Effects of combined oral contraceptives on hemostasis and biochemical risk indicators for venous thromboembolism and atherothrombosis

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MD

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Because enavid may produce uncontrollable vomiting and provoke pulmonary embolism and infarction, I would counsel caution in the use of this widely advertised drug.

WM Jordan. Lancet 1961

To my family
Abstract

Combined oral contraceptives (COCs) are one of the most common contraceptive methods in the world. Already a few years after their introduction, in the early 1960s, case reports were published showing associations between use of COCs and venous and arterial thromboembolism. Epidemiological studies have confirmed increased risk for these unwanted side effects, but the mechanisms behind the increase are still not fully evaluated.

The overarching aim of this thesis was to identify alterations in hemostasis as well as in established and novel risk markers for venous thromboembolism and atherothrombotic disease during treatment with COCs containing different types and doses of progestogen and estrogen.

In a prospective randomized cross-over study (papers I-III), two different monophasic low-dose COCs (one second-generation COC and one third-generation COC) were used for two two-month periods with a two-month wash-out period in between. Changes in hemostatic factors, lipoproteins and other risk indicators for atherothrombosis were examined before and during treatments. Changes in hemostatic factors occurred during both treatments towards a more hypercoagulable state, and were more pronounced with the third-generation COC than with the second. The observed alterations in serum cholesterol were more advantageous in women using third-generation COCs, but conversely the C-reactive protein (CRP) levels were enhanced more than during use of the second-generation COC. Other inflammatory markers investigated did not indicate occurrence of a general inflammatory response during use of COCs. A positive correlation between circulating levels of coagulation factor VII and triglycerides in COC users was also observed, with higher concentrations of both during use of third-generation COCs. In addition, users of COCs showed a significantly reduced sensitivity to the anticoagulant effect of activated protein C (APC), a well established risk factor for venous thrombosis, and this effect strongly correlated with changes in the circulating levels of sex-hormone binding globulin (SHBG). In study two (paper IV), 11 women used two kinds of emergency contraceptives (EC), one containing progestogen alone and one containing both progestogen and estrogen. Biochemical risk indicators were examined before and at frequent intervals after treatment. Already two hours after treatment, the plasma concentrations of some coagulation parameters differ from baseline concentrations, regardless of treatment. Increases in plasma concentrations of fibrinogen and prothrombin fragment 1+2 and decrease in antithrombin were observed. In the last study (paper V), APC resistance, measured by two methods with different sensitivities towards sex hormones, was evaluated in the luteal and follicular phases of the menstrual cycle. No differences in APC resistance between follicular and luteal phase were observed, even though the serum estrogen levels increased 200%.

In conclusion, although third generation COCs were developed to reduce the negative effects on lipoproteins and the associated risk of atherothrombosis with older COC preparations, their use may affect other risk indicators in a less advantageous direction. The raised serum CRP concentration during treatment with COCs appears to be related to a direct effect on hepatocyte CRP synthesis. SHBG is a potential surrogate marker for the prothrombotic risk state induced by different OC preparations. Even a very short exposure to rather high levels of exogenous sex hormones causes a prompt effect on hepatic protein synthesis and induces a rapid activation of hemostasis. However, the physiological increase in estradiol during the normal menstrual cycle is not large enough to affect the individual’s sensitivity to APC.

Key words: Combined oral contraceptives, emergency contraception, menstrual cycle, ethinylestradiol, levonorgestrel, desogestrel, lipoproteins, hemostasis, risk indicators, APC resistance, SHBG
Content

List of papers...................................................................................................................... 9
Abbreviations...................................................................................................................... 11
Introduction.............................................................................................................................. 13
   Sex hormones in fertile women ...................................................................................... 14
      Menstrual cycle ........................................................................................................... 14
      Estrogen ..................................................................................................................... 16
      Progesterone .............................................................................................................. 16
   Combined oral contraceptives ....................................................................................... 17
      Hormonal emergency contraception ........................................................................ 18
      Progestogen-only contraception .............................................................................. 18
Hemostasis ............................................................................................................................ 19
   The coagulation process ............................................................................................... 20
   Natural inhibition of blood coagulation ..................................................................... 23
   Fibrinolysis .................................................................................................................. 24
      Inhibitors of fibrinolysis ......................................................................................... 26
Risk indicators for venous thromboembolism ............................................................... 27
Risk indicators for atherothrombosis .............................................................................. 28
   Lipoproteins ................................................................................................................ 28
   Inflammation .............................................................................................................. 29
   Glucose metabolism ................................................................................................. 30
   Endothelial activation ............................................................................................... 31
Influence of COCs on hemostatic parameters and risk indicators for venous thromboembolism and atherothrombosis ................................................................................................. 31
   Hemostasis ................................................................................................................ 31
   Liver function ............................................................................................................. 32
   Lipoproteins .............................................................................................................. 33
   Glucose metabolism ................................................................................................. 33
   Endothelial activation ............................................................................................... 34
Risk of venous thromboembolism and cardiovascular disease in fertile women ................................................................................................................................. 34
Aims of the study............................................................................................................... 35
Subjects and methods......................................................................................... 39
  Subjects........................................................................................................ 39
  Treatments.................................................................................................... 40
  Study designs............................................................................................... 40
  Blood sampling........................................................................................... 41
  Laboratory methods .................................................................................... 42
    Hemostatic factors.................................................................................... 42
    Genotyping FV......................................................................................... 43
    Lipoproteins.............................................................................................. 43
    Inflammatory parameters.......................................................................... 43
    Markers of glucose metabolism............................................................. 43
    SHBG........................................................................................................ 43
    E-selectin................................................................................................. 43
  Statistical methods...................................................................................... 44

Results .............................................................................................................. 45
  Different effects of oral contraceptives containing levonorgestrel or desogestrel on plasma lipoproteins and coagulation factor VII (paper I)........................................................................ 45
  Sex hormone binding globulin – A surrogate marker for the prothrombotic effects of combined oral contraceptives (paper II).................................................................................................................. 46
  Treatment with combined oral contraceptives induces a rise in serum C-reactive protein in the absence of a general inflammatory response (paper III) .................................................................... 48
  Rapid activation of hemostasis after hormonal emergency contraception (paper IV).......................................................................................................................... 49
  APC resistance during the normal menstrual cycle (paper V)................... 52

General discussion............................................................................................ 53
  Effects on hemostasis and thromboembolic risk indicators...................... 53
  Effects on risk indicators for atherothrombosis......................................... 58
  COCs in the perspective of venous and arterial occlusive disease............ 60

Methodological considerations and future perspectives............................ 63
  Future perspectives....................................................................................... 64

Conclusions ...................................................................................................... 67

Acknowledgements.......................................................................................... 69

References....................................................................................................... 73

Papers I-V
List of papers


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Activated protein C</td>
</tr>
<tr>
<td>APCr</td>
<td>APC ratio</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>aPTT</td>
<td>Activated partial thromboplastin time</td>
</tr>
<tr>
<td>AT</td>
<td>Antithrombin</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>C4BP</td>
<td>C4b-binding protein</td>
</tr>
<tr>
<td>Ca++</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>COC</td>
<td>Combined oral contraceptive</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DSG</td>
<td>Desogestrel</td>
</tr>
<tr>
<td>E2</td>
<td>Estradiol</td>
</tr>
<tr>
<td>EC</td>
<td>Emergency contraception</td>
</tr>
<tr>
<td>EE</td>
<td>Ethinyl estradiol</td>
</tr>
<tr>
<td>EE-EC</td>
<td>Combined emergency contraception; EE and LNG</td>
</tr>
<tr>
<td>EPCR</td>
<td>Endothelial protein C receptor</td>
</tr>
<tr>
<td>ESHRE</td>
<td>European Society of Human Reproduction and Embryology</td>
</tr>
<tr>
<td>ETP</td>
<td>Endogenous thrombin potential</td>
</tr>
<tr>
<td>F 1+2</td>
<td>Prothrombin fragment 1+2</td>
</tr>
<tr>
<td>FDP</td>
<td>Fibrinogen degradation products</td>
</tr>
<tr>
<td>FV, FVII</td>
<td>Coagulation factor V, coagulation factor VII etc</td>
</tr>
<tr>
<td>FVa, FVIIa</td>
<td>The activated forms of FV, FVII etc</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormonal replacement therapy</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoprotein</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin-like growth factor I</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IS</td>
<td>Ischemic stroke</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LNG</td>
<td>Levonorgestrel</td>
</tr>
<tr>
<td>LNG-EC</td>
<td>Emergency contraception containing levonorgestrel alone</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>nAPCsr</td>
<td>Normalized APC sensitivity ratio</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidized LDL</td>
</tr>
<tr>
<td>PAI</td>
<td>Plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PAP</td>
<td>Plasmin – antiplasmin complex</td>
</tr>
<tr>
<td>PCI</td>
<td>Protein C inhibitor</td>
</tr>
<tr>
<td>POP</td>
<td>Progestogen-only pills</td>
</tr>
<tr>
<td>sE-sel</td>
<td>Soluble E-selectin</td>
</tr>
<tr>
<td>SAA</td>
<td>Serum amyloid A</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex hormone binding globulin</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TAFI</td>
<td>Thrombin activatable fibrinolysis inhibitor</td>
</tr>
<tr>
<td>TAT</td>
<td>Thrombin – antithrombin complex</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>TM</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>t-PA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>u-PA</td>
<td>Urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>VTE</td>
<td>Venous thromboembolism</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
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</table>
Introduction

It is now 50 years since the first clinical trial of combined oral contraceptives (COCs) took place in Puerto Rico (Pincus et al 1959). Since then, at least half a billion women have used “the pill” to protect themselves from unwanted pregnancies (Odlind et al 2002). In Sweden nearly 90% of all women will at some time during their fertile period have used a COC (Oddens & Milsom 1996, Larsson et al 1997).

COCs constitute a combination of two steroid hormones, estrogen and progestogen, given daily for three of four weeks. The mechanism of action is related to both hormones. Progestogen acts by suppressing luteinizing hormone (LH) and estrogen by suppressing follicle stimulating hormone (FSH), and in both cases the end effect is to inhibit ovulation. In addition, progestogen makes the endometrium thin, atrophic and non-receptive to ovum implantation, reduces the motility of the fallopian tubes, and makes the cervical mucus thick and impermeable to sperms. The estrogen component provides stability to the endometrium so that irregular bleeding is minimized, and the presence of estrogen potentiates the action of the progestogen.

Numerous studies over the years have improved our knowledge about advantages as well as risks for users of “the pill”. Beneficial effects of course include the good contraceptive effect but also reduced risk of endometrial and ovarian cancers, less anemia and dysmenorrhea. Serious but rare side effects of COC include venous thromboembolism (VTE), myocardial infarction (MI) and stroke (Sherif 1999).

Case reports on VTE have been published since the early 1960s (Jordan 1961, Boyce et al 1963) and since the late 1960s studies showing an association between the dose of estrogen (i.e. ethinyl estradiol, EE) and the risk of VTE have appeared in the literature (Böttiger et al 1980, Rosendaal et al 2003). The estrogen dose of the COCs has subsequently been decreased to reduce the risk of thromboembolic events, but treatment with modern, so-called, low-dose COCs (containing less than 50 µg EE/day) still increases the risk of VTE approximately two to four times (Jick et al 1995, Middeldorp 2005). In parallel with the reduction of the EE content, new progestins were developed. In the mid 1980s, the so-called third-generation
progestogens (desogestrel or gestodene) were introduced. They were developed to reduce some of the negative metabolic effects of the more androgen-like so-called second-generation progestogen (i.e. levonorgestrel).

In 1995, three independent epidemiological studies reported differences in the risk of VTE among users of COCs with the same amount of EE but different types of progestogen (WHO 1995, Jick et al 1995, Spitzer et al 1996). The risk for users of second-generation COCs was estimated to 2/10 000 and the risk for users of third-generation COCs to 3-4/10 000 compared with 1/10 000 for non-users.

In the late 1990s, the debate focused on possible differences in the risk of VTE between different types of preparations. The earlier suggestion that estrogen alone is responsible for the increased risk of VTE among pill users was questioned (WHO 1995, Jick et al 1995, Spitzer et al 1996, Herings et al 1999).

It is not only the differences in the risk of venous thromboembolic disease that have been under debate. The risk of arterial occlusive disease has also been discussed. A recent meta-analysis suggests that low-dose COCs increase the risk of both cardiac and vascular events (Baillargeon et al 2005). Some studies have made direct comparisons between third- and second-generation COCs regarding their effects on risk of MI (Tanis et al 2001, WHO 1997) or stroke (Kemmeren et al 2002, Lidegaard & Kreiner 2002). However, the results of these studies are not conclusive.

In the past decade much research effort has focused on the mechanisms behind the risk of VTE and arterial cardiovascular disease in relation to use of COCs. Since most users of COCs are healthy young women taking the compounds not to cure a life-threatening disease but to prevent pregnancies, it is of great importance to make the pills as safe as possible.

