oral contraceptives [148, 149], which may indicate a net antithrombotic effect. Progestagen-only-pills may thus be a safer method of contraception. However, during the study many women complained about irregular bleedings when these preparations were used (65% compared to 15% during the use of combined oral contraceptives).

The present study may provide an explanation for the observation that combined oral contraceptives with desogestrel cause more marked changes of the anticoagulant protein C system than levonorgestrel-containing oral contraceptives. We hypothesize that estrogens such as ethinylestradiol cause changes of anticoagulant parameters that are in the same direction, but more profound than observed with combined oral contraceptives. Due to their androgenic properties, progestagens induce changes in the anticoagulant system that are opposite to those of estrogen, and which, due to the higher androgenicity[34,35] are more pronounced with levonorgestrel than with desogestrel. Hence, we propose that combined oral contraceptives with desogestrel induce more profound changes of the anticoagulant system than levonorgestrel-containing oral contraceptives because the effects of ethinylestradiol on anticoagulant parameters are less well compensated by desogestrel than by levonorgestrel. This view is supported by the observation that APC resistance correlates inversely with the dose of levonorgestrel present in five different combined

oral contraceptives[132] suggesting that high concentrations of levonorgestrel counteract the increase in APC resistance.

In conclusion, our findings indicate that desogestrel-containing oral contraceptives have a more pronounced effect on the anticoagulant protein C system than levonorgestrel-containing oral contraceptives, especially in women with factor V_{Leiden}. Particularly the decrease in protein S and the profoundly increased resistance to APC might contribute to the elevated risk of venous thrombosis in carriers of factor V_{Leiden} who use third generation oral contraceptives. The differential effects of second and third generation oral contraceptives on the anticoagulant pathway can at least be partially explained by the observation that levonorgestrel is more effective than desogestrel in counteracting the thrombotic effect of ethinylestradiol.

References

1. Gerstman BB, Piper JM, Tomita DK, Ferguson WJ, Stadel BV, Lundin FE. Oral contraceptive estrogen dose and the risk of deep venous thromboembolic disease [see comments]. Am J Epidemiol 1991; 133:32-7.
2. WHO. World Health Organization Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception. Venous thromboembolic disease and combined oral contraceptives: results of international multicentre case-control study. Lancet 1995a; 346:1575-82.
3. Jick H, Jick SS, Gurewich V, Myers MW, Vasilakis C. Risk of idiopathic cardiovascular death and nonfatal venous thromboembolism in women using oral contraceptives with differing progestagen components. Lancet 1995; 346:1589-93.
4. Bloemenkamp KWM, Rosendaal FR, Helmerhorst FM, B□ller HR, Vandenbroucke JP. Enhancement by factor V Leiden mutation of risk of deep- vein thrombosis associated with oral contraceptives containing third- generation progestagen. Lancet 1995; 346:1593-1596.
5. Spitzer WO, Lewis MA, Heinemann LA, Thorogood M, MacRae KD. Third generation oral contraceptives and risk of venous thromboembolic disorders: an international case-control study. Transnational Research Group on Oral Contraceptives and the Health of Young Women [see comments]. British Medical Journal 1996; 312:83-8.
6. Farmer RD, Williams TJ, Simpson EL, Nightingale AL. Effect of 1995 pill scare on rates of venous thromboembolism among women taking combined oral contraceptives: analysis of general practice research database. Bmj 2000; 321:477-9.
7. Jick H, Kaye JA, Vasilakis-Scaramozza C, Jick SS. Risk of venous thromboembolism among users of third generation oral contraceptives compared with users of oral contraceptives with levonorgestrel before and after 1995: cohort and case-control analysis. Bmj 2000; 321:1190-1195.
8. Newton JR. Classification and comparison of oral contraceptives containing new generation progestagens. Hum Reprod Update 1995; 1:231-263.
9. Speroff L. Oral contraceptives and venous thromboembolism. Int J Gynaecol Obstet 1996; 54:45-50.
10. Kluft C, Lansink M. Effects of oral contraceptives on haemostasis variables. Thromb Haemost 1997; 78:315-326.
11. Rosing J, Tans G. Effects of oral contraceptives on hemostasis and thrombosis. American Journal of Obstetrics and Gynecology 1999; 180:S375-82.
12. Tans G, Curvers J, Middeldorp S, et al. A Randomized Cross-over Study on the Effects of Levonorgestrel- and Desogestrel-containing Oral Contraceptives on the Anticoagulant Pathways. Thrombosis and Haemostasis 2000; 84:15-21.
13. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood 1996; 88:3698-703.
14. Kraaijenhagen RA, in't Anker PS, Koopman MM, et al. High plasma concentration of factor VIIIc is a major risk factor for venous thromboembolism [see comments]. Thromb Haemost 2000; 83:5-9.
15. van Hylckama Vlieg A, van der Linden IK, Bertina RM, Rosendaal FR. High levels of factor IX increase the risk of venous thrombosis. Blood 2000; 95:3678-82.
16. Meijers JC, Tekelenburg WL, Bouma BN, Bertina RM, Rosendaal FR. High levels of coagulation factor XI as a risk factor for venous thrombosis. N Engl J Med 2000; 342:696-701.
17. van Tilburg NH, Rosendaal FR, Bertina RM. Thrombin activatable fibrinolysis inhibitor and the risk for deep vein thrombosis. Blood 2000; 95:2855-9.
18. Rosing J, Tans G, Nicolaes GAF, et al. Oral contraceptives and venous thrombosis: Different sensitivities to activated protein C in women using second- and third-generation oral contraceptives. British Journal of Haematology 1997; 97:233-238.
19. Lindqvist PG, Svensson PJ, Marsaal K, Grennert L, Luterkort M, Dahlback B. Activated protein C resistance (FV:Q506) and pregnancy. Thromb Haemost 1999; 81:532-7.
20. Rosendaal FR. Thrombosis in the young: epidemiology and risk factors. A focus on venous thrombosis. Thromb Haemost 1997; 78:1-6.
21. Walker ID. Inherited coagulation disorders and thrombophilia and pregnancy. In: Bonnar J, ed. Recent advances in Obstetrics and Gynaecology. Vol. 20. London: Churchill-Livingstone, 1998:35-64.

22. Rutherford S, Montoro M, McGhee W, Strong T. Thromboembolic disease associated with pregnancy: an 11 years review. Am Obstet Gynecol. 1991; 164 (suppl):286.
23. Macklon NS, Greer IA. Venous thromboembolic disease in obstetrics and gynaecology: the Scottish experience. Scott.Med. J. 1996; 41:83-86.
24. Rosendaal FR. Risk factors for venous thrombosis: prevalence, risk, and interaction. Semin Hematol 1997; 34:171-87.
25. Bonnar J, Green R, Norris L. Inherited thrombophilia and pregnancy: the obstetric perspective. Semin Thromb Hemost 1998; 24:49-53.
26. Stirling Y, Woolf L, North WR, Seghatchian MJ, Meade TW. Haemostasis in normal pregnancy. Thromb Haemost 1984; 52:176-82.
27. van Wersch JW, Ubachs JM. Blood coagulation and fibrinolysis during normal pregnancy. Eur J Clin Chem Clin Biochem 1991; 29:45-50.
28. Bremme K, Ostlund E, Almqvist I, Heinonen K, Blomback M. Enhanced thrombin generation and fibrinolytic activity in normal pregnancy and the puerperium. Obstet Gynecol 1992; 80:132-7.
29. Levine A, Teppa, J, McGough, B, Cowchock, F.S. Evaluation of the prethrombotic state in pregnancy and in women using OC. Contraception 1996; 53:255-257.
30. Mercelina-Roumans PE, Ubachs JM, van Wersch JW. Coagulation and fibrinolysis in smoking and nonsmoking pregnant women. Br J Obstet Gynaecol 1996; 103:789-94.
31. Kjellberg U, Andersson NE, Rosen S, Tengborn L, Hellgren M. APC resistance and other haemostatic variables during pregnancy and puerperium. Thromb Haemost 1999; 81:527-31.
32. Clark P, Brennand J, Conkie JA, McCall F, Greer IA, Walker ID. Activated protein C sensitivity, protein C, protein S and coagulation in normal pregnancy. Thromb Haemost 1998; 79:1166-70.
33. Comeglio P, Fedi S, Liotta AA, et al. Blood clotting activation during normal pregnancy. Thromb Res 1996; 84:199-202.
34. Schlit AF, Col-De Beys C, Moriau M, Lavenne-Pardonge E. Acquired activated protein C resistance in pregnancy. Thromb Res 1996; 84:203-6.
35. Comp PC, Thurnau GR, Welsh J, Esmon CT. Functional and immunologic protein S levels are decreased during pregnancy. Blood 1986; 68:881-5.
36. Faught W, Garner P, Jones G, Ivey B. Changes in protein C and protein S levels in normal pregnancy. Am J Obstet Gynecol 1995; 172:147-50.
37. Cumming AM, Tait RC, Fildes S, Yoong A, Keeney S, Hay CR. Development of resistance to activated protein C during pregnancy. Br J Haematol 1995; 90:725-7.
38. Mathonnet F, de Mazancourt P, Bastenaire B, et al. Activated protein C sensitivity ratio in pregnant women at delivery. Br J Haematol 1996; 92:244-6.
39. Bokarewa MI, Wramsby M, Bremme K, Blomback M. Variability of the response to activated protein C during normal pregnancy. Blood Coagul Fibrinolysis 1997; 8:239-44.
40. Meinardi JR, Henkens CMA, Heringa MP, vanderMeer J. Acquired APC resistance related to oral contraceptives and pregnancy and its possible implications for clinical practice. Blood Coagulation and Fibrinolysis 1997; 8:152-154.
41. Nicolaes GAF, Thomassen MCLGD, Tans G, Rosing J, Hemker HC. Effect of activated protein C on thrombin generation and on the thrombin potential in plasma of normal and APC-resistant individuals. Blood Coagulation and Fibrinolysis 1997; 8:28-38.
42. Thomassen MCLGD, Curvers J, Rimmer JE, et al. Influence of hormone replacement therapy, oral contraceptives and pregnancy on APC resistance. Thrombosis and Haemostasis 1999; 82 (Suppl):770-771.
43. Sugimura M, Kobayashi T, Kanayama N, Terao T. Detection of marked reduction of sensitivity to activated protein C prior to the onset of thrombosis during puerperium as detected by endogenous thrombin potential-based assay. Thromb Haemost 1999; 82:1364-1365.
44. Petitti DB, Wingerd J, Pellegrin F, Ramcharan S. Risk of vascular disease in women. Smoking, oral contraceptives, noncontraceptive estrogens, and other factors. Jama 1979; 242:1150-4.
45. Nachtigall LE, Nachtigall RH, Nachtigall RD, Beckman EM. Estrogen replacement therapy II: a prospective study in the relationship to carcinoma and cardiovascular and metabolic problems. Obstet Gynecol 1979; 54:74-9.
46. Devor M, Barrett-Connor E, Renvall M, Feigal D, Jr., Ramsdell J. Estrogen replacement therapy and the risk of venous thrombosis [see comments]. Am J Med 1992; 92:275-82.

