Hormonal contraceptives

and

venous thrombosis

Bernardine H. Stegeman

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	Prof. Dr. F.R. Rosendaal
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	Prof. Dr. J. Rosing (Maastricht University)

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Introduction

Steroid hormone use and venous thrombosis

In 1960, shortly after the introduction of combined oral contraceptive Enovid ® (150 µg mestranol, an estrogen, and 985 mg norethynodrel, a progestagen), the first case of venous thrombosis associated with contraceptive use was reported[1]. Since then many observational studies have been conducted to assess the association between combined oral contraceptives and venous thrombosis. Overall, combined oral contraceptive use is associated with a two-fold to six-fold increased risk of venous thrombosis[2–5]. Nowadays many women worldwide use oral contraceptives making the impact of oral contraceptive use on venous thrombosis risk large, despite the low incidence of venous thrombosis of about 3 per 10,000 woman-years among women of reproductive age[6].

The causality between hormone use and venous thrombosis can be discussed using Hill's criteria of causality[7]. A review by Vandenbroucke et al[8] showed that the association between combined oral contraceptive use and venous thrombosis was consistent and of the same strength over several observational studies. The plausibility of the association is strengthened by the effect of oral contraceptives on the levels of several coagulation factors and the resulting shift in the balance of coagulation towards a prothrombotic state. However, the mechanism behind this effect remains unclear.

An outline of the current state of literature is given on the risk of venous thrombosis associated with hormone use and the biological mechanism that may explain the prothrombotic effect. First, the association between hormone use and the risk of venous thrombosis will be evaluated. The following applications of estrogens or progestagens will be addressed; contraception, relieve of menopausal symptoms, restriction of tall stature and sex change. Secondly, a hypothesis will be derived on whether estrogen, progestagen or combination of estrogen and progestagen may lead to venous thrombosis. Thirdly, the effect of hormone

use on coagulation factors, activated protein C resistance and sex hormone binding globulin levels will be evaluated. Lastly, the outline of this thesis with the research questions will be proposed.

Hormonal contraception

Hormonal contraception is a birth-control method to prevent ovulation and thus pregnancy. Hormonal contraception consists of steroid hormone use in two main types of formulations; combined formulations which contain both estrogen and progestagen and progestagen-only formulations. Progestagen suppresses the surge in luteinizing hormone (LH) and thereby prevents ovulation. Estrogen reduces the secretion of follicle-stimulating hormone (FSH) and thereby inhibits folliculogenesis. The estrogen compound has a major role in drug compliance; by increasing the stability of the endometrium, breakthrough bleeding and spotting are reduced. Hormonal contraception is prescribed to regulate the uterine, menstrual cycle or for other hormonally dependent disorders as acne[9] and hirsutism[10].

The most commonly used estrogen in combined hormonal contraceptives is ethinylestradiol, whereas different types of progestagens are used in combined or progestagen-only contraceptives. Contraceptive progestagens can be categorised according to the time of their introduction (first, second and third generation, respectively introduced in the sixties, seventies, and eighties of the last century) or according to their tetracyclic structure[11], i.e. estranes (derivatives of testosterone), pregnanes (derived from the progesterone molecule) and gonanes. The following progestagens correspond to first generation progestagens: norethisterone (NET), ethynodiol diacetate, lynestrenol (LYN), and norethynodrel. Levonorgestrel (LNG) and norgestrel (NG) correspond to second generation progestagens and third generation progestagens are desogestrel (DSG) and its active metabolite etonogestrel, gestodene (GSD), and norgestimate (NGM) and

its active metabolite norgestromin (NGMN). Examples of other progestagens used in hormonal contraceptives are cyproterone acetate (CPA), chlormadinone acetate, nomegestrol, drospirenone (DRSP) and medroxyprogesterone acetate (MPA). These last progestagens are classified as pregnanes based on their structure. Estranes are comprised of the first generations progestagens, while the second and third generation progestagens belong to the gonanes.

Steroid hormones can be administered via different routes or applications, such as orally (pill), intrauterinely (intrauterine device (IUD)), transdermally (patch), subcutaneously (injectable or implant), or transvaginally (ring). The most commonly used route for combined formulations is orally and occasionally transdermally or transvaginally. Progestagen-only formulations are administered orally (mini pill) as well as subcutaneously or intrauterinely.

Combined hormonal contraceptives Several large studies[12–15] in the 1990s have confirmed the two-fold to four-fold increase in risk of venous thrombosis associated with oral contraceptive use, which was already shown in four studies[2–5] from the late 1960s. The risk of venous thrombosis is the highest in the first three months of combined oral contraceptive use, i.e., about twelve-fold increased compared with non-users[16–18]. With extended use the risk decreases to an approximately five-fold increased risk. Because the estrogen compound in combined oral contraceptives was thought to cause the increased risk of venous thrombosis, the dose of estrogen has been lowered from 150-100 µg after the introduction of the oral contraceptive to 50 µg in the 1960s to 30-35 µg and 20 µg in the 1970s[19,20]. The lower dose of ethinylestradiol in combined oral contraceptives was associated with a reduction in venous thrombosis risk[12,21–24]. The currently prescribed combined oral contraceptives containing 30 µg of ethinylestradiol are associated with a higher risk of venous thrombosis than contraceptives containing 20 µg[17,18].