**Sex hormones in fertile women**

**Menstrual cycle**

The menstrual cycle is divided into three different phases – the follicular phase, ovulation and the luteal phase. Hormones in the hypothalamus, the pituitary gland and the ovaries are all involved in the delicate regulation of this cyclic pattern. Their interactions are regulated by positive and negative feed-back systems, which are not discussed here. LH and FSH show cyclic variations during the menstrual cycle (figure 1).
During the follicular phase, sequential hormonal changes take place to make the follicle(s) ready for ovulation. In the early follicular phase, FSH is secreted in relatively large amounts, whereafter secretion slowly decreases until approximately 48 hours before ovulation. Plasma concentrations of FSH are highest 36 to 48 hours before ovulation. The plasma levels of LH are relatively constant during the menstrual cycle, except for the “LH peak” occurring 8-40 hours prior to ovulation.

The levels of estrogen and progesterone during the menstrual cycle are shown in figure 1. They are low during menstruation and early follicular phase, and increase prior to ovulation to a “pre-ovulatory peak”. After the ovulation the plasma estrogen concentration decreases transiently before it reaches a plateau during the follicular phase. In contrast, progestogen reacts with a slight increase at the LH peak, and thereafter reaches its highest levels during the luteal phase (figure 1).

<table>
<thead>
<tr>
<th>FSH/LH IU/l</th>
<th>Estradiol pmol/l</th>
<th>Progesterone nmo/l</th>
</tr>
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<tbody>
<tr>
<td>52</td>
<td>65</td>
<td></td>
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<tr>
<td>48</td>
<td>2400</td>
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</tr>
<tr>
<td>44</td>
<td></td>
<td>55</td>
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<tr>
<td>40</td>
<td>2000</td>
<td>50</td>
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<tr>
<td>36</td>
<td></td>
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<td>32</td>
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<td>8</td>
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<td>5</td>
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<tr>
<td>0</td>
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</table>

**Figure 1. The menstrual cycle.**
**Estrogen**

During the fertile period of a woman’s life, almost all biologically active estrogen, the 17β-estradiol (E2), is secreted from the granulosa cells in the ovary. After the follicle ruptures at ovulation, the corpus luteum secretes progesterone, but significant amounts of estrogen are released as well. The plasma levels of E2 vary during the menstrual cycle, with the lowest levels occurring in the early follicular phase and the highest levels at the time of ovulation. During the luteal phase, plasma concentrations are significantly higher than during the follicular phase (see figure 1). Ethinyl estradiol (EE) is a synthetic estrogen commonly used in COCs in doses of 20 to 50 µg. The ethinyl group on carbon 17 renders this estrogen a higher receptor affinity, longer duration and implies a change in biological transport. Thus, EE is to be regarded as a clearly more potent estrogen than E2.

In blood, the majority of estrogen is bound to a carrier protein, sex hormone-binding globulin (SHBG), produced in the liver. Another 10-30% is bound to albumin, and only about 1% remains free. Estrogen induces synthesis of SHBG, whereas progestins decrease the plasma SHBG concentration. The biological effects of estrogen and steroid hormones in general are largely exerted by the free fraction of the hormone.

**Progesterone**

Progesterone is produced and secreted by the corpus luteum in the ovaries of menstrual women. Small amounts of progesterone are also secreted by the adrenals. The synthesis and plasma level of progestogen also fluctuates during the normal menstrual cycle, with low levels before ovulation and the highest levels attained in the luteal phase. The main transport protein for progestogen is corticosteroid-binding globulin (CBG), and the free circulating fraction of progestogen only amounts to 2-4%. A rapid metabolism and a poor resorption have hampered the use of natural progesterone in COCs and instead numerous synthetic progestogens (gestagens, progestogens, progestins) have been developed. There are two major classes of progestogens, derived either from 19-nortestosterone or from 17-α-hydroxyprogesterone. In COCs, most frequently derivates from 19-nortestosterone are used, for example levonorgestrel (LNG) and desogestrel (DSG).
**Combined oral contraceptives**

COCs contain a combination of estrogen and progestogen. The combination pills prevent ovulation by inhibiting gonadotropin secretion via effects on both pituitary and hypothalamic centers. The progestogen component primarily suppresses LH secretion and thus prevents ovulation, and the estrogen component suppresses FSH and thus prevents the selection and emergence of the dominant follicle. Therefore, the estrogen component significantly contributes to the contraceptive efficacy.

The estrogen component in all COCs is EE while the progestogen component varies between different preparations. In the 1970s the so-called second-generation progestogens were introduced in contraceptives. These progestogens have slightly androgenic properties and counteract in many ways the biological effects of EE. LNG is the most frequently used second-generation progestogen. COCs with a combination of EE and LNG are analogously named second-generation COCs.

Following the introduction of COCs an evolution has taken place regarding doses and progestogen types. Since many of the unwanted side effects are dose-dependent, dosages have been decreased. Initially the daily EE dose was 100-150 µg or more, and the treatment regimen was one tablet daily for three weeks followed by a “pill-free” week. In the 70s the low-dose COCs were introduced, with a daily dose of 30 µg. Currently the daily EE dose is 30 to 35 µg in the commonly used preparations, although in newer preparations doses as low as 20 µg EE daily are used. Concerns about unwanted, androgenic, side-effects on e.g. the lipoprotein pattern and the association to atherothrombosis during use of second-generation COCs stimulated development of alternative dosage regimens and new progestogenic substances. The phasic preparations, where the progestogen doses vary during the cycle, reduce the total hormone dose during the treatment period. In the 1980s a new type of progestogens was developed for use in COCs; the so-called third-generation progestogen, i.e. desogestrel (DSG) and gestodene. These progestogens have a reduced androgenicity and less anti-estrogenic effects on liver metabolism. The third- as well as the second-generation progestogens are derived from 19-nortestosterone.

During recent years, new forms of administration have been developed including combined hormonal contraception such as vaginal rings and patches, and there is a trend toward extended use (i.e. use of the contraceptives for three or sex months without “pill-free” intervals in between). Still, the monophasic “low-dose” COCs are the most frequently used combined contraceptive method in Sweden.
Hormonal emergency contraception

Hormonal emergency contraception (EC) with a combination of estrogen and progestogen was first introduced in the 1970s. In the original method, known as Yuzpe’s method, a combination of 100 µg EE and 1 mg LNG was administered twice with an interval of twelve hours (Yuzpe & Lancee 1977). During the 1990s LNG alone was shown to be more effective and have fewer side effects than the Yuzpe regimen (Task Force 1998). Therefore, the recommended regimen today is 750 µg LNG given twice with an interval of twelve hours or 1.5 mg administered as a single dose. Irrespective of method, treatment has to be started within 72 hours after unprotected intercourse.

EC is freely available in many countries and given to all women who request it, without any medical investigation. There are no restrictions for women with risk of deep vein thrombosis, even though the effects of EC on the coagulation system remain to be fully evaluated. There is, as far as we know, only one published report on VTE in relation to EC – a case report on retinal vein thrombosis occurring the day after use of 1000 µg norgestrel and 100 µg EE twice within 12 hours (Lake & Vernon 1999). Conversely in a large American cohort study including 73,302 EC users without cardiovascular risk factors no case of VTE was reported (Vasilakis et al 1999a).

Progestogen-only contraception

Progestogen-only pills (POPs), sometimes called ”minipills”, were first licensed in 1973. They contain no estrogen and usually have a lower dose of progestogen than that in combined pills. In the last decades other progestogen-only contraceptives, e.g. intrauterine devices and implants, have been developed. Treatment with progestogen-only contraceptives causes only minor effects on coagulation and fibrinolysis (Kuhl 1996), and is not associated with thrombotic complications (Vasilakis et al 1999b). They seem to be a good contraceptive method for women with increased risk of VTE (Conard et al 2004). Data on CVD with the use of progestogen-only contraception are limited but to date, no increased risk has been demonstrated (ESHRE 2006).
Hemostasis

The hemostatic system counteracts loss of blood and disturbances of blood flow and participates in the repair of injured vessels. Hemostasis is also involved in revascularization and formation of new connective tissue. There are many important participants in hemostasis such as the vessel wall, platelets and leukocytes, coagulation factors and their inhibitors, fibrinolytic factors and their inhibitors, calcium ions (Ca$^{++}$) and phospholipids as well as the blood flow itself (Butenas & Mann 2002, Norris 2003). Most of the hemostatic interactions occur on surfaces, i.e. on activated platelets, on endothelial cells or on exposed collagen in the vessel wall (Mann 1999).

Blood contains a number of proteins, among them the coagulation factors and their inhibitors. The coagulation factors are either vitamin K-dependent (Factors II, VII, IX and X) or thrombin-dependent (fibrinogen, Factors V, VIII, XIII and XI). All of them are synthesized in the liver, except factor V (FV) (platelets) and factor VIII (FVIII) (endothelium). The coagulation proteins are involved in tightly controlled sequences of interactions resulting in the formation of thrombin and subsequently fibrin. Coagulation factors circulate in blood in an inactive form, as precursors (Butenas & Mann 2002). Coagulation begins with a series of events ultimately leading to clot formation; the activation process comprises a sequence of proteolytic cleavages at very specific sites on the precursor proteins. The activation causes conformal changes of the protein, and thus exposes the enzymatically active sites. The surface of the protein contain binding sites for phospholipids and other specific receptors or cofactors located on various cell surfaces (Norris 2003). In some situations, the precursor proteins can be activated not only by coagulation factors but also by other proteases, for example enzymes from tumor cells, microorganisms and leukocytes or proteins released from surfaces that have become exposed due to vessel damage.

Blood coagulation has traditionally been described as a cascade of distinct enzymatic reactions where proteolytic activation of one proenzyme leads to activation of the next proenzyme in the cascade (Mann 1999). The cascade model involves two somewhat independent pathways that converge to a final common pathway with thrombin generation as the endpoint (figure 3). This model is based on studies in vitro and can most accurately be described as a laboratory model. In the past few years, another model mirroring in vivo coagulation has evolved. This modern, cell-based model of coagulation is an improvement over the old model in that it includes the interactions between cellular and plasma factors.
The coagulation process

In the cell-based model the coagulation process can be divided into three distinct but overlapping steps, the initiation, the amplification and the propagation phases (Monroe & Hoffman 2006) (figure 2).

The initiation of blood coagulation

When hemostasis is initiated, by vascular damage, an immediate vaso-constriction occurs. This rapid process is effective enough to arrest bleeding from small vessels but cannot stop bleeding from larger blood vessels. During the initiation phase of hemostasis, the damage in the vessel wall brings plasma into contact with cells bearing tissue factor (TF). TF is a protein mainly found in subendothelial tissue, but it has been detected in almost all tissues in the body (Østerud & Björklid 2006). Monocytes/macrophages in an atherosclerotic plaque can express TF after stimulation. After an injury or after stimulation by inflammatory mediators, such as interleukins, TF can be released or synthesized. Circulating coagulation factor VII (FVII) binds to TF and is rapidly activated by coagulation proteases or non-coagulation proteases, depending on the cellular source of TF. A small amount of activated FVII (FVIIa) also circulates in normal plasma and is considered to have a priming function for initiating the coagulation process (Morrissey et al 1993). The FVIIa/TF complex, together with the co-factors Ca^{++} and phospholipids, cleaves inactive FX into the proteolytically active FXa (FXa) and cleaves FIX into FIXa. The activated forms of these two proteins play distinct roles in the subsequent coagulation reactions. FXa activates FV on the TF-bearing cells, and together the two activated proteins produce small amounts of thrombin. Depending on the strength of the stimuli, the initiating process will either proceed or be stopped through the action of the tissue factor pathway inhibitor (TFPI).

Figure 2. Cell-based model of coagulation.
Reproduced with permission from the authors [Monroe et al 2002]. In this scheme coagulation occurs in three phases: initiation, amplification and propagation. In the initiation (A) FVIIa bound to TF activates FIX and FX. Factor Xa then activates FV on the TF-bearing cell, complexes with FVa and converts a small amount of FII to FIIa. In the amplification phase (B), the small amount of initial FIIa activates platelets, causing release of α-granulae contents including FV, activates FV, activates FXI and activates FVIII by cleaving it from vWf. Cofactors bind to the platelet surface before their respective enzymes. The FVIIa/TF complex is shut down through the action of the TFPI in complex with FXa. In the propagation phase (C), FXIa generated by FVIIa/TF binds to the activated platelets and subsequently activates FX. This factor IXa is supplemented by FIXa generated on the platelet surface by factor XIa. FXa then moves directly into a protected complex with FVa resulting in a burst of thrombin generation.
Introduction
The amplification of blood coagulation

The small amounts of thrombin initially generated on the surface of the TF-bearing cells can activate circulating platelets and platelets adhering to the site of injury. Platelet adhesion to the extracellular matrix of the subendothelial surface on the endothelium is mediated by von Willebrand factor (vWf) which acts like a glue between platelets and collagen. Activated platelets express procoagulant-specific platelet receptors that localize both enzymes and cofactors (mainly negatively charged phospholipids) responsible for the amplification of the coagulation on the surface of the platelet. The main focus of coagulation now moves from TF-bearing cells to the platelets. Thrombin activation of the platelets also results in degranulation and release of FV from the platelets. Thrombin activates FVIII, releasing it from vWf. In addition, thrombin activates FV and also FXI bound to platelet surfaces.