47. Daly E, Vessey MP, Hawkins MM, Carson JL, Gough P, Marsh S. Risk of venous thromboembolism in users of hormone replacement therapy. Lancet 1996; 348:977-80.
48. Jick H, Derby LE, Myers MW, Vasilakis C, Newton KM. Risk of hospital admission for idiopathic venous thromboembolism among users of postmenopausal oestrogens [see comments]. Lancet 1996; 348:981-3.
49. Ernst E, Resch KL. Fibrinogen as a cardiovascular risk factor: a meta-analysis and review of the literature [see comments]. Ann Intern Med 1993; 118:956-63.
50. Thomas DP, Roberts HR. Hypercoagulability in venous and arterial thrombosis [see comments]. Ann Intern Med 1997; 126:638-44.
51. Nabulsi AA, Folsom AR, White A, et al. Association of hormone-replacement therapy with various cardiovascular risk factors in postmenopausal women. The Atherosclerosis Risk in Communities Study Investigators [see comments]. N Engl J Med 1993; 328:1069-75.
52. Lobo RA, Pickar JH, Wild RA, Walsh B, Hirvonen E. Metabolic impact of adding medroxyprogesterone acetate to conjugated estrogen therapy in postmenopausal women. The Menopause Study Group. Obstet Gynecol 1994; 84:987-95.
53. M.R.C.G.P.R.F. Randomised comparison of oestrogen versus oestrogen plus progestogen hormone replacement therapy in women with hysterectomy. Medical Research Council's General Practice Research Framework. Bmj 1996; 312:473-8.
54. Cushman M, Meilahn EN, Psaty BM, Kuller LH, Dobs AS, Tracy RP. Hormone replacement therapy, inflammation, and hemostasis in elderly women. Arterioscler Thromb Vasc Biol 1999; 19:893-9.
55. Scarabin PY, Vissac AM, Kirzin JM, et al. Population correlates of coagulation factor VII. Importance of age, sex, and menopausal status as determinants of activated factor VII. Arterioscler Thromb Vasc Biol 1996; 16:1170-6.
56. PEPI. Effects of estrogen or estrogen/progestin regimens on heart disease risk factors in postmenopausal women. The Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial. The Writing Group for the PEPI Trial [see comments]. Jama 1995; 273:199-208.
57. Folsom AR, Wu KK, Davis CE, Conlan MG, Sorlie PD, Szklo M. Population correlates of plasma fibrinogen and factor VII, putative cardiovascular risk factors. Atherosclerosis 1991; 91:191-205.
58. Lee AJ, Lowe GD, Smith WC, Tunstall-Pedoe H. Plasma fibrinogen in women: relationships with oral contraception, the menopause and hormone replacement therapy. Br J Haematol 1993; 83:616-21.
59. Meilahn EN, Kuller LH, Matthews KA, Kiss JE. Hemostatic factors according to menopausal status and use of hormone replacement therapy. Ann Epidemiol 1992; 2:445-55.
60. Andersen LF, Gram J, Skouby SO, Jespersen J. Effects of hormone replacement therapy on hemostatic cardiovascular risk factors. Am J Obstet Gynecol 1999; 180:283-9.
61. Lindberg UB, Crona N, Stigendal L, Teger-Nilsson AC, Silfverstolpe G. A comparison between effects of estradiol valerate and low dose ethinyl estradiol on haemostasis parameters. Thromb Haemost 1989; 61:65-9.
62. Kroon UB, Silfverstolpe G, Tengborn L. The effects of transdermal estradiol and oral conjugated estrogens on haemostasis variables. Thromb Haemost 1994; 71:420-3.
63. Gordon EM, Williams SR, Frenchek B, Mazur CA, Speroff L. Dose-dependent effects of postmenopausal estrogen and progestin on antithrombin III and factor XII. J Lab Clin Med 1988; 111:52-6.
64. Boschetti C, Cortellaro M, Nencioni T, Bertolli V, Della Volpe A, Zanussi C. Short- and long-term effects of hormone replacement therapy (transdermal estradiol vs oral conjugated equine estrogens, combined with medroxyprogesterone acetate) on blood coagulation factors in postmenopausal women. Thromb Res 1991; 62:1-8.
65. Sporrong T, Mattsson LA, Samsioe G, Stigendal L, Hellgren M. Haemostatic changes during continuous oestradiol-progestogen treatment of postmenopausal women. Br J Obstet Gynaecol 1990; 97:939-44.
66. Caine YG, Bauer KA, Barzegar S, et al. Coagulation activation following estrogen administration to postmenopausal women. Thromb Haemost 1992; 68:392-5.
67. Gilabert J, Estelles A, Cano A, et al. The effect of estrogen replacement therapy with or without progestogen on the fibrinolytic system and coagulation inhibitors in postmenopausal status. Am J Obstet Gynecol 1995; 173:1849-54.

68. van Baal WM, Emeis JJ, van der Mooren MJ, Kessel H, Kenemans P, Stehouwer CD. Impaired procoagulant-anticoagulant balance during hormone replacement therapy? A randomised, placebo-controlled 12-week study. Thromb Haemost 2000; 83:29-34.
69. Clarkson TB, Shively CA, Morgan TM, Koritnik DR, Adams MR, Kaplan JR. Oral contraceptives and coronary artery atherosclerosis of cynomolgus monkeys. Obstet Gynecol 1990; 75:217-22.
70. De Mitrio V, Marino R, Cicinelli E, et al. Beneficial effects of postmenopausal hormone replacement therapy with transdermal estradiol on sensitivity to activated protein C. Blood Coagul Fibrinolysis 2000; 11:175-82.
71. Marcucci R, Abbate R, Fedi S, et al. Acquired activated protein C resistance in postmenopausal women is dependent on factor VIII:c levels. Am J Clin Pathol 1999; 111:769-72.
72. Teede HJ, McGrath BP, Smolich JJ, et al. Postmenopausal hormone replacement therapy increases coagulation activity and fibrinolysis. Arterioscler Thromb Vasc Biol 2000; 20:1404-9.
73. Scarabin PY, Alhenc-Gelas M, Plu-Bureau G, Taisne P, Agher R, Aiach M. Effects of oral and transdermal estrogen/progesterone regimens on blood coagulation and fibrinolysis in postmenopausal women. A randomized controlled trial. Arterioscler Thromb Vasc Biol 1997; 17:3071-8.
74. Koh KK, Horne MK, 3rd, Cannon RO, 3rd. Effects of hormone replacement therapy on coagulation, fibrinolysis, and thrombosis risk in postmenopausal women. Thromb Haemost 1999; 82:626-33.
75. Stadel BV. Oral contraceptives and cardiovascular disease (first of two parts). N Engl J Med 1981; 305:612-8.
76. WHO. World Health Organization Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception. Effect of different progestagens in low oestrogen oral contraceptives on venous thromboembolic disease. World Health Organization Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception. Lancet 1995b; 346:1582-1588.
77. Lewis MA, Heinemann LA, MacRae KD, Bruppacher R, Spitzer WO. The increased risk of venous thromboembolism and the use of third generation progestagens: role of bias in observational research. The Transnational Research Group on Oral Contraceptives and the Health of Young Women. Contraception 1996; 54:5-13.
78. Farmer RD, Lawrenson RA, Thompson CR, Kennedy JG, Hambleton IR. Population-based study of risk of venous thromboembolism associated with various oral contraceptives [see comments]. Lancet 1997; 349:83-8.
79. Spitzer WO. The 1995 pill scare revisited: anatomy of a non-epidemic. Human Reproduction 1997; 12:2347-57.
80. Vandenbroucke JP, Helmerhorst FM, Bloemenkamp KWM, Rosendaal FR. Third-generation oral contraceptive and deep venous thrombosis: From epidemiologic controversy to new insight in coagulation. American Journal of Obstetrics and Gynaecology 1997; 177:887-891.
81. Walker AM. Newer oral contraceptives and the risk of venous thromboembolism. Contraception 1998; 57:169-181.
82. Mishell DR. A critical analysis of progestins and VT. Am J Obstet Gynecol 1998; 179:S37-S86.
83. Winkler UH. Effects on hemostatic variables of desogestrel- and gestodene-containing oral contraceptives in comparison with levonorgestrel-containing oral contraceptives: A review. Am J Obstet Gynecol 1998; 179:S51-S61.
84. Dahlbäck B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. Proc Natl Acad Sci U S A 1993; 90:1004-8.
85. Bertina RM, Koeleman BPC, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994; 369:64-67.
86. Koster T, Rosendaal FR, de Ronde H, Briet E, Vandenbroucke JP, Bertina RM. Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. Lancet 1993; 342:1503-6.
87. Vandenbroucke JP, Rosendaal FR. End of the line for "third-generation-pill" controversy? Lancet 1997; 349:1113-1114.
88. Schramm W, Heinemann LAJ. Oral contraceptives and venous thromboembolism: Acquired APC resistance? British Journal of Haematology 1997; 98:491-491.
89. Winkler UH. Blood Coagulation and Oral Contraceptives. A Critical Review. Contraception 1998; 57:203-209.