The propagation of blood coagulation

In this last phase of the coagulation process highly effective enzyme complexes are formed on the platelet surface. The so-called tenase (Xase) complex (FIXa/FVIIIa) activates FX and the prothrombinase (FXa/FVa) complex activates prothrombin (FII) to thrombin. The cleavage of prothrombin into thrombin occurs via a number of steps; one of them results in the active enzyme α-thrombin and prothrombin fragment 1+2 (F1+2). As circulating thrombin is difficult to measure, the more stable F1+2 is frequently used as a marker of prothrombin–thrombin conversion. With thrombin generated, fibrinogen is converted to fibrin monomer by splitting two small fibrinopeptides from the N-terminal end of the fibrinogen molecule (fibrinopeptides A and B). Fibrin monomers then form fibrin oligomers and subsequently polymers which make up an instable fibrin clot. The active form of FXIII acts as a transglutaminase and crosslinks the fibrin to an insoluble fibrin polymer, the fibrin network, which seals the site of injury and protects the damaged tissue during wound healing. FXIIIa also crosslinks other proteins to the formed clot. Among these are the two fibrinolysis inhibitors thrombin activatable fibrinolysis inhibitor (TAFI) and antiplasmin, which protect the clot from resolving too soon.

The regulation of the coagulation process is essential to avoid a generalized activation of the system resulting in massive fibrin deposition. The system must only be active at a local site of injury and must remain active only for a period of time sufficient for enough fibrin production to seal the wound. This regulation is very complex, with strong negative and positive feedback systems. Positive feedback is provided by activation of a proenzyme by the reaction product. For example in the presence of TF, Ca²⁺ and phospholipids, FVIIa triggers the process of cleaving FVII to its active form FVIIa. The most important positive feedback is the activation of FV by
thrombin. The activated FVa acts together with cofactors Ca$^{++}$ and phospholipids and amplifies the transformation of prothrombin to thrombin, which subsequently induces activation of FV to FVa. The most important regulatory pathways, however, contain a series of anticoagulant proteins and cofactors which bind to activated coagulation factors and limit their period of activity.

**Natural inhibition of blood coagulation**

A complex system of coagulation inhibitors exists to protect the body from uncontrolled coagulation and restrict the coagulation to specific areas. There are three major systems of natural coagulation inhibitors: TFPI, the heparin–antithrombin pathway and the protein C system.

TFPI inactivates the initial trigger of the initiation of the coagulation, namely FVIIa bound to TF. TFPI can only inactivate TF/FVIIa after a previous interaction with FXa. As soon as some FXa has been formed and bound to TFPI, a negative feedback loop starts. The effect is rapid inhibition of the TF-FVIIa complex activation of the cascade (Esmon 2005).

Antithrombin (AT) is the most important circulating inhibitor of the activated coagulation factors. Its most important target is thrombin, but it also binds and inhibits coagulation factors IXa, Xa, XIIa, and the TF-FVIIa complex (Kobayashi 2005). The ability of antithrombin to inhibit these factors is greatly accelerated by heparan sulphate, lining the endothelial layer, and heparin released from the granules of mast cells associated with the endothelium (Gomez et al 2005). Thrombin bound to AT forms the stable thrombin–antithrombin (TAT) complex. The plasma TAT complex concentration reflects thrombin production or functional state of the coagulation system.

The protein C anticoagulant pathway is thought to be the major mechanism by which thrombosis in the microcirculation is prevented (Figure 3). Protein C circulates in an inactive form in blood, and its activation is triggered when thrombin binds to thrombomodulin (TM) on the endothelial surface (Esmon 2005). The thrombin–TM complex activates protein C alone or protein C bound to the endothelial protein C receptor (EPCR). Activated protein C (APC) is inactive as an inhibitor while it remains bound to the EPCR. To be activated it binds to protein S and is released from the EPCR. Protein S exhibits both this APC cofactor activity and some direct anticoagulant activity, of which the APC cofactor activity probably is the most important. The protein S–APC complex proteolytically inactivates FVa and FVIIIa, which down-regulates formation of the prothrombinase and tenase complexes.
enzymes responsible for activation of thrombin and FX respectively (Dahlbäck & Villoutreix 2005). The thrombin-TM complex also accelerates the inactivation of thrombin by antithrombin (Rezaie et al 1995).

**Figure 3. Schematic representation of blood coagulation and the protein C anticoagulant system.** (Reproduced with permission from K Strandberg). The figure demonstrates a scheme of blood coagulation reactions together with the balancing anticoagulant reactions of the protein C pathway.

Together with antithrombin and TFPI, the protein C system is an important defence system against thrombosis. Deficiencies in the protein C system are associated with increased risk of VTE, as are inherited AT deficiency (Simioni et al 2006). Resistance to APC, usually caused by a mutation in the FV gene, is one of the most common inherited trombophilias. The impact of deficiencies of the TFPI is still unclear, even though it has been postulated that TFPI resistance may constitute a novel hemostatic genetic risk factor for VTE (Lwaleed & Bass 2006).

**Fibrinolysis**

The fibrinolytic system is involved in the removal of the clot from the vascular system and may also participate in thrombogenesis, restenosis and atherosclerosis. The central protein in fibrinolysis is plasminogen, which after enzymatic activation forms plasmin, dissolving the fibrin network into fibrin degradation products (FDP). FDPs exist in many different sizes – the smallest ones are fragment D and fragment E. Crosslinked D’s, D-dimers, are well established as markers of fibrinolysis activity (Siragusa 2006).
There are two major activators of plasmin formation; tissue plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Both circulate in blood as inactive precursors, and require proteolytic cleavage to be activated. t-PA is synthesized by endothelial cells and released into the blood stream. Fibrin formation enhances t-PA secretion, and there is strong affinity between t-PA and fibrin. Also, plasminogen is tightly bound to fibrin via specific binding sites. Fibrin acts as a cofactor for the t-PA-mediated enzymatic cleavage of plasminogen to active plasmin. Plasmin is a strong but unspecific enzyme, which also cleaves fibrinogen into smaller fragments. Some of these fibrinogen degradation products impair platelet aggregation and fibrin polymerization and function as anticoagulants (Cesarman-Maus & Hajjar 2005) (figure 4).

u-PA is activated by factor XIIa, kallikrein and plasmin (positive feedback). The role of u-PA in clot dissolution is not clear. However, it seems to be of greatest importance in other fibrinolysis-associated processes in the body, such as tissue remodelling and cell migration (Mazzieri & Blasi 2005).

**Figure 4. The fibrinolytic system** (Reproduced with permission from K Strandberg). Soluble fibrinogen is converted to insoluble fibrin in the final step of the coagulation process. The fibrin clot is stabilized by FXIIIa that covalently cross-links fibrin monomers. Plasmin can degrade the fibrin clot to soluble products. The most important activator of plasminogen is tPA, which is in turn inactivated by PAI-1. Thrombin not only activates FXIII to FXIIIa, but also activates TAFI that inhibits fibrinolysis.
Inhibitors of fibrinolysis

The primary inhibitor of active plasmin is antiplasmin. Antiplasmin binds irreversibly to circulating plasmin or plasmin on the surface of the clot. A stable complex, the plasmin–antiplasmin complex (PAP) is formed. This complex can be measured in plasma, and is used as a marker of plasmin production. Antiplasmin is bound to fibrin by FXIIIa during clot formation. It is clot bound antiplasmin which modulates fibrinolysis and interaction of free plasmin and antiplasmin is probably more important in preventing systemic lytic state (Coughlin 2005).

Plasminogen activator inhibitor type 1 (PAI-1) is found in plasma and in platelets, and functions as a specific inhibitor of t-PA and u-PA. The synthesis by endothelial cells is enhanced by proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor (TNF), by endotoxins (lipopolysaccharides) released during sepsis, and by thrombin (Huber 2001). PAI-1 acts as an acute-phase reactant; its concentrations rises during inflammation and thrombosis, and it has been proposed to be a strong risk factor for cardiovascular disease (CVD) (Feinbloom & Bauer 2005).

Thrombin activated fibrinolysis inhibitor (TAFI) is activated by thrombin and probably also by plasmin. Binding to thrombin and thrombomodulin strongly enhances the activation of TAFI. The antifibrinolytic mechanism of TAFI involves removal of the binding site for plasminogen on fibrin. Since this binding is necessary for the activation by t-PA, TAFI prevents fibrinolysis from occurring (Bouma & Meijers 2003).

There are also other inhibitors of fibrinolysis. Examples include α₂-macroglobulin, which is strongly anti-fibrinolytic, PAI-2 which increases significantly during pregnancy (from the placenta), and PAI-3, a weak fibrinolysis inhibitor which also gradually inhibits APC and therefore is called protein C inhibitor (PCI).
Risk indicators for venous thromboembolism

In contrast to atherothrombosis the discussion on factors for VTE has historically not focused on biochemical risk indicators, but more on the triad of Virchow (i.e., alterations in blood flow, injury to the vascular endothelium and alterations in the constitution of blood), although the phenomenon of biochemical risk indicator has been discussed since the 1960s. Individual risk factors for VTE include age, obesity, history of VTE and thrombophilia. Originally, thrombophilia was associated with a predisposition to thrombosis in carriers of AT deficiency (Egeberg 1965). Subsequently, the term thrombophilia was associated with clinical conditions characterized by increased tendency toward thrombosis in young patients in the absence of known diseases commonly related to thrombosis (e.g. cancer). At present the term thrombophilia includes any hereditary or acquired condition associated with an increase tendency to VTE (Simioni et al 2006). The thrombophilias are characterized by a deficiency of an anticoagulant protein or by an increased level or function of a coagulation factor. Deficiencies in AT, protein C and protein S are rare but results in a high thrombogenic potential, whereas some more common thrombophilias (e.g., increased levels of FVIII and homocysteine) increase the VTE risk to a lesser extent (Simioni et al 2006). Even dysfunction in certain factors or steps in the hemostatic process can result in thrombophilia, e.g., dysfibrinogenemia or APC resistance (Bick 2006).

During recent years, “new” thrombophilias which are potential risk indicators for VTE have been uncovered, such as increased levels of F IX or FXI (Meijers et al 2000b), or TAFI (Willemse & Hendriks 2007) and alterations in TFPI (Dahm et al 2003).

A variety of laboratory tests have been proposed for the detection of abnormal coagulation activity in VTE. These include measurement of TAT, F 1+2, fibrinopeptide A, fibrin degradation products, PAI-1 activity and D-dimer, among others. D-dimer, a fibrin degradation product that contains part of cross-linked fibrin strands, has gained attention because of its possible negative predictive value for exclusion of VTE, when it is under a certain cut-off level (Siragusa 2006). However, venous thrombogenesis may be regarded as the product of an unstable dynamic balance between coagulation and fibrinolysis and to get a picture of this haemostatic balance in a certain moment, global methods like APC resistance assays, thrombin generation assays and thrombelastographic methods are more adequate.

Besides intrinsic or disease related, the risk factors for VTE also include use of oral contraceptives. Therefore, in the present studies the effects of use of COCs on some of the laboratory tests associated with VTE were investigated.
Risk indicators for atherothrombosis

CVD and atherosclerosis have traditionally been viewed to simply reflect the deposition of lipids within the vessel wall of medium-sized and large arteries. This concept has changed in the past two decades. It is now assumed that the conditions involve a complex endothelial dysfunction induced by elevated and modified low-density lipoproteins (LDL), free radicals, infectious micro-organisms, shear stress, hypertension, toxins related to smoking or combinations of these and other factors, leading to a compensatory chronic inflammatory response (Ross 1999). Accordingly, during recent years, new biochemical risk markers have been identified in addition to the classical ones. Some of them will be addressed in the present thesis.

Lipoproteins

There is a firm association between high LDL cholesterol concentration in plasma and increased risk for CVD (Meagher 2004). Numerous studies have also identified high triglyceride (TG) (Austin et al 1998) and low high density lipoprotein (HDL) cholesterol (Meagher 2004) levels as risk factors for CVD. In the circulation, TG and cholesterol are transported as parts of circulating lipoproteins. These lipoprotein particles consist of a surface layer made up of apolipoproteins and a core containing hydrophobic lipid constituents. Depending on the size and density, lipoproteins are divided into subfractions designated chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoprotein (IDL), LDL and HDL. VLDL, IDL and LDL contain apolipoprotein B (apo-B) whereas HDL mainly contains apolipoprotein A1 (apo-A1).

Modification of LDL, and to a lesser extent of other lipoproteins, is essential for the initiation of the atherosclerotic process. Oxidation of LDL is believed to be most important. As a response to oxidation, LDL changes its configuration and new antigens are exposed on the surface of the oxidated LDL (oxLDL). OxLDL serves an antigen for the human immune system, and antibodies against oxLDL are present in atherosclerotic lesions and in the circulation of healthy individuals early in the atherosclerotic process (Tsimikas 2006). Levels of anti–oxLDL are raised in individuals with more advanced atherosclerosis, and they seem to correlate with the degree of atherosclerosis in such persons (Bergmark et al 1995, Wu et al 1997).

Lipoproteins promoting endothelial damage include for example LDL, VLDL and lipoprotein(a). OxLDL can damage the arterial endothelium, and there is much evidence to suggest an association between LDL oxidation and development
of atherosclerosis. HDL, on the other hand, is suggested to prevent or alleviate endothelial damage. In recent years, the ratio between apo B and apo A1 has been established as one of the best predictors for CVD (Walldius & Jungner 2006).

The effect of lipoproteins can also be mediated through coagulation factors. Lipoproteins have been suggested to increase FVII synthesis and activation. FVII levels are influenced by environmental and genetic factors and are associated with increased levels of TG, and therefore discussed as a risk factor for CVD. However, as FVII is associated also with other established risk factors such as obesity and postmenopausal state, its role as an independent risk factor is not confirmed.

**Inflammation**

As mentioned earlier, atherosclerosis has many features of an inflammatory process. Accordingly, markers of an inflammatory response such as C-reactive protein (CRP) and fibrinogen have proved to be useful in assessing the risk of CVD. The Women's Health Study has shown that CRP is a strong independent risk factor for CVD in females (Ridker et al 1998, Ridker et al 2000).

High-sensitivity assays have shown CRP levels <1, 1 to 3, and >3 mg/L to correspond to low, moderate, and high risk of future cardiovascular events, respectively (Ridker 2003). **Even a modest elevation of CRP is associated with an increased risk of atherothrombotic events up to 20 years after blood samples were obtained (Sakkinen et al 2002).** A recently published study indicates that an elevated serum CRP concentration is independently associated with aortic as well as coronary atherosclerosis even in young adults; aged 15-34 (Zieske et al 2005). Recent data also show that CRP can be produced inside the arterial plaque, supporting the concept that CRP might play an active pathophysiological role in atheromatous lesions (Yasojima et al 2001). In addition, CRP mediates the uptake of native LDL particles by macrophages via the CD32 receptor (Zwaka et al 2001).

Fibrinogen is also a well-known risk factor for atherothrombosis (Danesh et al 2005). Fibrinogen mediates its proatherogenic effects by increasing plasma viscosity, promoting platelet aggregability and by stimulating smooth muscle cell (SMC) proliferation (Ernst 1993). Fibrinogen affects platelet aggregation through its reaction with platelet receptors (glycoprotein complex IIb/IIIa), which is a key reaction in thrombus formation. In addition fibrinogen forms fibrin, the main component of the clot.
PAI-1 is classified as an acute-phase reactant, and a number of cytokines have been found to stimulate endothelial PAI-1 production. Studies demonstrate enhanced expression of PAI-1 in diseased vessels, and that the expression is proportional to the degree of atherosclerotic burden. In addition PAI-1 may impair normal vascular remodelling through its effects on integrin expression and cellular migration. However, even though levels of PAI-1 appear to positively correlate with atherothrombotic disease, it is not epidemiologically verified as an independent risk factor for atherothrombosis (Feinbloom & Bauer 2005).

**Glucose metabolism**

It is well established that diabetes is associated with a proatherosclerotic state. Results from many different studies indicate that insulin resistance is an important factor in the development of atherosclerosis in patients with type 2 diabetes, obesity, and/or metabolic syndrome (Cersosimo & DeFronzo 2006). Insulin causes proliferation of vascular smooth muscle cells, which is a hallmark lesion of the atherosclerotic process. Insulin also switches on the genes that are involved in connective tissue formation, an important part of the atherogenesis. Furthermore, insulin upregulates LDL-receptor activity and stimulates the production of growth factors (Defronzo 2006). While insulin resistance, by promoting dyslipidemia and other metabolic abnormalities, is part of the proatherogenic milieu, it is possible that insulin resistance in the vascular wall itself does not promote atherosclerosis.

In the past few years, insulin-like growth factor-I (IGF-I) and two of its binding proteins, IGFBP-1 and IGFBP-3, have been identified as putative mediators in CVD. Low serum IGF-I has been associated with coronary artery disease in both cross-sectional and prospective studies (Juul et al 2002, Spallarossa et al 1996), and a low circulating IGFBP-1 concentration has consistently been related to a less favorable cardiovascular risk profile (Heald et al 2001, Gibson et al 1996, Janssen et al 1998). High IGFBP-3 concentrations, on the other hand, could reduce the bioavailability of IGF-I, and therefore contribute to the negative effects of low IGF-I levels.

**Endothelial activation**

Structural and functional changes in the vascular endothelium, which could be interpreted as a response to cellular injury, may be involved in the development of vascular disease (Ross 1999). Hence, a method for assessing endothelial damage/dysfunction would be valuable. One approach is to quantify the plasma concentrations of various endothelial products, such as vWf or soluble E-selectin (sE-sel). Indeed,
vWF has been proposed as a marker of endothelial damage/dysfunction, as increased plasma levels have been found in inflammatory and atherosclerotic vascular diseases where the endothelium is likely to be affected (Blann & Taberner 1995). In contrast, it remains unclear whether elevation of sE-sel reflects activation, damage, or merely a physiological response (Roldán et al 2003).

Influence of COCs on hemostatic parameters and risk indicators for venous thromboembolism and atherothrombosis

Hemostasis
It has been known for many years that oral contraceptives influence hemostasis (Kluft & Lansink 1997). Following the epidemiological studies reported in the mid 1990s, suggesting a difference in risk of VTE according to progestogen content of the COCs, studies focused on differential effects on hemostasis with different COCs.

So-called low-dose COCs were shown to increase the plasma concentrations of FVII, FVIII, FX, F1+2 (Middeldorp et al 2000, Kluft & Lansink 1997). In a randomized study, more pronounced effects were found on prothrombin, FV and FVII with a third-generation COC than with a second-generation COC (Middeldorp et al 2000). Of note, moderately increased levels of prothrombin and FVIII have been associated with increased risk of VTE (Kraaijenhagen et al 2000, Bank et al 2005). Fibrinogen, a link between CVD and VTE, which is synthesized in the liver and – like CRP – behaves as an acute phase reactant, shows the same pattern of increase during COC treatment, and higher plasma concentrations during use of third generation COCs (Middeldorp et al 2000, Döhring et al 2004, Kluft & Lansink 1997).

COCs also influence the natural anticoagulant system, particularly the protein C pathway. Plasma from women using COCs is less sensitive to APC than plasma from non-users (Rosing et al 1997). Furthermore, women using third-generation COCs have a more pronounced APC resistance than women using second-generation COCs (Rosing et al 1999, Tans et al 2000). In some cases, APC resistance during COC use is of the same magnitude as that observed in carriers of the factor V<sub>Leiden</sub> mutation (Rosing et al 1997). Thrombelastography has been evaluated in women
using COCs. However, the estrogen component in those reports range in the majority of studies between 50 and 100 µg EE. In one study on low-dose COCs no trend towards hypercoagulability was found (Zahn et al 2003).

Studies on fibrinolytic parameters during COC use suggest an increased fibrinolytic activity (Kluft & Lansink 1997, Meijers 2000a). PAI-1, important in the regulation of the fibrinolytic activity and also a marker of endothelial activity, is highly sensitive to COC treatment; the plasma concentrations decrease significantly during use of second- as well as third-generation COCs. Conversely, COCs induce elevated plasma levels of TAFI, an effect that is more pronounced with third-generation COCs than with second-generation COCs (Meijers 2000a). Elevated plasma levels of TAFI are known to be a risk factor for VTE (van Tilburg et al 2000).

Liver function

Next after the reproductive organs, the liver is the organ most affected by sex steroids. The estrogen component in COCs increases the synthesis of serum enzymes formed in the liver and also some of the plasma proteins (Laurell et al 1968, von Schoultz et al 1989). Some of these plasma proteins are coagulation factors in their inactive form. The plasma protein most strongly affected by estrogen is SHBG.

Oral intake of EE alone results in a profound dose-dependent increase in SHBG (Mashchak et al 1982, Stege et al 1988). Conversely, progestogen intake results in varying degrees of decrease, depending on dose and type of progestogen. The differences might be due to the differences in anti-estrogenic effects of the progestogens (Mainwaring et al 1995). SHBG has been proposed as a marker of the total estrogenicity of a defined COC in the sense that the effect on SHBG is an expression of the net effect of the estrogenic influence of EE and the anti-estrogenic effect of progestogen (Odlind et al 2002)

CRP, another liver-synthesized protein, is also affected by steroid hormone treatment. Women using COCs present higher levels of CRP in plasma than non-users (Dreon et al 2003, Kluft et al 2002), and women treated with third-generation COCs display higher levels than those on second-generation COCs (Döhring et al 2004). In studies on women using hormone replacement therapy (HRT), results indicate a difference between oral and transdermal administration; transdermal formulations do not affect CRP whereas perorally given HRT increases the plasma concentration (Vehkavaara et al 2001, Lacut et al 2003). Therefore, the route of estrogen administration may be an important determinant of the serum CRP concentrations.
Lipoproteins

The many effects that the estrogens and progestogens used in oral contraceptives and postmenopausal HRT exert on lipoprotein metabolism are of importance because of the involvement of lipoproteins in endothelial dysfunction and atherogenesis.

The estrogenic component of COCs introduces a favorable lipoprotein pattern in the blood with increased HDL cholesterol and decreased LDL and VLDL (Kloosterboer & Rekers 1990). COCs containing the more androgenic progestins (i.e. second-generation progestogen) counteract the beneficial effects of estrogen on lipoproteins, i.e., they decrease serum levels of HDL and increase LDL and triglycerides (Kemmeren et al 2001). Third-generation COCs may either cause adverse but minor changes in lipoprotein or even induce an elevation in HDL, which is often accompanied by a reduction in LDL (Godsland et al 1990) and an increase in serum TGs (Kemmeren et al 2001). In combination with estrogens, the third-generation progestogen (i.e. DSG) seems to counteract the effects of estrogen to a lesser extent than levonorgestrel (Kemmeren et al 2001).

Studies on apolipoproteins during COC use report diverging results; some describe an increase in apo A1 levels (Knopp et al 2001), some a decrease (Tuppurainen et al 2004) and some could not detect any changes at all (Scharnagl et al 2004). Regarding apo B, most studies demonstrate increased serum levels (Knopp et al 2001, Tuppurainen et al 2004). However, one study could not determine any differences in the two apolipoproteins when two COC with different estrogen (20 vs 30µg) and LNG (100 vs 150µg) content were compared (Scharnagl et al 2004).

Glucose metabolism

Use of older high-dose COCs apparently led to impaired glucose tolerance in many women. Wynn and Doar (1966) described cases of “steroid diabetes” in women using OC containing >50 µg of EE. Such marked hyperglycemia is not seen with modern preparations. In fact, the observed changes in carbohydrate metabolism during use of modern low-dose COCs are so minimal that they are believed to have no clinical significance in relation to diabetes (Speroff & Frits 2005).
Endothelial activation

There are only a few studies on the effect of COCs on E-selectin (Seeger et al 2002, Ponthieux et al 2004). E-selectin levels appear to decrease during COC treatment in a manner resembling what had earlier been observed during HRT treatment (Vehkavaara et al 2001), without any difference between two preparations, one containing 20 µg EE and 100 µg levonorgestrel and the other containing 30 µg EE and 150 µg levonorgestrel (Seeger et al 2002). No comparative studies on different COCs effects on vWf are available. Different studies report either increased levels (Kluft & Lansink 1997, Kluft et al 2002) or no changes in the plasma concentrations of vWf (Prasad et al 1999) during COC use.

Risk of venous thromboembolism and cardiovascular disease in fertile women

The risk that a healthy fertile woman will suffer VTE is low. In a population-based study, Nordström et al (1992) observed an incidence of venous thrombosis of 0.11, 0.26 and 0.97 per 1000 women for the age categories 20 to 29, 30 to 39 and 40 to 49 years, respectively. In the age group <20 years the incidence was 0.05 per 1000 women. It is notable in this context that this population-based study did not discriminate between first and recurrent thrombosis, and might therefore give an overestimation of the risk of first-time thrombosis.