90. Spitzer WO. Bias versus causality: Interpreting recent evidence of oral contraceptive studies. Am J Obstet Gynecol 1998; 179:S43-S50.
91. Nicolaes GAF, Thomassen MCLGD, vanOerle R, et al. A prothrombinase-based assay for detection of resistance to activated protein C. Thombosis and Haemostasis 1996; 76:404-410.
92. Böttcher CJF, van Gent CM, Pries C. A Rapid and Sensitive Sub-micro Phosphorus Determination. Analitica Chimica Acta 1961; 24:203-207.
93. Elisen MG, Maseland MH, Church FC, Bouma BN, Meijers JC. Role of the A+ helix in heparin binding to protein C inhibitor. Thromb Haemost 1996; 75:760-6.
94. Sala N, Owen WG, Collen D. A functional assay of protein C in human plasma. Blood 1984; 63:671-675.
95. de Ronde H, Bertina RM. Laboratory diagnosis of APC-resistance: a critical evaluation of the test and the development of diagnostic criteria. Thrombosis and Haemostasis 1994; 72:880-886.
96. Pocock SJ. Clinical trials, a practical approach. Chichester: John Wiley & Sons, 1983:110-119.
97. Heeb MJ, Griffin JH. Physiologic inhibition of human activated protein C by alpha 1-antitrypsin. J Biol Chem 1988; 263:11613-6.
98. Heeb MJ, Gruber A, Griffin JH. Identification of divalent metal ion-dependent inhibition of activated protein C by alpha 2-macroglobulin and alpha 2-antiplasmin in blood and comparisons to inhibition of factor Xa, thrombin, and plasmin. J Biol Chem 1991; 266:17606-12.
99. Espana F, Gruber A, Heeb MJ, Hanson SR, Harker LA, Griffin JH. In vivo and in vitro complexes of activated protein C with two inhibitors in baboons. Blood 1991; 77:1754-60.
100. Jespersen J, Petersen KR, Skouby SO. Effects of newer oral contraceptives on the inhibition of coagulation and fibrinolysis in relation to dosage and type of steroid. Am J Obstet Gynecol 1990; 163:396-403.
101. Basdevant A, Conard J, Pelissier C, et al. Hemostatic and metabolic effects of lowering the ethinyl-estradiol dose from 30 mcg to 20 mcg in oral contraceptives containing desogestrel. Contraception 1993; 48:193-204.
102. Granata A, Sobbrio GA, D'Arrigo F, et al. Changes in the plasma levels of proteins C and S in young women on low-dose oestrogen oral contraceptives. Clin Exp Obstet Gynecol 1991; 18:9-12.
103. Cachrimanidou AC, Hellberg D, Nilsson S, von Schoulz B, Crona N, Siegbahn A. Hemostasis profile and lipid metabolism with long-interval use of a desogestrel-containing oral contraceptive. Contraception 1994; 50:153-65.
104. Petersen KR, Sidelmann J, Skouby SO, Jespersen J. Effects of monophasic low-dose oral contraceptives on fibrin formation and resolution in young women. Am J Obstet Gynecol 1993; 168:32-8.
105. Winkler UH, Holscher T, Schulte H, Zierleyn JP, Collet W, Schindler AE. Ethinylestradiol 20 versus 30 micrograms combined with 150 micrograms desogestrel: a large comparative study of the effects of two low-dose oral contraceptives on the hemostatic system. Gynecol Endocrinol 1996; 10:265-71.
106. Malm J, Laurell M, Dahlback B. Changes in the plasma levels of vitamin K-dependent proteins C and S and of C4b-binding protein during pregnancy and oral contraception. Br J Haematol 1988; 68:437-43.
107. Jespersen J, Nielsen MT. Levels of protein S during the normal menstrual cycle and in women on oral contraceptives low in estrogen. Gynecol Obstet Invest 1989; 28:82-6.
108. Curvers J, Thomassen MCLGD, Nicolaes GAF, et al. Acquired APC resistance and oral contraceptives: differences between two functional tests. British Journal of Haematology 1999; 105:88-94.
109. Olivieri O, Friso S, Manzato F, et al. Resistance to activated protein C in healthy women taking oral contraceptives. British Journal of Haematology 1995; 91:465-470.
110. Olivieri O, Friso S, Manzato F, et al. Resistance to activated protein C, associated with oral contraceptives use effect of formulations, duration of assumption, and doses of oestro-progestins. Contraception 1996; 54 (3):149-152.
111. Henkens CM, Bom VJ, Seinen AJ, van der Meer J. Sensitivity to activated protein C; influence of oral contraceptives and sex. Thrombosis and Haemostasis 1995; 73:402-4.
112. Rotteveel RC, Roozendaal KJ, Eijsman L, Hemker HC. The influence of oral contraceptives on the time-integral of thrombin generation (thrombin potential). Thromb Haemost 1993; 70:959-62.

113. Middeldorp S, Meijers JCM, van den End AE, et al. Effects on Coagulation of Levonorgestrel- and Desogestrel-containing Low Dose Oral Contraceptives: a Cross-over Study. Thrombosis and Haemostasis 2000; 84:4-8.
114. Zöller B, Berntsdotter A, Garcia de Frutos P, Dahlbäck B. Resistance to activated protein C as an additional genetic risk factor in hereditary deficiency of protein S. Blood 1995; 85:3518-23.
115. Freyburger G, Javorschi S, Labrouche S, Bernard P. Proposal for objective evaluation of the performance of various functional APC-resistance tests in genotyped patients. Thombosis and Haemostasis 1997; 78 (5):1360-1365.
116. Letsky E, de Swiet M. Annotation. Thromboembolism in pregnancy and its management. Br J Haematol 1984; 57:543-52.
117. Speroff L, DeCherney A. Evaluation of a new generation of oral contraceptives. The Advisory Board for the New Progestins. Obstet Gynecol 1993; 81:1034-47.
118. Meijers JCM, Middeldorp S, Tekelenburg W, et al. Increased Fibrinolytic Activity during Use of Oral Contraceptives Is Counteracted by an Enhanced Factor XI-independent down Regulation of Fibrinolysis: A Randomized Cross-over Study of Two Low-dose Oral Contraceptives. Thrombosis and Haemostasis 2000; 84:9-14.
119. Rosendaal FR, Koster T, Vandenbroucke JP, Reitsma PH. High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). Blood 1995; 85:1504-1508.
120. Rodeghiero F, Tosetto A. Activated protein C resistance and factor V Leiden mutation are independent risk factors for venous thromboembolism. Ann Intern Med 1999; 130:643-50.
121. de Visser MC, Rosendaal FR, Bertina RM. A reduced sensitivity for activated protein C in the absence of factor V Leiden increases the risk of venous thrombosis. Blood 1999; 93:1271-6.
122. Rosing J, Middeldorp S, Curvers J, et al. Low-dose oral contraceptives and acquired resistance to activated protein C: a randomised cross-over study. Lancet 1999; 354:2036-40.
123. Aurousseau MH, Samama MM, Belhassen A, Herve F, Hugues JN. Risk of thromboembolism in relation to an in-vitro fertilization programme: three case reports. Human Reproduction 1995; 10:94-97.
124. Stewart JA, Hamilton PJ, Murdoch AP. Thromboembolic disease associated with ovarian stimulation and assisted conception techniques. Human Reproduction 1997; 12:2167-2173.
125. Bremme K, Wramsby H, Andersson O, Wallin M, Blomback M. Do lowered factor VII levels at extremely high endogenous oestradiol levels protect against thrombin formation? Blood Coagul Fibrinolysis 1994; 5:205-10.
126. Lox C, Canez M, DeLeon F, Dorsett J, Prien S. Hyperestrogenism induced by menotropins alone or in conjunction with luprolide acetate in in vitro fertilization cycles: the impact on hemostasis. Fertil Steril 1995; 63:566-70.
127. Biron C, Galtier-Dereure F, Rabesandratana H, et al. Hemostasis parameters during ovarian stimulation for in vitro fertilization: results of a prospective study. Fertil Steril 1997; 67:104-9.
128. Lox C, Canez M, Prien S. The influence of hyperestrogenism during in vitro fertilization on the fibrinolytic mechanism. Int J Fertil Womens Med 1998; 43:34-9.
129. Brechmann J, Unterberg C. [Superior vena cava thrombosis after in vitro fertilization]. Dtsch Med Wochenschr 2000; 125:1429-1432.
130. Ludwig M, Felberbaum RE, Diedrich K. Deep vein thrombosis during administration of hMG for ovarian stimulation. Arch Gynecol Obstet 2000; 263:139-141.
131. Wramsby ML, Bokarewa MI, Blomback M, Bremme AK. Response to activated protein C during normal menstrual cycle and ovarian stimulation. Hum Reprod 2000; 15:795-7.
132. Kluft C, de Maat MP, Heinemann LA, Spannagl M, Schramm W. Importance of levonorgestrel dose in oral contraceptives for effects on coagulation [letter]. Lancet 1999; 354:832-3.
133. Gillmer MD. Progestogen potency in oral contraceptive pills. Am J Obstet Gynecol 1987; 157:1048-52.
134. Vandenbroucke JP, Koster T, Briet E, Reitsma PH, Bertina RM, Rosendaal FR. Increased risk of venous thrombosis in oral-contraceptive users who are carriers of factor V Leiden mutation [see comments]. Lancet 1994; 344:1453-7.
135. Nicolaes GAF, Tans G, Thomassen MCLGD, et al. Peptide bond cleavages and loss of functional activity during inactivation of factor Va and factor VaR506Q by activated protein C. Journal of Biological Chemistry 1995; 270:21158-66.
136. Kalafatis M, Bertina RM, Rand MD, Mann KG. Characterization of the molecular defect in factor VR506Q. Journal of Biological Chemistry 1995; 270:4053-7.

137. Aparicio C, Dahlbäck B. Molecular mechanisms of activated protein C resistance. Properties of factor V isolated from an individual with homozygosity for the Arg506 to Gln mutation in the factor V gene. Biochemical Journal 1996; 313:467-72.
138. Clouse LH, Comp PC. The regulation of hemostasis: the protein C system. New England Journal of Medicine 1986; 314:1298-1304.
139. van 't Veer C, Hackeng TM, Biesbroeck D, Sixma JJ, Bouma BN. Increased prothrombin activation in protein S-deficient plasma under flow conditions on endothelial cell matrix: an independent anticoagulant function of protein S in plasma. Blood 1995; 85:1815-21.
140. Griffin JH, Gruber A, Fernandez JA. Reevaluation of total, free, and bound protein S and C4b-binding protein levels in plasma anticoagulated with citrate or hirudin. Blood 1992; 79:3203-11.
141. Dahlbäck B, Stenflo J. High molecular weight complex in human plasma between vitamin-K dependent protein S and complement component C4b-binding protein. Proc Natl Acad Sci U S A 1981; 78:2512-2516.
142. Bertina RM. Molecular risk factors for thrombosis. Thromb Haemost 1999; 82:601-609.
143. Lemus AE, Zaga V, Santillan R, et al. The oestrogenic effects of gestodene, a potent contraceptive progestin, are mediated by its A-ring reduced metabolites. J Endocrinol 2000; 165:693-702.
144. Voorberg J, Roelse J, Koopman R, et al. Association of idiopathic venous thromboembolism with single point-mutation at Arg506 of factor V. Lancet 1994; 343:1535-6.
145. Quehenberger P, Loner U, Kapiotis S, et al. Increased levels of activated factor VII and decreased plasma protein S activity and circulating thrombomodulin during use of oral contraceptives. Thromb Haemost 1996; 76:729-34.
146. Boerger LM, Morris PC, Thurnau GR, Esmon CT, Comp PC. Oral contraceptives and gender affect protein S status. Blood 1987; 69:692-4.
147. Bloemenkamp KW, Rosendaal FR, Buller HR, Helmerhorst FM, Colly LP, Vandenbroucke JP. Risk of venous thrombosis with use of current low-dose oral contraceptives is not explained by diagnostic suspicion and referral bias. Arch Intern Med 1999; 159:65-70.
148. Winkler UH, Howie H, Buhler K, Korver T, Geurts TB, Coelingh Bennink HJ. A randomized controlled double-blind study of the effects on hemostasis of two progestogen-only pills containing 75 microgram desogestrel or 30 microgram levonorgestrel. Contraception 1998; 57:385-92.
149. Egberg N, van Beek A, Gunnervik C, et al. Effects on the hemostatic system and liver function in relation to Implanon and Norplant. A prospective randomized clinical trial. Contraception 1998; 58:93-8.

Chapter 8

General summary and conclusions

Venous thrombosis is recognized to be a multicausal disease. In recent years more and more genetic and acquired risk factors have been identified that play a role in the development of venous thrombosis. One major risk factor that was discovered in 1993 is activated protein C (APC) resistance. Hereditary APC resistance results from a single point mutation in coagulation factor V. The mutated molecule, often called FV_{Leiden}, lacks an APC cleavage site and therefore, its procoagulant activity cannot be efficiently down-regulated by APC. As a consequence, the plasma of carriers of the FV_{Leiden} mutation is APC resistant. However, 10% of the APC resistant individuals are not carrying the FV_{Leiden} mutation and hence have a different, as yet unknown cause for APC resistance[1]. Moreover, APC resistance without the FV_{Leiden} mutation has also been established as a risk factor for venous thrombosis[2]. Theoretically, APC resistance not caused by FV-Leiden could be induced by altered levels in haemostatic factors that enhance thrombin generation or that hamper the action of the anticoagulant protein C pathway, i.e. 1) high concentrations of procoagulant proteins (*e.g.* prothrombin, factor VIII), 2) low concentrations of inhibitors of coagulation (*e.g.* tissue factor pathway inhibitor), 3) low concentrations of anticoagulant proteins (*e.g.* protein S) or 4) high concentrations of inhibitors of anticoagulant proteins (*e.g.* protein C inhibitor). It is also possible that combinations of these occur and act in concert to render an individual's plasma APC resistant.

Rather than trying to identify single contributing risk factors by a combination of individual measurements, it is useful to assess a possible prothrombotic condition via a single overall assay. The APC resistance test used throughout the investigations described in this thesis, is such a measurement. This assay quantifies the effect of APC on thrombin generation initiated in plasma via the extrinsic pathway. The first results obtained with this test, revealed that women using oral contraceptives (OC) were more resistant to APC than women not using OC. Moreover, users of so-called third generation OC exhibited responses to APC equal to that of individuals with the FV_{Leiden} mutation[3]. Since the observed APCsr's correlated with earlier reported risks of venous thrombosis for carriers of FV-Leiden and of users of different types of OC (i.e. second and third generation), it was proposed that acquired APC resistance could provide a biological explanation for the increased risk of thrombosis associated with the use of OC.

In Chapters 2, 3 and 4 of this thesis the use of this assay for the assessment of APC resistance is described. In Chapters 5, 6 and 7, the effects of changes of female sex hormones on the anticoagulant pathway are reported. In this discussion a summary will be given of the different investigations described in this thesis.

Characteristics of the APC resistance test

In Chapter 2 we have described the effects of analytical and pre-analytical variables on APCsr determined with our APC resistance test. The effects of variation of the concentration of the different reactants used in the test (calcium, phospholipids, tissue factor and APC) on thrombin formation were determined. We found that APC inhibits by thrombin formation in a dose-dependent manner. The inhibition by APC was affected by the concentration of tissue factor, calcium and phospholipids. In order to obtain reproducible APCsr, the concentrations of these reactants should therefore, be standardized. We have chosen the conditions of this assay such, that normal pooled plasma has a residual thrombin formation in the presence of APC of approximately 10%. Small variations in residual thrombin formation, due to variations of reactants, will influence the outcome of the assay in both normal and patient plasma. This may result in a day-to-day variation that can be minimized by normalisation i.e. by dividing the APCsr of a particular plasma sample by the APCsr obtained in normal pooled plasma[4].

The influence of different procedures to process whole blood to plasma, was also investigated. These included 1) concentration of citrate used to anticoagulate whole blood, 2) time before processing, 3) time and speed of centrifugation, 4) storage temperature of plasma and 5) an additional thawing of a frozen plasma sample. Differences in centrifugation and additional thawing of plasma had no effect on the APCsr, whereas a minor effect was observed for the time waited before sample processing as well as for the storage temperature. However, variation in the concentration of sodium citrate, used to anticoagulate whole blood upon drawing, has considerable influence on the APCsr. This is likely due to the fact that the anticoagulant action of APC is critically dependent on the calcium concentration. In different laboratories the sodium citrate concentration that is used to anticoagulate whole blood varies between 0.105 and 0.129M. Thus, the citrate concentration appears to influence the APCsr, an effect that has also been reported for APCsr

values determined with clotting times in the aPTT[5] . To at least partially reduce citrate effects, it is recommended to normalize APCsr against a pooled normal plasma that is collected on the same concentration of anticoagulant.

Hereditary and acquired APC resistance

The APC resistance assay described in this thesis is sensitive to both hereditary and acquired risk factors for venous thrombosis (Chapter 3). Carriers of the prothrombin mutation (PT G20210A), as well as individuals with protein S deficiency had higher APCsr than controls. APC resistance in carriers of the prothrombin mutation, which is associated with high levels of prothrombin, may be explained by an inhibition of APC-dependent factor Va inactivation[6]. Moreover, high levels of prothrombin might increase the amount of thrombin formed, leading to excessive formation factor Va. This poses an increasing demand on APC to down-regulate thrombin formation, which may lead to an APC resistant state of plasma.

Protein S is a cofactor of APC in the inactivation of factor Va. Hence, at low levels of protein S down-regulation of thrombin formation is impaired, which results in APC resistance. Protein S deficiency can be both of hereditary and acquired origin. Hereditary protein S deficiency can be caused by mutations in the protein S molecule. Acquired protein S deficiency, on the other hand, is associated with the use of hormone preparations (OC), and with pregnancy.

In chapter 3 it is also described that co-existence of risk factors for venous thrombosis that increase the APCsr, cause an even higher APCsr and in some cases render the plasma of affected individual almost completely resistant to the action of APC. The finding that more than one risk factor needs to be present to eventually cause thrombosis, is thus reflected in the APCsr values. This idea is supported in Fig3.5, which shows that there is a good correlation between the APCsr of individuals with hereditary and/or acquired risk factors for venous thrombosis and the reported odds ratios.

Clotting or thrombin formation

In chapter 3 it is shown that acquired APC resistance is especially observed in women who are using OC or who are pregnant. Depending on the APC resistance test used, there appears to be a variable number of individuals who are acquired

APC resistant. The molecular basis of this acquired APC resistance is, however, unclear. With the intention to gain more insight in this, we have assessed the sensitivity to APC of FV_{Leiden} carriers and OC users with two different APC resistance assays (Chapter 4): 1) the thrombin generation assay, which is very sensitive to hormonal influences (e.g. OC, HRT and pregnancy) and thus to acquired APC resistance and with 2) an APC resistance assay that is routinely used in hospital laboratories and that evaluates the effect of APC on the clotting times of plasma in which coagulation is initiated via the intrinsic pathway (*i.e.* the activated partial thromboplastin time, aPTT).

Both assays were equally sensitive and specific in determining the FV_{Leiden} mutation. However, the increased APC resistance during OC use is hardly observed in the classical clotting assay. Thus, apart from the ability to detect the FV_{Leiden} mutation, the correlation between the two assays is rather poor. There are at least two possible explanations for these observed differences:

1) In the two assays coagulation is initiated with activators which activate different pathways of coagulation (tissue factor versus kaolin). Tissue factor-induced coagulation mainly proceeds via the extrinsic pathway, whereas kaolin activates the intrinsic pathway. Plasma proteins of each pathway may be differentially modulated during OC use and this can lead to different sensitivities of the plasma for APC. For example, the APCsr measured in the aPTT is influenced by high levels of factor VIII, whereas the APCsr determined in the ETP-based test is sensitive to changes in protein S.

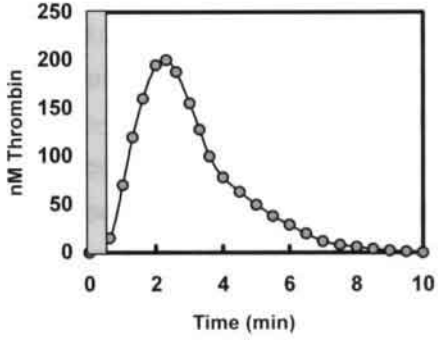


Figure 8.1 Already after formation of the first traces of thrombin, clotting is achieved, as indicated by the grey bar. However, measurement of thrombin generation represents the total amount of thrombinthat has been formed during coagulation.

2) The endpoint determination of the two assays is different *i.e.* clotting time versus thrombin formation. Clotting occurs already after the first traces of thrombin are formed (i.e. in approximately 30 sec), whereas the measurement of the endogenous thrombin potential reflects the total amount of thrombin that has been formed over a prolonged time period (Fig 8.1). It can be hypothesized that the first phase of coagulation (*i.e.* when the first traces of thrombin are generated and the clot is formed) is less sensitive to modulation by (anti)coagulation factors than the endogenous thrombin potential which quantifies the amount of thrombin that has been formed over a much longer time period. However, further experiments are needed to test these hypotheses.

APC resistance during the use of oral contraceptives

In PART II of this thesis the effects of changes in female sex steroid hormones on APC resistance protein C system are described. From the very early beginning on, the use of oral contraceptives has been associated with an increased risk for venous thrombosis. However a satisfying biological explanation for the observed increased thrombotic risk was lacking, until 1997 when acquired APC resistance [3] was proposed as a possible mechanism to explain the occurrence of venous thromboembolism in OC-using women.The study published in 1997 concerned a cross-sectional, non-cycle controlled design the results of which could have been influenced by uncontrolled pill effects and selection bias. Therefore, a double blind randomized cross-over trial of two oral contraceptive preparations was performed. The cross-over study showed that a large number of haemostatic variables changed during the use of OC (procoagulant, anticoagulant and fibrinolytic). The changes of most of the coagulation factors were more pronounced during the use of desogestrel-containing OC than during the use of levonorgestrel-containing OC. Notably, more pronounced APC resistance was observed during the use of the desogestrel-containing preparation than on levonorgestrel-containing OC.

Differences between the effects of second and third generation pills on the level of anticoagulant proteins were only found for protein S. Decreases in protein S were more pronounced during the use of third generation OC. Moreover, changes of total and free protein S correlated negatively with the increase of the APCsr determined with the ETP-based APC resistance assay (r= -0.44 / -0.60), which

indicates that the decrease in protein S can, at least partially, explain increases in APCsr. However, other mechanisms leading to acquired APC resistance must also be involved, since protein S did not significantly change during the use of second generation OC, whereas the APCsr increased considerably. Particularly, the combination of higher levels of factor VII, X and prothrombin[7] will result in an increased procoagulant pressure that may further contribute to the APC resistant phenotype found in OC using women.

Considering the discussion about OC use and the risk of venous thrombosis, it was proposed that the increased thrombin formation and decreased anticoagulant action could partially be counteracted by an increased fibrinolysis. The cross-over study confirmed that levels of fibrinolytic proteins increase during the use of OC, however, no shortened clot lysis time was found in these samples. Meijers et al.[8] report a progestagen specific difference for TAFI (thrombin activatable fibrinolysis inhibitor). TAFI was increased during the use of OC, especially during third generation OC. The increase in TAFI together with the elevated thrombin formation, probably yields more activated TAFI that can counterbalance the effects of increased levels of fibrinolytic proteins by inhibiting fibrinolysis.