Since the introduction of COCs in the early 1960s, the risk of VTE has been studied. There is a relation between the dosage of estrogen and the risk of VTE with a trend towards decreasing incidence of VTE with decreasing estrogen component in the oral contraceptive (Inman et al 1970, Rosendaal et al 2003). Accordingly, the estrogen content of COCs has been gradually reduced during the last several decades. Simultaneously, the progestogen component of the pills has been developed to be more favorable in terms of metabolic side-effects. Today, the risk of developing VTE during use of COCs is estimated to 2-4 per 10 000 women annually, with the higher rates attained among users of third generation COCs (Jick et al 1995, Farmer et al 1997). However, the absolute risk of VTE associated with OC use is low (one in 3000 to 5000) and smaller than the risk of pregnancy-associated VTE (Godsland et al 2000) (figure 5). The risk of VTE is influenced by duration of use; it is highest the first year, goes down by more than 50% in subsequent years (Bloemenkamp et al 2000, Lidegaard et al 2002), and returns to the same level as that among non-users after discontinuation of the drug (Hannaford 2000).
During use of progestogen only contraceptives no increase in VTE is observed (Collins & Crosignani 2003) but some studies indicate that during use of progestogen for gynecological disorders, it might be an association to VTE (Poulter et al 1999, Vasilakis et al 1999b).

Figure 5. Incidence of venous thromboembolism in young women during pregnancy and according to type of oral contraceptives. [Reproduced with permission from author (Godsland et al 2000)]

In women of fertile age the risk of venous thrombosis is low and the risk of arterial cardiovascular disease is even lower. According to WHO statistics for industrialized countries from the period 1992 to 1996, the estimated rate of MI among women who were non-smokers and not taking oral contraceptives in the age ranges 20 to 24, 30 to 34 and 40 to 44 years was 0.14, 1.70 and 21.28 per million and year, and the estimated rate of ischemic stroke (IS) among the same groups of women was 6.0, 9.8 and 16.1 per million per year, respectively (Hannaford 2000).

Since the basal incidence of arterial occlusive disease is so low, it is hard to get reliable epidemiological data about whether the risk increases during use of COCs. A recent meta analysis found a summary odds ratio (OR) for MI of 1.84 and a summary OR for IS of 2.12 (Baillargeon et al 2005). Subgroup analysis based on type of progestogen showed that second-generation OCs increased the risk of MI events 1.9-fold and the risk of IS events 2.5-fold. Third-generation OCs doubled the
risk of IS outcome and increased (nonsignificantly) the risk of MI by 28%. Some studies have made direct comparisons between third- and second-generation COCs regarding their effects on risk of MI (WHO 1997, Dunn et al 1999, Tanis et al 2001) or stroke (Kemmeren et al 2002, Lidegaard & Kreiner 2002). However, the results of these studies are not conclusive with respect to demonstrating a reduced risk among users of third-generation COCs. Also, the risk of peripheral arterial disease appears to be equally elevated in users of second- and third-generation COCs (van den Bosch et al 2003). Thus, although the third-generation OCs were developed to reduce the risk of arterial thrombosis, no such effect has been unequivocally demonstrated.

Smoking and hypertension both substantially increase the risk of MI among COC users, and some data suggest an increased risk among women with diabetes, hypercholesterolemia or a history of pregnancy-induced hypertension or preeclampsia. There is no epidemiological evidence for increased risk for MI with past use of COCs (Stamfer et al 1990, Beral et al 1999, Colditz 1994). One large follow-up study on mortality related to past COC use describes a trend towards increased risk of cerebrovascular events (Colditz 1994).
Aims of the study

The overarching aim of this thesis was to identify alterations in hemostasis as well as in established and novel risk markers for atherothrombotic disease during treatment with COCs and to compare different types and doses of progestogen and estrogen.

Specifically, the aims of the individual studies were:

- To compare the change in the plasma lipoprotein pattern and the association to FVII during use of two different COCs.
- To identify a plausible biological mechanism to explain why SHBG could act as a “surrogate marker” for the risk for VTE during use of COCs.
- To investigate the alterations in CRP during use of COCs and to find a biological explanation for the expected increase.
- To elucidate the effects on hemostasis and atherosclerotic risk markers during the short term exposition to sex hormones provided by EC pills.
- To clarify whether there is any change in APCresistance during the normal menstrual cycle.
Subjects

The present thesis is based on three different groups of women. A total of 160 women between 18-51 years of age volunteered for the studies. All subjects were healthy and without any kind of medication; they had normal menstrual periods and had not been pregnant, breast-feeding or used any hormonal contraceptives within two months prior to study start. Use of non-steroidal anti-inflammatory drugs (NSAID) was not allowed for two weeks prior to blood sampling in any of the studies.

In study I (papers I-III, van Rooijen et al 2006) the subjects were nulliparous non-smokers. Their age ranged from 18 to 33 years (median 24.6). Nine women out of 44 included withdrew their consent to participate, eight for personal reasons and one due to severe migraine during use of one of the study preparations.

In study II (paper IV) 11 of the 12 included volunteers completed the trial. One woman discontinued the trial as she became pregnant in the wash-out period. The remaining participants had normal body mass index (BMI) and their age ranged from 21 to 34 years (median 27.2).

In the last study (paper V) 104 women with regular menstrual cycles (cycle length 21 to 35 days) and without any hormonal treatment were included. There were no restrictions regarding body mass index (BMI), smoking or parity, but the participants were not allowed to take any medication at all. Of 104 women two were excluded; one for personal reasons and one due to severe problems with the blood sampling. The remaining 102 had a median age of 31.8 years (range 18 to 51), median BMI 22.7 (range 17.4 to 37.2). Nine of them were smokers.

The separate studies were approved by the local ethics committee and all patients gave their informed consent to their participation.
Treatments

In study I (papers I-III), two different kinds of monophasic COCs were used: one so-called second generation COC with a daily dosage of 30 µg EE/150 µg LNG (Follimin®) and one so-called third-generation COC with a daily dosage of 30 µg EE /150 µg DSG (Desolett®).

In study II (paper IV), two different methods of hormonal EC were used; one method in which two pills containing 50 µg EE and 250 µg LNG each (Follinett®) were given twice with an interval of twelve hours, and one method in which 750 µg LNG (Postinor®) was given in the same manner. As general indisposition and vomiting are common side effects of the combined EC method, participants were assigned to take an antiemetic drug (6.5 mg Torecan®; Tietylperazine) at the same time as they took the second treatment dosage.

Study designs

The first study was a randomized, prospective cross-over study, in which the women were randomly assigned to start with one of the two study medications, and use it for two months. After the first treatment period, there was a wash-out period of two months before participants switched to the alternative study medication for two (more) months. In the menstrual cycle prior to the first treatment period blood samples were drawn twice; once in the follicular phase (cycle day (cd) 5-8) and once in the luteal phase (cd 22-25). During the last week of each treatment period, blood samples were drawn once again. See figure 6.

Figure 6. Flow chart for treatments and blood samplings in study I. A: second-generation COC, B: third-generation COC.
In the second study, we also used a randomized, prospective cross-over study design, in which the women were assigned to use one of the two EC methods at ovulation time. An ovulation test was performed to determine ovulation time. Since EC might cause menstrual bleeding disturbances, participants were required to have one normal menstrual cycle in between the two treatment cycles. In the second treatment cycle, they used the alternative EC method. Three weeks after each treatment, a pregnancy test was performed to exclude pregnancy. Blood sampling took place before administration of the medication and 2, 4, 8, 12, 24 and 48 hours after the second dose. The first dosage was given at 8 pm on day one, the second dosage at 8 am in the next morning (i.e. day two). Remaining blood samples were collected at 10 am, noon and 8 pm day 2, at 8 am day 3 and at 8 am study day 4. See figure 7.

**Figure 7. Flow chart for treatment and blood sampling in study II**

The third study is a descriptive study on changes in resistance to APC during the normal menstrual cycle. Blood samples were drawn twice in a normal menstrual cycle: once in early follicular phase (cd 3-5) and once in the luteal phase (cd 22-25).

**Blood sampling**

Venous blood samples were drawn from an antecubital vein after 15 minutes rest in the sitting or supine position. In studies I and III all samples were drawn in the morning after an overnight fast. In study II, the samples taken in the morning, i.e., before the second dosage and after 24 and 48 hours, were fasting samples.

Blood samples for analysis of coagulation and fibrinolytic factors were collected in vacutainer tubes (Becton Dickinson) containing citrate (0.13 mmol/L) and immediately centrifuged. In the COC study (papers I-III), the tubes were centrifuged twice at 3000 rpm (1500 g) for 20 minutes, in the study on emergency contraceptives (paper IV) twice at 2000g for 20 minutes and in the last study (paper V) the two centrifugations were at 2000 g for 15 minutes. Samples for determination of PAI-1
activity were drawn into acidified citrate tubes (Stabilyte, Biopool, Sweden) and treated in the same manner as described above.

Blood for serum preparation was collected in plain vacutainer tubes without anticoagulants and kept at room temperature for one hour before centrifugation at 3000 rpm (1500 g) for 20 minutes in Study I and for 10 minutes at 2000 g in studies II and III. Cell-free plasma and serum samples were stored at -70°C until analyzed.

Blood samples for lipid and lipoprotein determinations were drawn into precooled sterile tubes (Vacutainer Becton Dickinson) containing Na₂EDTA (final concentration 4 mmol/L), which were instantly put into ice water. Plasma was then removed by low-speed centrifugation (1750 g, 20 minutes, 4°C).

**Laboratory methods**

**Hemostatic factors**

FVII mass concentration was determined as factor VII antigen (FVIIag) with the Factor VII EIA kit (Dako A/S, Glostrup, Denmark). Activated factor VII (FVIIa) was determined according to Morrissey (*Morrissey et al 1993*). F1+2 and plasmin–antiplasmin (PAP) complexes were determined by enzyme immunoassays (Dade Behring, Marburg GmbH, Marburg, Germany). Fibrinogen was determined using the IL Test Fibrinogen C kit from Instrumentation Laboratory Spa, Milan, Italy. TAFI activity was measured with the Actichrome kit from American Diagnostica Inc., Greenwich, CT, USA. PAI-1 activity and tPA antigen were measured by immunoassays (Chromolize PAI-1 and TintElize tPA, Biopool International) Protein S was determined with the Coaliza Free Protein S kit from Chromogenix Instrumentation Laboratory Spa Milan, Italy. The heparin cofactor activity of antithrombin was quantified with the Coamatic antithrombin kit from Chromogenix. Factor VIII was analyzed by a chromogenic assay with the Coamatic FVIII kit from Chromogenix. vWFag was measured by an in-house enzyme immunoassay with antibodies from Dako A/S.

APC sensitivity ratios (APCsr) were determined with the APTT-based Coatest APC Resistance C from Chromogenix Laboratory Spa. Normalized APCsr (nAPCsr) was determined with the ETP-based APC-resistance test, in which the effect of APC on the time integral of thrombin generation (the endogenous thrombin potential, ETP) was quantified via measurement of end-point levels of α₂-macroglobulin-thrombin complex (*Nicolaes et al 1997*).
Genotyping FV
For genotyping of the FV<sub>Ledden</sub> mutation we used the pyrosequencing assay described by Holmberg et al (2005).

Lipoproteins
The major plasma lipoproteins, VLDL, LDL and HDL, were determined by a combination of preparative ultracentrifugation and precipitation of apolipoprotein B-containing lipoproteins followed by lipid analyses (Carlsson 1973). Cholesterol and triglycerides were determined by enzymatic methods with reagents from Merck, Darmstadt, Germany, and Wako Chemicals GmBH, Neuss, Germany. Apo A1 and apo B were analyzed in serum using particle-enhanced immunonephelometry (Behring Nephelometer Analyzer Dade Behring GmBH, Marburg, Germany).

Immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies against oxLDL were determined by an ELISA method, essentially as described (Wu et al 1999).

Inflammatory parameters
CRP and serum amyloid A (SAA) were analyzed using particle-enhanced immunonephelometry (Behring Nephelometer Analyzer). IL-6 and TNF-α were analyzed in serum using commercially available high sensitivity ELISA kits (R&D Systems, Minneapolis, MN, USA).

Markers of glucose metabolism
Insulin, IGFBP-3 and C-peptide in serum were measured using chemiluminiscent immunometric assays (Immulite 1000, Diagnostic Products Corporation, Los Angeles, CA, USA) and free IGF-1 and IGFBP-1 were determined by an ELISA (Diagnostic Systems Laboratories Inc, Webster, TX, USA). Glucose levels in serum were determined after enzymatic oxidation in the presence of glucose oxidase with reagents from Randox Laboratories Ltd, Antrim, UK.

SHBG
SHBG was measured using the Immulite 1000 assay (Diagnostic Product Corporation).

E-selectin
E-selectin in plasma was analyzed with an ELISA kit (R&D Systems).
**Statistical methods**

Normally distributed data are presented as arithmetic means and standard deviations (SD) or 95% confidence intervals (CIs), or as median and range. Some of the variables were log-transformed or reciprocally transformed before the formal analyses because of their positively skewed distribution.

In the first study (papers I-III) measurements were first analyzed by two-way analysis of variance (ANOVA), and as no significant period- or carry-over effect was found, they were thereafter analyzed by one-way repeated measures ANOVA. Associations were expressed using Pearson correlation coefficients (papers I and II). In the first paper Bonferroni corrections were performed to handle multiple comparisons between groups.

In paper III, differences from baseline on COC treatment and differences between COC treatments were presented by median values together with 95% CIs. The p-values were based on the results from the variance analyses and from the post-hoc pairwise comparisons between treatments based on the estimates from the ANOVA. Thus, the interpretation of the 95% CIs for the median can, in some instances, be different from these results.

In paper IV the data were analyzed using the Mixed procedure in SAS®. A two-way repeated measure ANOVA with treatment and time as within-subjects variable was used. The treatment interaction refers to the statistical test of whether the mean change over time is the same for the two treatments. In case of a significant interaction, simple effects were examined, i.e., effects of one factor while the other factor is held fixed.

In the final paper V comparisons between the two menstrual phases were performed with the Mixed procedure in SAS®, as the variables contain missing values. A one-way repeated measures ANOVA was performed with menstrual phase (follicle, luteal) as the within-subjects variable.

P-values of <0.05 were considered statistically significant.
Results

_Different effects of oral contraceptives containing levonorgestrel or desogestrel on plasma lipoproteins and coagulation factor VII (paper I)_

VLDL and LDL cholesterol fractions did not differ from baseline during treatments, but there was a significant increase in the serum concentration of HDL cholesterol during use of the third-generation COC (p<0.01), and conversely a slight (nonsignificant) decrease during use of second-generation COC. There was also a significant difference in effect on total plasma cholesterol between the two treatment regimens (p<0.01) (table I).

Both treatments increased LDL and HDL triglycerides, and the third-generation COC also resulted in an increase in VLDL TG. The increases in HDL triglycerides and total concentration of triglycerides were significantly more pronounced with the third-generation COC (p<0.005).

Use of second-generation COC did not significantly affect the plasma levels of FVIIag or FVIIa, but use of third-generation COC increased both FVIIa (42%) and FVIIag levels (44%). The changes in FVIIag and FVIIa showed positive associations with both treatments regimens. In addition, we found a positive correlation between plasma TG and FVIIag levels before and during the two treatments (r<sub>p</sub> = 0.411-0.587).
Table I. Concentrations (median value (range)) of cholesterol and triglycerides in plasma and major lipoprotein fractions before and during COC use. P-values denote differences between treatment and baseline and between the two treatments (*=p<0.05, **=p<0.01, ***=p<0.001).

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol (mmol/L)</th>
<th>Triglycerides (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Second-generation COC (LNG/EE)</td>
</tr>
<tr>
<td>Plasma</td>
<td>3.91 (2.9-5.4)</td>
<td>3.82 (2.62-5.27)</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.15 (0.04-0.68)</td>
<td>0.18 (0.40-0.54)</td>
</tr>
<tr>
<td>LDL</td>
<td>2.15 (1.49-3.66)</td>
<td>2.32 (1.32-3.74)</td>
</tr>
<tr>
<td>HDL</td>
<td>1.34 (0.71-2.07)</td>
<td>1.20 (0.86-2.48)</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.83 (0.38-1.88)</td>
<td>1.09** (0.62-2.27)</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.43 (0.21-1.35)</td>
<td>0.64 (0.20-1.52)</td>
</tr>
<tr>
<td>LDL</td>
<td>0.19 (0.09-0.34)</td>
<td>0.30*** (0.16-0.57)</td>
</tr>
<tr>
<td>HDL</td>
<td>0.14 (0.07-0.30)</td>
<td>0.20*** (0.11-0.30)</td>
</tr>
</tbody>
</table>

Sex hormone binding globulin – A surrogate marker for the prothrombotic effects of combined oral contraceptives (paper II)

In this study, we evaluated the effects of the two COCs on coagulation and fibrinolytic markers as well as on serum SHBG concentration. The changes in coagulation factors were all in a more procoagulant direction, except for the free fraction of protein S and the fibrinogen concentration during second-generation COC use. During treatment with the third-generation COC, the level of the free fraction of protein S decreased in contrast to the increase observed during use of the second-generation COC. The plasma fibrinogen concentration decreased during use of third-generation COC, but did not change during use of second-generation COC. The plasma concentration of F1+2 – regarded as a marker of coagulation...
activity – increased and the activity of AT – the important inhibitor of coagulation – decreased during both treatments with no differences between them.

The APC ratio, which is sometimes regarded as a global indicator of coagulation status, demonstrated a more unfavorable level during the use of third-generation COC. The baseline level of 3.40 (1.80-4.10) decreased down to 3.20 (1.90-4.10) with the second-generation pills and even lower, to 3.0 (1.70-3.80), during use of the third-generation pills.

The direction of the changes in the fibrinolytic markers PAI-1, tPA and PAP indicated an increased endogenous fibrinolytic activity with highly increased plasma PAP concentrations and decreased PAI-1 levels. All changes were of the same order of magnitude regardless of treatment regimen. Increase in TAFI was demonstrated with the third-generation COC only.

The serum levels of SHBG increased by 56% during use of the second-generation preparation and by 278% during use of the third-generation compound. The median baseline serum SHBG concentration was 46.3 nmol/L. It increased during treatment to 72.4 and 175 nmol/L respectively. An association was present between the changes in SHBG and in APC ratio during both treatments (figure 8).

![Graphs](image)

**Figure 8. Correlations between changes in plasma concentrations of steroid hormone binding globulin (SHBG) and changes in APC ratio during use of a combined oral contraceptive containing levonorgestrel (A) or desogestrel (B).** There were significant negative linear correlations between the changes in SHBG and APC ratio during use of both levonorgestrel ($r_p = -0.400, p=0.021$) and desogestrel ($r_p = -0.431, p=0.012$).
**Treatment with combined oral contraceptives induces a rise in serum C-reactive protein in the absence of a general inflammatory response (paper III)**

With both treatment regimens, the serum concentrations of CRP increased from a baseline level of 0.45 mg/L. During use of the second-generation COC the median level was 1.48 mg/L and during use of the third-generation COC the increase was even larger: 2.02 mg/L. In percent these increases are considerable. Seven women (20%) reached a serum CRP level above 3 mg/L during use of the second-generation pill and 12 (34%) women during use of the third-generation pill. SAA increased during both treatment regimens but statistical significance was only attained during treatment with third-generation COCs. No treatment effect was noted on IL-6 or TNF-α levels (table II).

Analyses of glucose metabolism did not reveal any signs of decreased insulin sensitivity since serum levels of insulin, glucose and C-peptide did not change during treatments. However, there was a decrease in serum IGF-I and an increase in the two binding proteins IGFBP-1 and IGFBP-3. The changes in IGF-I and IGFBP-3 were significantly more pronounced with the third-generation COC.

The serum level of E-selectin decreased from median baseline level of 36.4 µg/mL to 24.7 µg/mL during treatment with the third-generation COC and to 22.6 µg/mL during use of the second-generation COC. The difference in treatment effect was significant. Other markers of endothelial activity measured, i.e. vWF, F VIII and antibodies against oxLDL, were not affected by treatment.
Table II. Effects of COC treatment on inflammatory markers.

Values are median (range). P-values refer to differences from baseline on COC treatment or to differences between COC treatments. Post-hoc comparisons were performed when the F-test for treatment was significant. Before statistical analyses were concluded, marked variables were transformed according to †reciprocal transformation -1/x or #log transformation (*=p<0.05, **=p<0.01, ***=p<0.001).

Rapid activation of hemostasis after hormonal emergency contraception (paper IV)

In this study, the effects on hemostatic markers, SHBG, CRP and the apoB/apo A1 ratio after intake of a high dose of steroid hormone for emergency contraception were evaluated. Treatment consisted of two doses of estrogen and progestogen, or of progestogen only administered with a 12 hour interval.

A rapid hemostatic activation was induced with combined (estrogen and progestogen) emergency contraception (EE-EC) as well as with the preparation containing only levonorgestrel (LNG-EC) (table III). However, the effect was more pronounced with EE-EC. Already two hours after completed EC treatment (i.e., two doses), the plasma concentrations of AT, free protein S and F 1+2 differed significantly from baseline concentrations, regardless of treatment regimen (table III). All coagulation parameters changed in a more procoagulant direction except for free protein S, which increased slightly during both treatments. The plasma levels of PAI-1 were decreased at eight hours after treatment, from a baseline median level of 1.47 IU/mL down to a minimum plasma concentration of 1.13 IU/mL.
Two different methods to evaluate the sensitivity to APC were used in the study; one aPTT (activated partial thromboplastin time)-based and one ETP (endogenous thrombin potential) based. APC resistance increased at twelve hours after completed treatment with both tests after intake of EE-EC, as reflected by decreased APCsr values with the aPTT-based test and increased nAPCsr values with the ETP-based test. The aPTT-based assay revealed no difference in response between the different EC regimens. The median ratios reached their lowest values after 12 hours of 2.89 for LNG-EC and of 2.77 for EE-EC, and were normalized again at 24 hours.

In contrast, a difference between treatment regimens was evident with the ETP-based test. The nAPCsr had decreased at 12 hours after the second dose of LNG-EC and was normalized at 24 hours. At twelve hours after the EE-EC the median nAPCsr had increased to 2.38, and peaked at 2.59 at 24 hours. The nAPCsr remained different from baseline throughout the study period (figure 9).
SHBG reflected the different hormone treatments as expected, with decreased plasma concentration after progestogen-only treatment and increased plasma concentrations after treatment with the estrogen-containing preparation. The effect on SHBG was rapid with LNG-EC, a decrease being present already at two hours after treatment. The increase related to estrogen intake was slower, with the increase not occurring until after 24 hours.

The apoB/apo A1 ratio was affected in a favorable direction with EE-EC (a significant decrease in the ratio observed after eight hours), whereas there was no change in the ratio after use of the progestogen-only preparation. CRP increased regardless of treatment, from a median pre-treatment level of about 0.5 g/L to a peak median level at 24 hours after treatment; 1.40 g/L for LNG-EE and 0.91 g/L for EE (no significant difference between treatments).
**APC resistance during the normal menstrual cycle (paper V)**

In this descriptive study blood samples were collected twice during a normal menstrual cycle to evaluate whether there are any differences between the follicular and the luteal phases in terms of the sensitivity to APC. Two different APCsr assay were used, i.e. one aPTT based assay and one ETB based assay.

Serum concentrations of estradiol and progesterone were analyzed to assess whether ovulation took place in the studied menstrual cycles. For 72 women, changes in estradiol and progestogen indicated ovulation. Dividing the participants into two groups – all women or only those in whom ovulation occurred – did not change the results. Estrogen and progesterone increased as expected during the menstrual cycle, with higher levels being attained in the luteal phase. The sensitivity to APC did not change between the two menstrual phases regardless of assay used despite the fact that estrogen levels increased from 113±80 pmol/l to 347±203 pmol/L (200%). Twelve out of the 102 women included proved to be heterozygous carriers of the FV<sub>Leiden</sub> mutation. The change in APC resistance between the two time points did not differ between women with or without the mutation. However, there were differences (p<0.001) in APC resistance between carriers and non-carriers with both assays.

Table IV. APC resistance during follicular and luteal phases of healthy fertile women (n= 102). Values are mean ± SD FVL ; FV<sub>Leiden</sub> mutation.

<table>
<thead>
<tr>
<th></th>
<th>Follicular phase</th>
<th>Luteal phase</th>
<th>p-value</th>
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<tbody>
<tr>
<td><strong>APCsr , no FVL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(aPPT based)</td>
<td>3.7±0.4</td>
<td>3.7±0.5</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>APCsr , FVL</strong></td>
<td></td>
<td></td>
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<tr>
<td>(aPPT based)</td>
<td>2.5±0.29</td>
<td>2.6±0.39</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>nPACr , no FVL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ETP based)</td>
<td>2.1±0.7</td>
<td>2.2±0.9</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>nPACr, FVL</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(ETP based)</td>
<td>4.5±1.2</td>
<td>4.5±1.0</td>
<td>n.s</td>
</tr>
</tbody>
</table>

There were no detectable correlations between serum estradiol concentrations and APC resistance levels, nor were there any correlations between changes in the two parameters. Similarly, there were no correlations with progesterone levels.
General discussion

The epidemiology of vascular disease in oral contraceptive users has been reviewed frequently, but there have been few attempts to place the epidemiological data in the context of pathology and pathogenesis. Epidemiological investigation of occlusive vascular disease in oral contraceptive users has been hampered by the low incidence of vascular disease among women of child-bearing age. The most common condition is VTE, the incidence of which is less than 11 per 100 000 women per year (Nordström et al 1992) among young non-pregnant women, who are not using oral contraceptives. MI and stroke are even rarer (Hannaford 2000).

The aim of this thesis was to evaluate effects of COCs on a range of identified risk indicators for VTE and atherothrombotic disease. This was achieved by letting healthy volunteers use COCs in the same way as COCs would be used for ordinary or for emergency contraception, the latter involving short-time exposition to steroid sex hormones. Both treatments were studied using a randomized cross-over design.