Acquired APC resistance during HRT, pregnancy and IVF

The synthetic hormones in oral contraceptives cause changes in procoagulant (e.g. prothrombin, factor VII and factor X), anticoagulant (protein S total and free, Chapter 5) and fibrinolytic proteins (TAFI). Pregnancy, during which endogenous estradiol and progesterone are substantially increased, also induces major changes in the haemostatic system (factor VIII, protein S and antithrombin). Moreover, pregnancy, the use of OC and hormone replacement therapy lead to acquired protein C resistance (Chapter 3). Although, the hormones administered during OC therapy differ from the naturally occurring hormones (such as during pregnancy) the effects on haemostatic parameters are similar.

In chapter 6 the effect of changes in endogenous estradiol en progesterone on the protein C pathway are described. In plasma of women who followed an in vitro fertilisation (IVF) protocol (n=31) and subsequently became pregnant (n=6) the levels of hormones and anticoagulant proteins were determined. During IVF the levels of endogenous estradiol and progesterone increase within a short period of time.

During IVF treatment, no changes in protein S, protein C and protein C inhibitor, but an increase in APC resistance was observed. The estradiol levels correlated well with the APC sensitivity ratios. Moreover, the increase in APC resistance showed a significant correlation with the increase in 17ß-estradiol (r=0.7, p=0.001). In this study six women became pregnant following IVF. In these pregnant women a rapid increase in estradiol and progesterone was observed, concurrent with a rise in APCsr and a decrease in protein S. Although hormone levels during early pregnancy were similar to those during hyperstimulation (E2) and luteal support (P), the effects on APCsr and protein S were more pronounced. This might be caused by an up-regulation of hormone-receptors already early in pregnancy.

Despite considerable changes in estradiol and progesterone, the levels of hemostatic factors and the APCsr changed only minimally and less than observed during OC use. A possible explanation for this might be that synthetic hormones exhibit higher affinity for and have more pronounced effects on hormone receptors than natural hormones[9, 10].

Progesterone may counterbalance the prothrombotic effects of estrogen

Since lowering of the estrogen content in (second and third generation) OC led to an overall decrease in the risk for VTE as compared to first generation preparations, estrogen was held responsible for thrombosis associated with hormonal changes in women. However, since second and third generation OC preparations contain the same amount of ethinyl estradiol (30µg) this suggests that progestagens are, at least partially, responsible for the increased risk of VTE during use of third generation OC. Therefore we have compared the effects of desogestrel and levonorgestrel on the protein C pathway during the use of combined OC, containing ethinyl estradiol (EE) and progestagen, and progestagen-only preparations in women with and without the FV_{Leiden} mutation (Chapter 7). In both populations desogestrel-containing (DSG) combined preparations had more pronounced effects on the anticoagulant pathway than levonorgestrel-containing (LEV) OC. Effects of desogestrel-containing combined OC were more pronounced in carriers than in non-carriers of the FV_{Leiden} mutation. During the use of progestagen-only preparations reverse effects on anticoagulant proteins were found, which in general were more pronounced for levonorgestrel than for desogestrel. The findings presented in this

chapter can explain the increased risk for desogestrel users, especially in the case of carriership of the FV_{Leiden} mutation combined with OC use[11].

In a small study it was proposed that progestagens had a "compensating" effect on coagulation, since APC sensitivity ratios declined with increasing progestagen content in triphasic OC[12]. If progestagens indeed compensate the action of estrogens on hemostasis, it is appealing to reduce the level of ethinyl estradiol in oral contraceptives (so called sub-30 pills) and at the same time maintain or even increase the level of progestagen. The results described in this thesis relate to two different kinds of OC preparations, both containing 150µg progestagen (either levonorgestrel or desogestrel). It is interesting to investigate the influence on hemostasis of OC containing norgestimate, a progestagen that is third generation, but which is metabolised to levonorgestrel. Furthermore, it would be interesting to investigate the changes in haemostasis during the use of OC that contain the same amount of progestagen in combination with a lower EE content e.g. Mercilon® (20µg EE and 150µg DSG). However, also the newest sub-30 pills that contain gestodene (Meliane®, Minulet®) should be compared to their older counterparts that contain more EE and the same amount of gestodene (Femodeen® and Harmonet®, respectively).

In 1997 the Committee for Proprietary Medicinal Products (CPMP) of the European Medicines Evaluation Agency (EMEA) requested the oral contraceptive manufacturers to perform a study to evaluate the effects of seven monophasic oral contraceptives on the haemostatic system. In this study the effect of seven different combination preparations on a large number of haemostatic variables have been investigated[13]. Also a comparison of the effect of different progestagens (gestodene, DSG, LEV and NGM) and different concentrations of EE (20 vs 30 vs 50 µg EE) was made in this study. Although there are indications that this study has been completed, the detailed results of this investigation have as yet not been published.

Can changes of haemostatic parameters explain pill thrombosis?

We have shown in all our studies (cross-sectional, cross-over and longitudinal designs) that the use of combined oral contraceptives is associated with a decreased anticoagulant potency of the protein C pathway as reflected by an increased APC resistance. Moreover, the effects of oral contraceptives containing desogestrel are

more pronounced than levonorgestrel-containing OC. Our studies and other reports have indicated that the changes during OC use in levels of factor VII, factor V, prothrombin and protein S represent an important modulatory effect that in part can explain the observed difference in risk of venous thrombosis between users of second and third generation OC use. Our studies further indicate that individuals with FV_{Leiden} (who already have impaired APC-dependent down regulation of coagulation) become even more resistant to APC during OC use (especially when they use desogestrel-containing OC), and this can explain the high risk for venous thrombosis in carriers of the FV_{Leiden} mutation who use OC.

Since changes in haemostatic variables during OC use are relatively small and the plasma levels of coagulation factors in most women stay within normal ranges, the relevance of changes during OC use has been questioned. For a number of reasons we would like to challenge this interpretation.

Recent reports have indicated that elevated levels of prothrombin[14], factor VIII[15, 16], factor IX[17] factor XI[18] and TAFI[19] are independent risk factors for venous thrombosis. Even when the changes in levels of these proteins are relatively small, the risk of VTE is significantly increased. The effects of OC on the coagulation system are generally judged on the basis of mean changes of haemostatic parameters relative to the mean level of that parameter in the population. However, it is important to realise that, although a change in population average can stay within the normal range, such changes result in an increased number of individuals at the extreme of the normal population distribution.

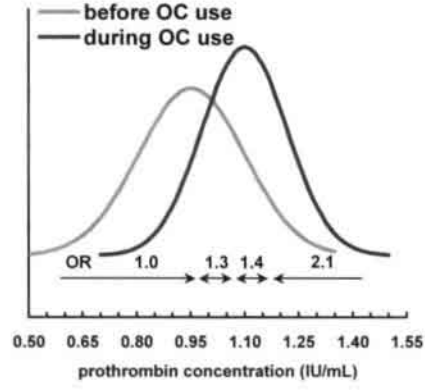


Figure 8.2 Normal distribution of the level of prothrombin before and during the use of OC. Odds ratio's of venous thrombosis reported for the level of prothrombin[12] are indicated in the figure along the x-axis.

Fig 8.2 illustrates this for the level of prothrombin before and during the use of OC. It has been shown that individuals with prothrombin levels >1.15 IU/mL have a 2.1-fold increased risk for developing VTE[14]. The normal distribution of levels of prothrombin, i.e. without OC, are indicated in light-gray. Of the population only 12% has a prothrombin level above 1.15 IU/mL. However, during the use of OC this curve is shifted to the right and the number of individuals exposed to such high levels is increased substantially to 42%. It is unfortunate that in the discussion with regard to the effects of OC use, this phenomenon, which will lead to an increased number of women at risk, is underscored.

In most women the changes in hemostatic parameters during OC use will result in a moderate disbalance, however, the few individuals that already have a mild prothrombotic condition before they use OC (*e.g.* elevated levels of factor II, VIII, IX, XI or low levels of protein S, factor V), may be put in extreme danger during the use of OC, because the concentration of coagulation factors will further change during use. In these women OC may further impair the coagulation system and aggrevate the prothrombotic condition, a situation that in a small number of pill users (7/10000 women years) may culminate in the development of venous thrombosis.

References

1. Tosetto A, Castaman G, Cappellari A, Rodeghiero F. The VITA Project: Heritability of resistance to activated protein C. Thromb and Haemost 2000; 84:811-814.
2. de Visser MC, Rosendaal FR, Bertina RM. A reduced sensitivity for activated protein C in the absence of factor V Leiden increases the risk of venous thrombosis. Blood 1999; 93:1271-6.
3. Rosing J, Tans G, Nicolaes GAF, et al. Oral contraceptives and venous thrombosis: Different sensitivities to activated protein C in women using second- and third-generation oral contraceptives. British Journal of Haematology 1997; 97:233-238.
4. de Ronde H, Bertina RM. Laboratory diagnosis of APC-resistance: a critical evaluation of the test and the development of diagnostic criteria. Thrombosis and Haemostasis 1994; 72:880-886.
5. van den Besselaar AM, Chantarangkul V, Tripodi A. A comparison of two sodium citrate concentrations in two evacuated blood collection systems for prothrombin time and ISI determination [In Process Citation]. Thromb Haemost 2000; 84:664-7.
6. Smirnov MD, Safa O, Esmon NL, Esmon CT. Inhibition of activated protein C anticoagulant activity by prothrombin. Blood 1999; 94:3839-3846.
7. Middeldorp S, Meijers JCM, van den End AE, et al. Effects on Coagulation of Levonorgestrel- and Desogestrel-containing Low Dose Oral Contraceptives: a Cross-over Study. Thrombosis and Haemostasis 2000; 84:4-8.
8. Meijers JCM, Middeldorp S, Tekelenburg W, et al. Increased Fibrinolytic Activity during Use of Oral Contraceptives Is Counteracted by an Enhanced Factor XI-independent down Regulation of Fibrinolysis: A Randomized Cross-over Study of Two Low-dose Oral Contraceptives. Thrombosis and Haemostasis 2000; 84:9-14.
9. Mashchak CA, Lobo RA, Dozono-Takano R, et al. Comparison of pharmacodynamic properties of various estrogen formulations. Am J Obstet Gynecol 1982; 144:511-8.
10. Gillmer MD. Progestogen potency in oral contraceptive pills. Am J Obstet Gynecol 1987; 157:1048-52.
11. Bloemenkamp KWM, Rosendaal FR, Helmerhorst FM, B□ller HR, Vandenbroucke JP. Enhancement by factor V Leiden mutation of risk of deep- vein thrombosis associated with oral contraceptives containing third- generation progestagen. Lancet 1995; 346:1593-1596.
12. Kluft C, de Maat MP, Heinemann LA, Spannagl M, Schramm W. Importance of levonorgestrel dose in oral contraceptives for effects on coagulation [letter]. Lancet 1999; 354:832-3.
13. OCHSG. An open label, randomized study to evaluate the effects of seven monophasic oral contraceptive regimens on hemostatic variables. Outline of the protocol. Oral Contraceptive and Hemostasis Study Group. Contraception 1999; 59:345-55.
14. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood 1996; 88:3698-703.
15. Kamphuisen PW, Lensen R, Houwing-Duistermaat JJ, et al. Heritability of elevated factor VIII antigen levels in factor V Leiden families with thrombophilia. Br J Haematol 2000; 109:519-22.
16. Kraaijenhagen RA, in't Anker PS, Koopman MM, et al. High plasma concentration of factor VIIIc is a major risk factor for venous thromboembolism [see comments]. Thromb Haemost 2000; 83:5-9.
17. van Hylckama Vlieg A, van der Linden IK, Bertina RM, Rosendaal FR. High levels of factor IX increase the risk of venous thrombosis. Blood 2000; 95:3678-82.
18. Meijers JC, Tekelenburg WL, Bouma BN, Bertina RM, Rosendaal FR. High levels of coagulation factor XI as a risk factor for venous thrombosis. N Engl J Med 2000; 342:696-701.
19. van Tilburg NH, Rosendaal FR, Bertina RM. Thrombin activatable fibrinolysis inhibitor and the risk for deep vein thrombosis. Blood 2000; 95:2855-9.