**Effects on hemostasis and thromboembolic risk indicators**

Earlier studies have shown increased coagulation activity during use of COCs, with increased plasma concentrations of fibrinogen, FV, FVII, FIX, FX and F XIII as well as of vWF (Godsland et al 2000) and decreased levels of the important coagulation inhibitors AT and protein S (Kluft & Lansink 1997). Fibrinolysis is also affected during use of COC, as reflected by increased plasma concentration of plasminogen and decreased PAI-1 activity (Kluft & Lansink 1997).

Until the beginning of this millennium, there had only been few direct comparisons between second- and third-generation COCs, containing the same amount of estrogen and different progestogens, regarding their effect on hemostasis. In addition, differences between laboratories and assays, heterogeneous patient groups and use of a complex mixture of monophasic and triphasic preparations have
complicated the interpretation of results from previous studies (Kluft & Lansink 1997, Winkler 1998). Significant differences between the effects of levonorgestrel- and desogestrel-containing COCs on some coagulation and fibrinolytic factors were first reported from a randomized cross-over study (Middeldorp et al 2000, Tans et al 2000, Meijers et al 2000). Our results, reported in papers I and II, provide overall confirmation of these previous results, showing activation of the coagulation and a slightly increased fibrinolysis. Apparently, third-generation COCs have a stronger effect on the vitamin K-dependent coagulation factors (II, VII, IX and X) all synthesized in the liver.

SHBG is a steroid sensitive plasma protein synthesized in the liver. It is an important regulator of the amounts of bioavailable sex steroids in that it binds estrogen and testosterone with high affinity. Most of the circulating sex steroids are bound to albumin or SHBG and therefore biologically inactive. Oral administration of estrogen is known to increase SHBG levels in a dose dependent manner (Stege et al 1988, Mashchak et al 1982). The synthesis of SHBG in hepatocytes is strongly related to estrogen concentrations in blood (Mashchak et al 1982). Treatment with progestogens results in a decrease that is related to both the dose and the type of progestogen (Mainwaring et al 1995). The serum concentration of SHBG can be regarded as a marker for a woman’s estrogen/androgen status (Anderson 1974, Kahn et al 2002). Recently the possibility to use SHBG as a marker for the risk for VTE during COC treatment has been suggested (Odlind et al 2002). In paper II we present, for the first time, a biological plausible explanation for the suggested correlation between SHBG and VTE. There is a significant correlation between changes in APC resistance, the well accepted risk marker for VTE, and changes in plasma SHBG levels during use of the second as well as the third generation COC. SHBG is a stable and easily measured plasma protein for which there are reliable and cheap assays, and therefore a possible “surrogate marker” for the VTE risk during estrogen treatments.

In addition, in our study, the level of the free fraction of protein S increased during treatment with the second-generation COC in contrast to the decrease observed during use of the third-generation COC. The mechanism(s) underlying the opposing patterns remains unclear. Protein S circulates in plasma both as free protein S and also bound to C4b-binding (C4BP) protein. One comparative study on second- and third-generation COCs describes a decrease in C4BP during use of both COCs, more pronounced with the second-generation COC, and a decrease in the total protein S fraction with third-generation COC, whereas the total protein S concentration hardly changes at all with second-generation COC. These changes result in a decreased
free protein S concentration during use of third-generation COCs as opposed to an increased concentration during use of second-generation COCs (Kemmeren et al 2004).

EC is generally considered as harmless, and in many countries available without any medical examination or investigation of medical history. We found that use of EC also had significant effects on some coagulation parameters. Studies on contraceptives containing only progestogen, and given either as pills (Kemmeren et al 2004, Kuhl 1996) or as implants or injectables (Egberg et al 1998) mainly show no effects on hemostasis or effects opposite to the those observed during use COCs, Thus, for example contraceptives based on progestogen alone caused decreased nAPCsr, decreased FVII activity and increased AT activity. Surprisingly, we found signs of increased coagulation activity during use of progestogen alone as EC: plasma FVII and fibrinogen concentrations rose and AT concentration fell. In addition, we found increased levels of F 1+2 after both EC treatments, without any difference between the regimens. This has not been described previously. In fact, Egberg et al (1998) demonstrated decreased prothrombin activity during use of a progestogen-only implant. The reason for the activation of hemostasis after LNG-EC might be of the relatively high dose of LNG administered. However, in studies where progestogen is used in very high doses, e.g., in cancer therapy, the effects on hemostatic parameters are not conclusive. In some studies plasma AT, plasminogen and FX concentrations increase and fibrinogen, tPA and D-dimer concentrations decrease (Abe et al 1995, Kaibara et al 2001), in others no changes in these analytes have been observed (Oberhoff et al 2001). This discrepancy between different studies clearly indicates the need for further evaluation of EC use effects on hemostasis to prevent negative effects of the high availability of the treatment.

Since the phenomenon of APC resistance, acquired as well as inherited, has been of great interest in the field of obstetrics and gynecology during recent years, this was also addressed in the present thesis. Associations between increased levels of sex steroid hormones and acquired APC resistance are now well established. Pregnancy (Kjellberg et al 1999), hyperstimulation during in vitro fertilization (IVF) treatment (Curvers et al 2001) and COC (Curvers et al 2002) use all decrease the sensitivity to APC. Of note, APC resistance in the presence (Koster et al 1993) and absence (de Visser et al 1999, Rodeghiero et al 1999) of factor V\textsubscript{Leiden} mutation as well as acquired APC resistance during OC use (Tans et al 2003) increases the risk of DVT.

In the comparative study on COCs (paper II), we identified resistance to APC as one factor responding to the more estrogenic profile of the third-generation COC.
Here, we assessed APC resistance with an aPTT-based assay known as the original method for detecting APC resistance. With this method, the median APC ratio for non-treated women was 3.40, and it decreased to 3.20 with the second-generation COC and to 3.00 with the third-generation COC. The levels attained during COC use are well within the range seen in normal women without FV_{Leiden}.

Although it is fairly clear that APC resistance is related to estrogen levels in plasma, the various assay methods that have been used differ in their sensitivity to steroid hormone concentrations and thus the reported changes in APC resistance related to hormone levels also vary (Curvers et al 1999, Curvers et al 2002). An ETP-based assay (Nicolaes et al 1997) has turned out to be more sensitive than the original aPTT-based assay. Studies using the ETP-based assay have demonstrated that plasma from women using more estrogenic COCs (third-generation COCs) is less sensitive to the anticoagulant effect of APC i.e. is more APC-resistant than that of women using less estrogenic COCs (second-generation COCs) (Rosing et al 1999, Tans et al 2000).

In the EC study (paper IV), we used both assays and found an increase in APC resistance with both methods, although the aPTT-based kit could not discriminate between the estrogen-containing and the estrogen-free preparations, which the ETP-base assay could. With the ETP-based assay, one woman had a nAPCsr value of the same magnitude as women who are heterozygous for the factor V_{Leiden} mutation, but this was only seen at a single time point and consequently is probably without significance. Similarly, two participants, at two different time-points, had APCr below 2.20 with the aPTT-based assay.

In the study on APC resistance in women with a normal menstrual cycle, 12 women proved to be heterozygous carriers of the FV_{LEIDEN} mutation, three of whom had APCsr rates (aPTT-based assay) above the laboratory cut-off for abnormal APC resistance (2.50). One of them also had a nAPCsr (ETP-based) considerably lower than other carriers. These somewhat diverging results on APC resistance in relation to the presence of the FV-mutation raise concerns about which parameter most accurately identifies women at risk for VTE.

It is obvious from previous studies on associations between hemostatic risk indicators and risk of vascular disease that it is not yet possible to ascribe absolute levels of risk to a given level of a specific risk marker (Godsland et al 2000), not least since the interpretation of the hemostatic effects of COCs has frequently been based on relative changes from baseline rather than absolute levels. In our studies as
well in those of others (Middeldorp et al 2000, Meijers et al 2000a), essentially all participants had changes in coagulation and fibrinolytic markers that were confined to the normal reference intervals. This highlights the need for better risk indicators, or surrogate markers, for risk of cardiovascular events. During recent years, the need for a method describing the global hemostatic balance has become obvious; the old ones including aPTT and PK/INR, are not informative enough. Such new methods are now emerging, for example thrombin generation assays (Hemker et al 2003) and thrombelastographic methods (Sorensen et al 2003). Methods for quantifying APC resistance are sometimes regarded as global hemostatic methods, and as mentioned above different methods for measuring APC resistance are now available. APC in complex with the protein C inhibitor (PCI), i.e. the APC-PCI complex, serves as a marker of thrombin generation, so increased plasma levels of the complex were indicated to be a marker of hypercoagulability (Strandberg et al 2005). We have recently analyzed the APC-PCI complex in plasma samples from the COC study together (van Rooijen et al, unpublished data) (figure 10). An increased plasma concentration of the APC-PCI complex was seen during treatment with both COC regimens (p<0.001), as well as a significant difference between the two treatments (p<0.01). However, this method, as the other global methods, has no absolute cut-off for the risk for VTE.

Figure 10. Plasma levels of the ACP-PCI complex during luteal phases and COC use. Box-plots representing the median value with 50% of all data falling within the box, whiskers represent 10th, 25th, 50th, 75th and 90th percentile. Circles denote “outliers”
**Effects on risk indicators for atherothrombosis**

Atherosclerosis with superimposed thrombosis represents a complex process, involving many genetic and environmental factors. Use of COCs affects many of the established biochemical risk indicators detected in the past decades. One reason for introducing third-generation COCs was their superior effects on LDL and HDL, which at that point in time were believed to be the main pathogenetic risk factors involved in atherogenesis.

Changes in plasma lipoproteins induced by OC use have been investigated extensively (Crook & Godsland 1998). In agreement with earlier studies, we found that use of a third-generation COC induces a more favorable lipoprotein pattern regarding the HDL and LDL cholesterol concentrations. Simultaneously, we also describe a potentially harmful increase in total plasma triglyceride levels, which was more pronounced with the third-generation preparation. Estrogen in HRT reduces the development of early atherosclerotic lesions, in part through effects on lipid metabolism with accompanying beneficial consequences for the endothelium (Appt et al 2006, Topcuoclou et al 2005), and oxLDL seems to be less abundant in postmenopausal women using peroral estrogen replacement therapy (Ahotupa et al 2004). The reason is not clear, but it seems that estrogen itself has some advantageous effects on the oxidation process of LDL. The presence or generation of antibodies to oxLDL during use of COCs has not been studied earlier. In our study no changes in the levels of oxLDL antibodies were detected. The treatment period was probably too short to allow influences on oxidation, and the women studied may be too young to have oxLDL antibody levels amenable to reduction during such a short treatment period. The effect on the apolipoproteins of EC is an expected consequence of the prompt effect of steroid hormones on the liver protein synthesis. The clinical relevance of the changes in the perspective of atherosclerosis is probably negligible, but they highlight that steroid-induced changes in liver protein synthesis occur rapidly even after a very brief exposure.

The concept of atherosclerosis as a process related mostly to lipid metabolism, has now changed to a view of atherosclerosis as a chronic inflammatory response, induced mainly by LDL deposition in the arterial wall, but also through free radicals, micro-organisms, shear stress and environmental factors such as obesity and cigarette smoking. CRP has proved to be an important predictive factor for future CVD in women (Ridker et al 2000) and is therefore in focus in many studies of steroid therapy. During use of HRT as well as COCs, a slight but significant increase in CRP occurs (Silvestri et al 2003, Dreon et al 2003, Döring et al 2004). Although the magnitude of the increase is low, some women reach a level of 3 mg/L.
This level has been suggested as a cut-off for high risk of future CVD (Ridker 2003). Some studies indicate that estrogens may directly stimulate hepatic synthesis of CRP, rather than eliciting a systemic proinflammatory response (Silvestri et al 2003), which may be differentially modulated by different progestogens. Our study confirms this theory as no signs of activation of IL-6 or TNF-α were detected. The increase in SAA might also reflect an increased hepatic protein synthesis. We conclude that the rise in serum CRP concentration during treatment with COCs appears to result from a direct effect on hepatocyte synthesis of CRP, whereas it does not reflect IL-6 mediated inflammation, endothelial activation or induction of insulin resistance.

The time course of the CRP increase, in the study of EC treatment, is similar to the one seen in experimental endotoxin-induced CRP synthesis (Engelhardt et al 1990), or after initiation of an inflammatory process (MacIntyre et al 1982). This strongly indicates that the increase in serum CRP is secondary to de novo synthesis of CRP.

Whether an increase in CRP concentration, independent of inflammation, contributes to the atherosclerotic process is not fully clear. However, some data suggest that CRP is not merely a risk marker for arterial occlusive disease, but also a causative factor. The CRP molecule can be taken up into an existing plaque, or be synthesized in the plaque, and mediate the uptake of both native LDL particles and oxLDL into the plaque (Zwaka et al 2001). The uptake of LDL induces a transformation of macrophages into “foam cells”, which are characteristic of evolving atherosclerotic plaques. Thus, CRP itself may contribute to plaque formation. Epidemiological data also provide evidence that inflammatory diseases increase the risk of CVD (Ross 1999, Chung et al 2007).