Nederlandse Samenvatting

Veneuze trombose is een ziekte die door meerdere faktoren wordt veroorzaakt. In de laatste jaren is een groot aantal genetische en verworven risicofaktoren, die een rol spelen bij het ontstaan van veneuze trombose, geïdentificeerd. Een van de belangrijkste risicofaktoren voor veneuze trombose is ontdekt in 1993 en heet APC resistentie. Geaktiveerd protein C (APC) is een enzyme dat ervoor zorgt, dat stolling van bloed tijdig geremd wordt, zodat er geen trombose optreedt. Maar in een groot aantal patiënten met veneuze trombose blijkt dat APC minder goed te werken (het plasma is APC resistent). APC resistentie kan op twee manieren worden veroorzaakt: door een genetische afwijking (erfelijk) en/of door omgevingsfaktoren die tijdelijk aanwezig zijn (verworven). Erfelijke APC resistentie is het gevolg van een puntmutatie in stolfaktor V. Het gemuteerde molekuul, FV_{Leiden} genaamd, mist een belangrijke knipplaats voor APC, waardoor de stollingsbevorderende werking van FV niet goed gereguleerd kan worden. Plasma van FV_{Leiden}-dragers is dus minder gevoelig voor APC (APC resistent). De oorzaken van verworven APC resistentie zijn minder eenduidig. Theoretisch kan verworven APC resistentie het gevolg zijn van een verhoogde neiging tot trombinevorming veroorzaakt door een verandering in niveau van een of meerdere stolfaktoren. Dit kan een gevolg zijn van 1) een verhoogde concentratie van stollingsbevorderende eiwitten (bijv. protrombine of faktor VII), 2) een verlaagde concentratie van remmers van de stolling (bijv. tissue faktor pathway inhibitor), 3) lage concentratie van antistollingseiwitten (bijv. protein S) en/of 4) een hoge concentratie van remmers van het antistollingssysteem (bijv. protein C inhibitor).

Het is daarom niet eenvoudig aan te geven welke hemostase testen de voorkeur verdienen voor het diagnostiseren van verworven APC resistentie. Eén algemene test, waarvan het resultaat een samengestelde meting van pro- en anticoagulante effecten (een "protrombotische toestand") weergeeft, zou daarom heel nuttig zijn. De in dit proefschrift beschreven APC resistentie test kan in een aantal gevallen als zo'n algemene test beschouwd worden. Deze test kwantificeert het effect van APC op de totale trombinevorming na aktivatie van bloedplasma via het extrinsieke stolsysteem.

In Deel I van het proefschrift zijn achtereenvolgens beschreven: de experimentele omstandigheden waaronder de in Maastricht ontwikkelde APC resistentiemeting dient te worden uitgevoerd (hoofdstuk 2); voor welke veneuze

trombose risicofaktoren (erfelijk danwel verworven) de meting gevoelig is (hoofdstuk 3); alsmede een vergelijking van de resultaten met de meer algemeen in gebruik zijnde methode, waarin APC resistentie bepaald wordt door meting van het effect van APC op de stoltijd (hoofdstuk 4). In deel II (hoofdstukken 5-7) wordt vervolgens nader ingegaan op de effecten van veranderingen in de vrouwelijke hormoon huishouding op stollingsparameters, die de werking van het zgn. "protein C pathway" bepalen.

Deel I Eigenschappen van de APC resistentie-test

In hoofdstuk 2 wordt de invloed beschreven van variatie in de concentratie van de verschillende "ingrediënten" van de test (calcium, fosfolipiden, tissue faktor en APC) op de trombinevorming. De remming van de trombinevorming door APC blijkt afhankelijk te zijn van de concentratie tissue faktor, calcium en fosfolipiden. Om reproduceerbare getallen te verkrijgen, moeten de concentraties van deze reactanten daarom worden gestandaardiseerd. De dag tot dag variatie die desondanks aanwezig blijft, kan worden geminimaliseerd door de APCsr (APC sensitivity ratio) van een bepaald plasmamonster te delen door de APCsr van normaal plasma. Het effect van variaties in plasmabereiding uit volbloed is ook bepaald. De geteste variabelen zijn 1) de concentratie van het antistollingsmiddel citraat, 2) de tijd voordat begonnen wordt met de opwerking; 3) centrifugatietijd en -temperatuur gebruikt bij verwijdering van de cellulaire fraktie uit het bloedplasma; 4) opslagtemperatuur van het plasmamonster en 5) een extra keer ontdooien alvorens te meten. Het blijkt dat eigenlijk alleen de concentratie van citraat de APCsr waarden beïnvloedt. Om een goede vergelijking te waarborgen is het dan ook van belang dat de APC resistentie meting uitgevoerd wordt met plasmamonsters die in gelijke concentratie citraat afgenomen zijn.

De resultaten in hoofdstuk 3 maken duidelijk dat de APC resistentietest, zoals beschreven in dit proefschrift, niet alleen gevoelig is voor de faktor V_{Leiden} mutatie, maar ook voor andere risicofaktoren voor veneuze trombose. Dit kunnen zowel erfelijke (de protrombine G20210A mutatie; protein S deficientie) als verworven (zwangerschap, pilgebruik, hormoonsubstitutie therapie) risicofaktoren zijn. Tevens blijken de effecten van de risicofaktoren op APC resistentie in veel gevallen additief te zijn, zodat bij het gelijktijdig voorkomen van risicofaktoren extra

hoge APCsr waarden gevonden worden. Het feit dat de gemeten APCsr waarden correleren met in de literatuur gerapporteerde risico's van trombose suggereert dat de door ons ontwikkelde meting klinisch relevante uitkomsten oplevert.

In het laatste hoofdstuk van deel I (hoofdstuk 4) zijn de resultaten vergeleken met een meer algemeen in gebruik zijnde APC resistentie test (de zgn. aPTT), waarin het effect van APC gemeten wordt op basis van stoltijdmetingen in plasma geïnitieerd via de intrinsieke weg . Beide testen zijn even specifiek en gevoelig voor de aanwezigheid van de FV_{Leiden} mutatie, maar afgezien hiervan blijken de uitkomsten van beide testen verder nauwelijks gecorreleerd. Dit wijst erop dat beide APC resistentie meting via deze testen door verschillende faktoren beïnvloedt word en dat deze verschillende effecten teweeg brengen in de beide APC resistentie testen.

Deel II Hormonale beïnvloeding van APC resistentie

Reeds vanaf de beginjaren '60 is het gebruik van orale contraceptie geassocieerd met een verhoogd risico op veneuze trombose. Echter, totdat in 1997 APC resistentie als mogelijk mechanisme werd voorgesteld, ontbrak een biologische verklaring. In dat jaar werd namelijk gerapporteerd, dat de -in Maastricht ontwikkelde- test liet zien dat pilgebruik niet alleen tot APC resistentie leidt maar ook dat het gebruik van de zgn. derde generatie (bv desogestrel bevattende) pil tot een duidelijk hogere resistentie leidt dan het gebruik van tweede generatie (levonorgestrel bevattende) preparaten. Aangezien deze eerste resultaten sterk bekritiseerd werden is een tweede studie opgezet waarin het pileffect gemeten werd in een dubbel-geblindeerd gerandomiseerd cross-over experiment met twee combinatiepreparaten (hoofdstuk 5). Deze studie liet zien dat een groot aantal hemostase variabelen veranderen tijdens pilgebruik (stollingsbevorderende, antistollende en fibrinolytische eiwitten). De veranderingen waren in het algemeen sterker tijdens het gebruik van desogestrel bevattende pillen dan tijdens het gebruik van contraceptiva met levonorgestrel. Tevens werd aangetoond dat de verworven APC resistentie meer uitgesproken was bij het gebruik van desogestrel-bevattende (derde generatie) preparaten dan bij de levonorgestrel-bevattende preparaten. Verschillen in de effecten van de twee pilpreparaten op de antistollende eiwitten werden alleen

gevonden voor protein S. Protein S niveaus daalden sterker tijdens desogestrel gebruik. Bovendien correleerde de daling in zowel totaal als vrij protein S negatief met de stijging in APCsr waarde (r= -0.44 / -0.60). Dit wijst erop, dat de stijging in APC resistentie voor een deel verklaard kan worden door de daling in protein S. Maar andere mechanismen moeten ook bijdragen aan de gestegen APC resistentie, want tijdens het gebruik van de levonorgestrel bevattende pil veranderde de protein S concentratie nauwelijks terwijl de APCsr significant steeg.

Niet alleen synthetische hormonen (zoals in de anticonceptiepil) zijn in staat het hemostatische evenwicht te veranderen. Ook zwangerschap, wat gepaard gaat met een forse toename van de endogene estradiol- en progesteronspiegels, leidt tot significante veranderingen in stollingsparameters(zie ook hoofdstuk 3). In hoofdstuk 6 zijn de veranderingen in het protein C systeem als gevolg van de veranderingen in endogeen estradiol en progesteron beschreven. De concentratie van hormonen en stollingseiwitten werden bepaald in plasma van vrouwen die een in vitro fertilisatie (IVF) protocol volgden (n=31) en vervolgens zwanger raakten (n=6). Tijdens een IVF-cyclus veranderen de spiegels van endogeen estradiol en progesteron aanzienlijk in een kort tijdsbestek. In deze studie vonden we geen of weinig verandering in protein S, protein C en protein C inhibitor, maar wel een (lichte) verhoging van de APC resistentie. Estradiol spiegels bleken te correleren met APCsr waarden en bovendien was de verandering in 17β-estradiol gecorreleerd met de verandering in APC resistentie. Zes vrouwen werden zwanger na de IVF behandeling. In deze vrouwen werden hoge estradiol en progesteron waarden gevonden gelijktijdig met een stijging van de APCsr waarden en een verlaging van protein S. De hormoonspiegels gemeten in de vroege zwangerschap kwamen overeen met die tijdens hyperstimulatie en luteaal support, maar de effecten op protein S en APC resistentie waren beduidend sterker. Dit duidt erop dat tijdens zwangerschap nog andere faktoren bijdragen aan de ontstane APC resistentie.

Gedurende lange tijd werd verondersteld dat estrogeen in belangrijke mate verantwoordelijk is voor het ontstaan van veneuze trombose (geassocieerd met de pil). Het feit dat tweede en derde generatie pillen hetzelfde synthetische estrogeen in dezelfde hoeveelheid (30µg ethinyl estradiol) bevatten, is echter een aanwijzing dat de progestageen-component tenminste deels verantwoordelijk is voor het verhoogde tromboserisico tijdens derde generatie pilgebruik. Hoofdstuk 7 schetst de resultaten

van een studie waarin de effecten van de progestagenen, desogestrel en levonorgestrel vergeleken zijn op stollingsparameters in vrouwen zonder en met de FV_{Leiden} mutatie (hoofdstuk 7). In zowel draagsters als niet-draagsters van de FV_{Leiden} mutatie had de desogestrel bevattende combinatiepil een duidelijker effect op het antistollingsysteem dan de levonorgestrel bevattende pil. De effecten van het desogestrel bevattende combinatiepreparaat waren groter in draagsters van de FV_{Leiden} mutatie dan in niet-draagsters. Echter de belangwekkendste bevinding was dat tijdens het gebruik van preparaten met alleen maar progestageen, effecten gevonden werden die tegengesteld waren aan de effecten waargenomen bij de combinatiepreparaten. Het lijkt er dus sterk op dat de progestagen-component in het combinatiepreparaat de (protrombotische) werking van de estrogeen-component tegengaat. En aangezien het effect van levonorgestrel alleen in het algemeen groter was dan het effect van desogestrel alleen, kunnen deze waarnemingen de sterkere protrombotische effecten van de derde generatie (desogestrel-bevattende) pil ten opzichte van de tweede generatie (levonorgestrel-bevattende) pil mogelijk verklaren.

Kunnen veranderingen in het niveau van stolfaktoren piltrombose verklaren?

Wij hebben in al onze studies (cross-sectionele, cross-over en longitudinale opzet) laten zien dat het gebruik van combinatiepreparaten geassocieerd is met een verminderde werking van het antistollende protein C systeem, weerspiegeld in een verhoogde APC resistentie. Bovendien waren de effecten op de hemostase duidelijker bij gebruik van desogestrel bevattende pillen dan bij gebruik van levonorgestrel bevattende pillen. Onze en andere studies hebben laten zien dat het gebruik van de pil de niveaus van stollingsparameters op een belangrijke manier beïnvloedt en dat de verschillen in risico voor veneuze trombose tussen tweede en derde generatie pilgebruik hierdoor, tenminste deels, verklaard kunnen worden. Tevens hebben we laten zien dat personen met de FV_{Leiden} mutatie (die reeds een verslechterde remming van de stolling hebben) resistenter tegen APC worden tijdens pilgebruik (zeker in tijdens gebruik van desogestrel bevattende preparaten), wat het hoge risico voor veneuze trombose in FV_{Leiden} draagsters die de pil gebruiken kan verklaren.

Omdat de gerapporteerde veranderingen tijdens pilgebruik bij de meeste vrouwen voor een groot gedeelte binnen het normale gebied blijven, is de relevantie van deze veranderingen vaak ter discussie gesteld. Echter, het wordt de laatste jaren meer en meer duidelijk dat ook kleine veranderingen kunnen bijdragen aan het risico op trombose. Vaak wordt namelijk vergeten dat een kleine gemiddelde verandering van een reeds ongustige waarde net voldoende kan zijn om tot trombose te leiden. Maar ook dient er rekening mee gehouden te worden dat niet in alle personen de gemiddelde verandering optreedt. Wanneer een (uiteraard minder vaak voorkomende) grote verandering ook nog eens voorkomt bij een persoon met een ongunstige beginwaarde kan dat dit wel degelijk leiden tot trombose.

Curriculum Vitae

De auteur van dit proefschrift is geboren op vrijdag 25 mei 1973 in het ziekenhuis te Boxmeer. Zij doorliep al kwebbelend de basisschool "de Springplank" en iets harder werkend het VWO op het Kruisheren Kollege te Uden.

In 1991 vertrok zij naar Maastricht alwaar aangevangen werd met de studie Gezondheidswetenschappen aan wat toen de Rijksuniversiteit Limburg heette. Na het kiezen van Biologische Gezondheidskunde (BGK) als afstudeerrichting, deed zij haar eerste organisatorische vaardigheden op door (met een tal actievelingen) een studievereniging voor BGK'ers op te richten (BGK-Helix).

De eerste praktische onderzoeksvaardigheden werden opgedaan bij Prof. J. Smits, Farmacologie (onderzoek naar de aldosteron-geïnduceerde veranderingen in bloeddruk bij ratten). Haar afstudeerstage over het groei- en sterfgedrag van vetzuur-gesupplementeerde endotheelcellen voltooide zij bij Dr. C.P.M. Reutelingsperger en Dr. J.W.M. Heemskerk, Biochemie, Universiteit Maastricht. En na een buitenlandse stage in het noordelijke IJsland bij Prof. Dr. Sigmundur Guðbjarnason in Reykjavík (onderzoek naar de verandering van de vetzuur samenstelling van het rattenhart na adrenerge stimulatie), ontving zij in augustus 1996 haar diploma.

Vanaf 1 oktober 1996 was Joyce Curvers werkzaam als assistent in opleiding bij de (vakgroep of) capaciteitsgroep Biochemie binnen de onderzoeksschool hart- en vaatziekten (CARIM) aan de Universiteit Maastricht, onder de bezielende leiding van Prof. Dr. J. Rosing en Dr. G. Tans, hetgeen heeft geleid tot dit proefschrift.

Momenteel profiteert zij van een stimulerende baan als wetenschappelijk medewerker van de afdeling onderzoek en ontwikkeling bij Sanquin Bloedvoorzieningen, Bloedbank divisie Limburg te Maastricht.

List of Publications

Research articles

1 V.E. Benediktsdottir, **J. Curvers** and S. Guðbjarnasson. Time course of alterations in phospholipid fatty acids and number of β-adrenoceptors in the rat heart during adrenergic stimulation *in vivo*. Journal of Molecular and Cellular Cardiology, 1999, 31, 1105-1115.

2 **J. Curvers**, M.C.L.G.D. Thomassen, G.A.F. Nicolaes, R. van Oerle, K. Hamulyák, H.C. Hemker, G. Tans and J. Rosing. Acquired APC resistance and oral contraceptives: Differences between two functional tests. British Journal of Haematology, 1999, 105, 88-94.

3 J. Rosing, S. Middeldorp, **J. Curvers**, M.C.L.G.D. Thomassen, G.A.F. Nicolaes, J.C.M. Meijers, B.N. Bouma, H.R. Büller, M.H. Prins and G. Tans. Low-dose oral conraceptives and acquired resistance to activated protein C: a randomised cross-over study. Lancet, 1999, 354, 2036-2040.

4 G. Tans, **J. Curvers**, S. Middeldorp, M.C.L.G.D. Thomassen, J.C.M. Meijers, M.H. Prins, B.N. Bouma, H.R. Büller and J. Rosing. A randomised cross-over study on the effects of levonorgestrel- and desogestrel-containing oral conraceptives on the anticoagulant pathways. Thrombosis and Haemostasis, 2000, 84 (1), 15-21.

5 **J. Curvers**, A.W. Nap, M.C.L.G.D. Thomassen, S.A. Nienhuis, K. Hamulyák, J.L.H. Evers, G. Tans and J. Rosing. Effect of In Vitro Fertilisation treatment and subsequent pregnancy on the protein C pathway. British Journal of Haematology, in press.

6 **J. Curvers**, M.C.L.G.D. Thomassen, H. de Ronde. R.M. Bertina, F.Rosendaal, G. Tans and J. Rosing. Influence of the (pre-)analytical variables in the ETP-based APC resistance test. *Submitted.*

7 **J. Curvers**, M.C.L.G.D. Thomassen, J.E. Rimmer, K. Hamulyák, J. van der Meer, G. Tans, E.F. Preston and J. Rosing. Effects of hereditary and acquired risk factors of venous thrombosis on a tissue factor-based APC resistance test. *Submitted.*

8 J.M. Kemmeren, A. Algra, B.N. Bouma, G. Tans, J.C.M. Meijers, **J. Curvers**, J. Rosing and D.E. Grobbee. Effect of second and third generation oral contraceptives on the protein C system in women with and without FV_{Leiden}. *Submitted.*

Review articles

1 J. Rosing, **J. Curvers** and G. Tans. Oral contraceptives, thrombosis and haemostasis. European Journal of Obstetrics, Gynecology and Reproductive Biology 2001, 95 (2), 193-197

2 **J. Curvers**, G. Tans and J. Rosing. Sex steroids and blood coagulation. Pantheon publishing, in press

Abstracts

XVIIth Congress on Thrombosis and Haemostasis, Washington DC Augustus 1999

1 **J. Curvers**, M.C.L.G.D. Thomassen, G. Tans and J. Rosing. Oral contraceptives and acquired activated protein C resistance. Thombosis and Haemostasis 1999, 82 (Suppl), 770.

2 M.C.L.G.D. Thomassen, **J.Curvers**, J.E. Rimmer, F.E. Preston, J.W.J. van Wersch, G. Tans and J. Rosing. Influence of hormone replacement therapy, oral contraceptives and pregnancy on APC resistance. Thrombosis and Haemostasis 1999, 82 (Suppl), 770-771.

3 J. Rosing, S. Middeldorp, **J. Curvers**, M.C.L.G.D. Thomassen, G.A.F. Nicolaes, J.C.M. Meijers, B.N. Bouma, H.R. Büller, M.H. Prins and G. Tans. Different effects of levonorgestrel- and desogestrel-containing oral contraceptives on thrombin generation in the presence of activated protein C. Thrombosis and Haemostasis 1999, 82 (Suppl), 204.

4 G.Tans, F.R. Rosendaal, **J. Curvers**, M.C.L.G.D. Thomassen, R. Bertina and J. Rosing. APC resistance determined with the endogenous thrombin potential is associated with venous thrombosis: a blinded clinical evaluation. Thrombosis and Haemostasis 1999, 82 (Suppl), 202-203.

Nederlands Gynaecologen congres, Papendal. November 1999

5 S.A. Nienhuis, **J. Curvers**, J. Rosing, K. Hamulyák, J.L.J. Evers. IVF en bloedstolling. Nederlands Tijdschrift voor Obstetrie en Gynaecologie 1999, 112, 33-34.