The effects of COCs on lipoprotein metabolism are of importance because of the involvement of lipoproteins in endothelial dysfunction, atherogenesis and development of CVD. Two of the markers for endothelial activity chosen for this study, vWF and FVIII, did not show any changes during treatments, nor were any differences observed between the two studied COC preparations. However, other studies have demonstrated increased levels of vWF and FVIII during COC treatment (Kluft & Lansink 1997, Middeldorp et al 2000). There was a decrease in E-selectin during use of COCs, slightly more pronounced with the third-generation COC. However, healthy young women without any risk factors for atherosclerosis have a low E-selectin concentration, the further lowering of which probably lacks clinical significance. Estrogen is known to have beneficial effects on the body’s response to inflammatory stimuli. Maybe the decreased plasma E-selectin concentrations in the study population mirror this effect rather than a direct effect on the endothelium.
COCs in the perspective of venous and arterial occlusive disease

Vascular diseases in COC users have usually been interpreted in relation to the general features of the most common occlusive disorders, i.e. VTE and atherosclerosis. However, these manifestations are end stages after what is sometimes a long period of dysfunctional interactions between the vascular endothelium and the hemostatic system.

The increase in risk of VTE during use of COCs is unquestionable. Simultaneously, it is clear that the ways currently used for identifying individuals at risk (i.e. a detailed case history including family history of VTE and atherothrombosis and a clinical examination) are inadequate predictors of which women will develop VTE. A potential relationship between incidence of VTE and increase in SHBG based on reports to the Swedish Medical Products Agency was demonstrated in a study by Odlind et al (2002). Our findings of the correlation between SHBG and APCresistance, as confirmed in a later study (van Vliet et al 2005), might be a biological plausible explanation for the link between SHBG and VTE. Measurement of changes in SHBG or global methods after a trial period might be helpful tools to indicate which hormonal contraceptive methods might be associated with increase in risk of VTE for users of the specific preparation.

The risk markers for arterial occlusive disease evaluated in this thesis point towards an unfavorable pattern during use of COCs (i.e., prothrombotic effects on the hemostasis, increased TG levels accompanied by increase in FVII, decrease in IGF-I along with increase in its binding proteins IGFBP-1 and IGFBP-3 and raised plasma CRP concentration) and the effects appear more pronounced in women using third-generation COCs. However, experimental studies suggest that estrogen increases serum concentrations of nitric oxide and flow mediated vasodilatation during treatment of menopausal women. Also, a favourable reduction of carotid artery intima–media thickness and coronary arterial calcifications are described in studies on estrogen treated menopausal women (Miller et al 2006).

The facts that coagulation abnormalities subsides when women stop taking them and that past users of oral contraceptives have no increased risk of VTE (Hannaford 2000), MI or stroke (Stamfer et al 1990, Colditz 1994) support the view that COCs influence the risk of CVD through prothrombotic rather than proatherogenic mechanisms. It has been shown that arterial thrombosis during COC use is closely
related to changes in lipoprotein metabolism (Kemmeren et al 2001), whereas VTE is associated with changes in the hemostatic balance. Previous studies suggested an association between coagulation factors and serum lipids. Hypertriglyceridemia is frequently accompanied by high levels of fibrinogen and FX, as well as by low fibrinolytic activity (Simpson et al 1983). The correlation between TG and FVIIa is demonstrated in paper I. As the presence of FVIIa increase the readiness for activation of the coagulation and the thrombus formation, FVII and FVIIa might be one of the links between arterial and venous occlusive disease. In the process of acute arterial occlusion, a plaque rupture occurs and TF is expressed on the surface of the damaged vessel wall (Hansson 2005). During COC use the level of TG related FVIIa is increased and hemostasis is tilted toward a more procoagulant state that might favor formation of a thrombus at the ruptured plaque, even though the situation in a non-COC user would stay silent and subclinical. The liver-related changes in lipids and plasma proteins synthesized in the liver, along with the possibility that CRP is merely a marker of enhanced protein synthesis support this theory that procoagulant mechanisms underlie the transiently increased risk of arteriothrombotic disease during COC use.

Judging from the contradictory results of individual studies, the risk of developing arterial occlusive disease during COC remains unclear. However, meta-analysis showed an enhanced risk of MI and stroke during use of COCs (Baillargeon et al 2005). Nonetheless, in direct comparisons between second- and third-generation COCs no significant differences were detected for risk of MI (WHO 1997, Dunn et al 1999, Tanis et al 2001) or stroke (Kemmeren et al 2002, Lidegaard & Kreiner 2002). Thus, although the third-generation oral contraceptives were developed to reduce the risk of arterial thrombosis, no such effect has been unequivocally demonstrated.

The rapid activation of hemostasis and liver-synthesized plasma proteins such as fibrinogen, CRP and SHBG, after use of EC reminds us of the importance of being aware of the risk for VTE whenever exogenous steroid hormones are administered, even in situations involving short treatment times. It is probably particularly important to monitor individuals or patients with a genetic predisposition or a transiently disturbed hemostatic balance in whom even small changes like the ones seen in the present study might pose a threat. Still, the finding of activation of hemostasis after progestogen alone EC treatment needs further evaluation. In view of the fact that the large epidemiological study on VTE in conjunction with EC could not detect any increased risk and that there is only one published case report on thrombosis
in relation to the treatment, one must regard it as safe. In most countries, today, the recommended EC is the regimen that uses progestogen alone. It is regarded as more efficient and has less unwanted side effects, i.e., nausea and vomiting.

Even though there are negative effects during use of COCs, one has to take into consideration both the very low absolute risk for occlusive vascular disease; which in the case of VTE is lower than that observed during pregnancy. Additionally, COCs have many beneficial effects, which are not present during use of estrogen–free/progestogen-only methods, i.e., reduced risk of endometrial and ovarian cancer, reduced incidence of benign breast neoplasms and an improved menstrual regularity.

Different administration forms for COCs, such as patches, injectables or vaginal rings, new types of progestins, and in a near future also other types of estrogens, might alter the risks of VTE and CVD in COC-users. So far, however, epidemiological studies have not yet proven that the latest preparations offer any significant improvement in benefit.

For women at high risk of venous or arterial occlusive disease the alternative of an estrogen-free contraceptive method must obviously be available. To determine whether or not a woman is at risk one must take into account all the common/additive genetic risk factors (known thrombophilias or heredity) as well as the well-known environmental risk factors that have not been mentioned here, for example obesity, smoking and hypertension.
Methodological considerations and future perspectives

Some limitations of the studies in this thesis should be addressed. In the two treatment studies the number of participants was low and certainly the results must be interpreted with caution. Also in study I (papers 1-3) the drop out rate was 9 out of 44 women originally included. However, using a cross-over design all women served as their own control. All samples from each individual woman were run simultaneously. The lack of baseline blood samples after wash out could be regarded as a weakness, but no significant carry-over effect were detected at statistical analysis.

The EC study (IV) lacked a “placebo” or control group in which the participants were sampled without medication to control for the circadian variations of the factors analysed. When the study protocol was prepared the use of progestogen-only EC was not registered for use in Sweden, and we were not aware that the use of the EE containing preparation was going to decline only few years later. Therefore we attributed great importance to the comparison of the two different regimens.

Some coagulation factors and lipids display a circadian variation, and may also be interfered by food intake and physical activity. In study I (papers 1-3) and III (paper V) all samples were obtained in the morning after an overnight fasting. In the EC study (paper IV) according to the design samples were drawn at different hours and a complete fasting was not feasible. A larger patient material and the inclusion of a control group would have corroborated the results from this first pilot study. Five out of eleven women in this study were smokers, but there were no indications of a change in this respect during the study period. Still, all individual women were their own controls and all samples were obtained after 20 minutes rest in the supine position. There were distinct differences between the two treatment regimens indicating only a limited effect of circadian variations and food intake. However, the activation of hemostasis after EC with progestogen alone needs further confirmation.
The fact that there were no restrictions about age or weight in the study examining the menstrual cycle (paper V) meant that some women were “old” and some obese. Narrowing the inclusion criteria might have given a study population containing fewer non-ovulating women. However, the ones without ovulation were dispersed regarding age and BMI, and the results did not significantly change when they were excluded.

**Future perspectives**

Improved insight into the mechanisms of steroid hormone-induced venous and arterial thromboembolism will enhance the possibility to prevent these serious, and sometimes fatal, side-effects of treatment with estrogen and progestogen in different therapeutic situations.

One way to increase knowledge about the hemostatic balance in a specific situation is the development of different global methods. For example, APC resistance and the APC-PCI complex are demonstrated to reflect the increased risk for VTE during use of third- versus second-generation COCs as well as the increased risk compared to that in non-users of COC in study populations. However, even though some hemostatic risk indicators seem to be well correlated to the risk of developing VTE, there are other contributing factors making the risk estimation more complex. Many women with inherited or acquired risk factors use COCs for years without developing VTE. Therefore, other methods for identifying specific women with high risk for VTE, not only during COC use, but in all situations of increased estrogen levels, are needed. The individual sensitivity to estrogen, and the associated changes in plasma proteins and hemostatic factors probably vary. To study women with previous or present VTE during estrogen treatment is therefore a valuable way to learn more about the individual differences.

As previous discussed, the risk of atherothrombosis seems to be related more to a procoagulant state than to a proatherogenic state, but classic environmental risk factors for atherosclerosis, i.e., hypertension, smoking and diabetes, strongly increase the risk of MI and stroke in women using COCs. This indicates the need for studies of the endothelial function during COC use.

Furthermore, hemostatic factors as well as the individual response to inflammatory stimuli and other risk factors for atherothrombosis are genetically determined. In the future when we know more about the polymorphisms, or combinations of
polymorphisms, involved in the atherothrombotic and venous thromboembolic process, the genetic background should be taken into account when COCs (or other medications) are prescribed. It is also of importance to investigate the extent to which environmental and inherited risk factors interact in the process of atherothrombogenesis and venous thromboembolism.

As many hemostatic and metabolic effects of COCs are mediated through a direct effect on the liver and the hepatic protein synthesis, new parenteral administration of combined contraceptive preparations have been introduced on the market with the purpose to avoid this first passage. However, the few reports on hemostatic effects during use of patches and vaginal rings do not yet prove the superiority of these alternative means of administrations.

Modern medicine is obliged to base its recommendations on scientific evidence concerning the benefits and risks involved in different treatments. In the EC study we observed rapid hemostatic activation not only after use of estrogen-containing EC, but also after progestogen alone. Large studies on the risk of VTE and atherothrombotic disease during the use of “high dose” progestogen-only contraceptive methods and new “combined contraceptives” (i.e., new combinations, doses and ways of administration) are therefore necessary. In addition, it is important to compare the effects of extended use of combined preparations versus the usual treatment regimen designed to allow monthly wash-out periods, to see if the two treatment patterns have different effects on risk indicators for CVD and VTE.

The last study on normal healthy non-treated women was performed in attempt to detect alterations in hemostasis related to physiological increase in endogenous estrogen levels. The same situation, although more pronounced, is present in pregnancy and hyperstimulation during IVF. Further studies on the difference between high endogenous estradiol levels and high levels of exogenous administered estrogens on endothelial function and other risk indicators for CVD would be of interest. The menstrual cycle study will expand and proceed into studies on pregnant women with and without preeclampsia.

Methodological considerations and future perspectives
Conclusions

- Combined oral contraceptives with the same amount of estrogen but either levonorgestrel or desogestrel as progestogen display clear differences in their effects on hemostasis and lipoproteins. The increases in APC resistance, fibrinogen and TGs are more pronounced during use of the third-generation COC.

- There is a positive correlation between the amounts of circulating FVII and TG in plasma of COC users. Furthermore, both these risk factors show higher concentrations during use of the third-generation as compared to the second-generation COC. On the other hand, estrogen in combination with desogestrel exerts a beneficial effect increasing the levels of HDL cholesterol.

- For the first time we demonstrated an association between the well-established risk factor acquired APC resistance and plasma SHBG concentrations. During use of COCs the change in APC resistance occurs in parallel to changes in SHBG. These results support the notion that SHBG may serve as surrogate marker for an the prothrombotic risk state induced by different COC preparations.

- The increased serum CRP concentration during treatment with COCs appears to reflect a direct effect on hepatocyte CRP synthesis rather than an IL-6 mediated inflammation, endothelial activation or induction of insulin resistance.

- Even after a very short exposure to high levels of exogenous sex hormones there is a prompt effect on hepatic protein synthesis. Already two hours after administration of EC there is a marked change in AT, fibrinogen and F1+2 as well as SHBG. Activation of protein synthesis also involves an increase in the concentration of some important procoagulant factors, which trigger the coagulation cascade and initiate an activation of coagulation and fibrinolysis. This rapid effect on the hemostasis might be of importance for individuals with a genetic predisposition or transiently disturbed hemostatic balance or in various clinical situations.

- The physiological fluctuations in endogenous sex steroid hormones during menstrual cycle do not significantly affect the APC resistance.
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