Derde Louis Schellekens Symposium, Den Haag. Juni 2000

6 **J. Curvers**, S.J. Nienhuis, A.W. Nap, K. Hamulyák, J.L.H. Evers, J. Rosing. APC resistance during IVF treatment. European Journal of Obstetrics and Gynecology and Reproductive Biology 2001, 95 (2), 222-224. *Award winning presentation.*

First North Sea Conference on Thrombosis and Haemostasis, Maastricht. Juni 2000

7 **J. Curvers**, M.C.L.G.D. Thomassen, S. Middeldorp, J.C.M. Meijers, M.H. Prins, B.N. Bouma, H.R. Büller, G. Tans and J. Rosing. Effects on second and third generation oral contraceptives on the anticoagulant pathways: A randomized cross-over study. Haemostasis 2000, 30, 94

XVIIIth Congress on Thrombosis and Haemostasis, Parijs. Juli 2001

8 J.M. Kemmeren, A. Algra, J.C.M. Meijers, G. Tans, B.N. Bouma, **J. Curvers**, J. Rosing and D.E. Grobbee. Third generation oral contraceptives, but not the respective progestagen, affect the protein C pathway in absence and presence of the FV_{Leiden}-mutation. Thrombosis and Haemostasis 2001, 86 (Suppl)

9 **J. Curvers**, M.C.L.G.D. Thomassen, J.E. Rimmer, K. Hamulyák, J. Van der Meer, G. Tans, F.E. Preston and J. Rosing. Effects of hereditary and acquired risk factors of venous thrombosis on APC resistance quantified by the influence of APC on the endogenous thrombin potential (ETP). Thrombosis and Haemostasis 2001, 86 (Suppl)

10 P. Clark, J. Rosing, **J. Curvers**, G. Tans, J. Conkie, I.D. Walker, I.A. Greer. The Glasogow outcome, APC resistance and lipid (GOAL) pregnancy study: Pregnancy associated APC resistance. Thrombosis and Haemostasis 2001, 86 (Suppl)

54th Congress of the American Association of Blood Banks, San Antonio Texas, Oktober 2001

11 **J. Curvers**, E. Rombout, P. Giesen, J.W.M. Heemskerk, E.C.M. van Pampus. Thrombin generation in thrombocyte-apheresis products. Transfusion 2002 (Suppl),

12 E. Rombout, **J. Curvers**, E.C.M. van Pampus, J.W.M. Heemskerk. The ability of platelets to become activated decreases during storage. Transfusion 2002 (Suppl)

43rd American Society of Hematology Annual Meeting, Orlando, December 2001

13 E.C.M. van Pampus, **J. Curvers**, E. Rombout, P. Giesen, J.W.M. Heemskerk. Reduction in activation capacity of platelet-rich apheresis products during storage is not accompanied by a reduction in thrombin forming capacity. Blood 2002 (Suppl)

Bedankt...

...en als je dan een bijna-af versie in je handen hebt, dan besef je eens te meer dat dit proefschrift tot stand kwam in ruim vier jaar. Vier jaar waarin ik met zoveel mensen heb samen gewerkt, gedacht, geprikt, geborreld. Al die mensen hebben direct en indirect een bijdrage geleverd aan dit "kindje" van mij (vruchtbare periode). Ik bedank er een hoop, maar ben er ongetwijfeld vergeten, ook diegenen bedankt!

Professor Rosing, voor velen Jan, voorzitter en eerste man van deze kernploeg.
Ik mocht voor jou komen werken en van jou leren wetenschappen, maar achteraf bleek dat vooral leren abstraheren. Het continue hoge niveau wat je nastreeft, de gedachte dat je alles voor de wetenschap geeft, siert je als wetenschapper. Je optimisme, als ik het niet meer zag zitten (minder frequent) en je kritiek als ik weer eens als een idealistiese jonge hond te kort door de bocht vloog. Teamwork, dat is het! De laatste twee jaar heb je het steeds drukker gekregen, maar het ijs bleef glad en de wakken ontwijkbaar. In Utrecht aten we de maatjes van Vishandel (piep). Dr. Tans, Guido, trainer/coach, de vruchtbare discussies, je opmerkingen en opvliegers, krities, maar ook serieus, je luisterend oor en adviezen, daar kon ik mee door. Jij was de eerste filter voor mijn artikelen, voordat ze bij Jan op zijn bureau afgedaan konden worden als "vreemde perikelen". Vaak gaf je een verhelderende kijk op experimenten, maar je wist ook eenieder van zijn stuk te kletsen. Als er een vreemd onderwerp opdook, wist jij erover te zwetsen.
Stella, mijn dank aan jou is ontzettend groot. Alle technieken en praktische vaardigheden legde jij voor mij bloot. Vanaf dag 1 pakte je me bij de hand (of liever duwde je me een pipet in de hand). Op het laatst ging het pipetteren aan de lopende band. Door jou leerde ik dat " *iech aon de weeg kon zeen of het kindj kak houw*", of *"dat diech een kindj houw"*. Achter het waterbad leerde ik ook over je muziekvoorkeur, ik genoot van de avondjes Jazz (of fusion), shagjes rollen tijdens tijdschema's, schilderkunst en Belgische gewoonten ("sorry, maar eigenlijk wilde ik morgen 3 weken met vakantie, kan dat?"), Hollandse gewoonten (regeltjes), onze gezamenlijke voorkeur voor "het vest", unbeschreiblich weiblich... en ik vergeef je dat je niet meer wilde prikken! We begonnen vier jaar geleden met 20 samples op één dag en eindigde met 800 samples in één week.
Ook José bedankt. Samen op glad ijs, voor onze komputer (hij deed het zelden) betaalde we een hoge prijs. Gelukkig was je over te halen voor een door-Jan-gevreesde "vrouwenlunch" waar we ge-empowerd van terug sjeesden. Gerry, je bent zelf vlug weggegaan toen ik kwam, maar jij hebt me (mede) weten te overtuigen om meer dan vier jaar in dit lab te staan. Het zinkende schip heeft koers gehouden en blijkt bestand tegen wat storm en nu ben je

terug... Tilman, is het al borreltijd? Jou veelzijdigheid heeft mij gestimuleerd; het lot van de jonge honden is bezweert! Dankzij het rariteiten-kabinet uit de stuffbak heb ik er een paar leuke kastjes bij (hoezo peptide synthese!). Kristien, je versterkt het Belgische front, de stille kracht, Protein S multimeren in plasma, dat staat!!! (Als het maar niet te vroeg is 's ochtends, trouwens, daar heb je aan Gerry ook een goede). Gaan we nog eens naar de film? Rory, als little drummer boy en smartlapartiest, de NVTH ziet je komen, jij bent degene die altijd voor eerlijkheid kiest. Lico, als Fries, goede muziek (van vóór de Kast), maar een beetje koppig, bedankt voor je thesis-komputer. Rob, mensen zijn niet te vervangen door Roboclots, je bent voor een minder spannende klus binnen gekomen, maar hebt nu het heft bij farmaco in eigen hand genomen. Wilbert, helaas, al veel te snel trok jij aan de commerciële bel.

Iedereen van de vakgroep Biochemie en biomaterialen dank voor jullie eeuwige vriendelijkheid en interesse. Het lab, als het ging om het inplannen van vrouwen voor het (tot in den treuren) verzamelen van de nieuwe pilpool en mijn geweldige bereikbaarheid. Het duurde helaas langer dan gepland en bijna raakten we eraan gewend (of toch niet).
Alle OBP. Aio's, WP, Oio's, stageo's en andere rare afkortingen (BV's?) van de vakgroep: BEDANKT, snel vergeten zal ik jullie niet! Met plezier heb ik jullie dagjes uit (mee-) georganiseerd.
Het was verhelderend jullie creativiteit, doorzettingsvermogen en eetlust te aanschouwen om over de dans en drank mijn kop maar te houden. Ik heb me heerlijk thuis gevoeld bij jullie.

Speciële haemostase; Karly, René, Carina, Carol, Mariël en Dave met raad en daad altijd paraat, dank. Ik heb geleerd van jullie expertise en hoop nog vaak jullie kennis en handen te kunnen "leasen".
Gynaecologie en Obstetrie; Hans, Saskia, Annemiek en het IVF-inplanningsteam (Germaine, Laurence en Cécile) als trouwe achterban voor het enthousiasmeren, van de bijzondere IVF-vrouwen. Zelfs voor meerdere keren!
Dank ook voor alle coauteurs uit Utrecht, Amsterdam, Groningen, gelukkig is e-mail sneller dan de trein!
Thanks to the foreign colleagues (Janet Rimmer, Eric Preston, Peter Clark and Isobel Walker) for the excellent collaborations. We often had problems with planning because of the amount of samples that kept arriving. But it always was exciting to interpret the results, although the articles are much delayed. I hope to keep in touch and improve my knowledge in Scottish (whiskey)!

The foreign medical students that have gained some lab-skills and helped reducing the workload, Signe and Lise, I wonder why both of you came from Scandinavia and wanted to work on FV-Leiden?
Elisabetta, we have never worked together, but I think by this time you are like furniture to the lab. You are a wonderful person and I regret that we have not yet thought of a joint experiment (FVII-deficient pill users?).
Natuurlijk verdienen de vele, vele vrouwen (en die enkele man) die ik heb mogen prikken voor het onderzoek een extra reep chocola of bioscoopbon; bedankt!

Speciale dank voor vrienden (en ook nog collega's) Jacco, Irene, Lavienja, Cyriel, Paul en Harold voor de late avondjes waarop de gesprekken hun ware diepgang kregen, maar snel ook weer verloren. Ook voor de niet-biochemie gewijde vriend(inn)en, BGK-vriend(inn)en, Uden-vriend(inn)en, Noord-Holland (HHW)-vriend(inn)en en natuurlijk familie (zowel warm als koud) die voor mij heel belangrijk bleven. Het trombotische FV_{Leiden}-karakter van de familie Curfs heeft gezorgd voor een extra-stukje stimulans. En natuurlijk Dave, wij konden menig avondje achter meerdere pilsjes de wetenschap virtueel op zijn kop zetten, ons leven nog spannender maken, zeker in de ogen (of het hoofd) van de biochemisten. Alles is rech gekomme.
Bedankt dat jij en ook Leon mijn paranimfen willen zijn.

José en Cor, dank jullie voor de interesse, de winderige duinwandelingen, het trotse-oudergevoel en heerlijke discussies.
Pap en mam, dankjulliewel. Jullie vertrouwen, de onaflatende interesse, het trots zijn, me los laten en jullie warmte hebben mij altijd gestimuleerd. Helaas pap, hebben wetenschap en theologie elkaar hier niet kunnen ontmoeten. Thim en Karin, nog steeds ligt Maastricht niet dichter bij Brabant (ze bouwen richting het zuiden?!), maar voor *dit* boekje was het, dat ik voor *zo weinig* geld, *zo ver* weg, *zo hard* werken wilde.

Spike, twee wetenschappers op één kussen, daar past nix tussen. Resistance is futile, but the force is with us. Ik heb je thuis vaak genoeg verveeld met mijn beroepsdeformaties. Jij wist me, soms vaker dan mij lief leek, af te leiden met kleine klusjes (?!), zodat ik met frisse moed en nieuwe ideeën -wel weer wat later- aan de slag kon.
Deze is voor jou want mi gustas tu!