Besides adjustments in the dose of ethinylestradiol, the progestagen compound was changed to reduce side effects of oral contraceptive use. After the introduction of the third generation progestagens as part of the combined oral contraceptives in the eighties, the risk of venous thrombosis among users of those compounds was investigated. It was shown that third generation oral contraceptive users have a higher risk of venous thrombosis compared with second generation users[8,16–18]. However, these results were disputed by reasoning that bias or confounding could explain the difference in risk between these progestagens. These issues were addressed in an opinion article[25] and a meta-analysis[16] in which it was shown that the presence of bias or confounding could not explain the observed results. Other progestagens have been developed after the introduction of the third generation progestagens, e.g., drospirenone (introduced in 2001) and dienogest (introduced in 1995). The use of drospirenone in a combined oral contraceptive has been shown to increase the risk of venous thrombosis[17,18] compared with non-use and compared with second generation contraceptive use[26,27]. No information concerning the risk of venous thrombosis is available for the contraceptive containing dienogest because this contraceptive is mainly prescribed in Germany[28].

To diminish the risk of venous thrombosis, it was attempted to prevent the first-pass effect of steroids. Because of oral intake of steroid hormones, the metabolism in the liver was thought to be important in the pathogenesis of venous thrombosis. After a drug is ingested, it is absorbed by the digestive system and enters the liver through the hepatic portal system[29]. The liver metabolizes many drugs before they enter the remaining circulation. The first-pass through the liver may greatly affect the bioavailability of an ingested drug[29], hence the name first-pass metabolism. This led to the hypothesis that transdermal and transvaginal administration of estrogens and progestagens may reduce venous thrombosis risk. The application of hormones via the skin or vagina bypass the first-pass effect in the liver be-

cause the hormones are first distributed to other organs and later, diluted, to the liver[30].

The information concerning the risk of venous thrombosis with vaginal ring or transdermal patch use is lacking due to their fairly recent introduction onto the market in 2001 and 2002, respectively and due to a limited number of users of these types of contraceptives. However, two case reports concerning mesenteric vein thrombosis[31] and cerebral venous sinus thrombosis[32] were reported in two vaginal ring users suggesting a potential association between vaginal ring use and thrombosis. Furthermore, a deep vein thrombosis was reported as a serious adverse event in vaginal ring users in each of two trials (of which one randomized controlled trial (RCT)) evaluating the efficacy and tolerability of the vaginal ring compared with a second generation combined oral contraceptive[33,34]. The FDA showed in a large cohort study that vaginal ring use increased the risk of venous thrombosis compared with combined oral contraceptive users[35]. This result was confirmed by another large cohort study conducted in Denmark[36].

The risk of venous thrombosis in transdermal patch users has been assessed in two observational studies using health insurance databases. Both studies were supported by the manufacturers. The first study reported no difference in venous thrombosis risk between transdermal patch users and users of a third generation combined oral contraceptive[37]. The second study, published one year later, reported an increased risk of venous thrombosis in patch users compared with third generation oral contraceptive users[38]. These results were confirmed in updated analyses of both studies[39,40]. After these results the US Food and Drug Administration (FDA) issued the following warning on 22 January 2008: "FDA believes that Ortho Evra is a safe and effective method of contraception when used according to the labeling, which recommends that women with concerns or risk factors for serious blood clots talk with their health care provider about using Ortho Evra versus other contraceptive option"[41]. In 2011,

the FDA performed their own study and showed that the use of the transdermal patch increased the risk of venous thrombosis compared with combined oral contraceptive users[35]. This result was confirmed in a large cohort study from Denmark[36].

In summary, the use of combined hormonal contraceptives is associated with an increased risk of venous thrombosis whether the hormones are administered in a pill, in a vaginal ring or in a transdermal patch.

Progestagen-only contraceptives Injectable progestagen-only contraceptives were developed in the late 1950s and early 1960s as a result of a growing understanding of steroid hormones and the research into oral contraceptives. In the late 1960s, the first oral progestagen-only contraceptive was developed when concerns were raised about the side-effects of combined oral contraceptives. Progestagen-only implants were developed in 1960s and 1970s and the hormone-releasing IUD was developed in the early 1970s.

Four case-control studies have assessed the risk of venous thrombosis associated with progestagen-only pills (POP) for contraceptive use: three studies[23,42,43] reported a potential increase in risk compared with either non-users or users of combined oral contraceptives containing levonorgestrel, whereas one study[44] reported a decrease in risk compared with non-users. The risk of venous thrombosis was not separately assessed per type of progestagen. A cohort study reported no increased risk of venous thrombosis in users of levonorgestrel or norethisterone or in users of desogestrel, although a relatively small number of women were using these contraceptives[17].

Information about the risk of venous thrombosis in implant users is lacking, probably due to the low number of women using this type of contraceptive. A large cohort study showed that the use of an implant was associated with an increased risk of venous thrombosis compared with non-use[36]. Three studies[42,45,46] have investigated the use of injectables containing MPA and all three

reported an increased risk of venous thrombosis relative to non-users. Regarding IUD use, two observational studies showed that the use of an IUD containing levonorgestrel was not associated with venous thrombosis[17,45].

In a nested case-control study, the risk of venous thrombosis was not increased in users of progestagen-only contraceptives (i.e., POP, injectable, and implant combined) compared with non-users[47]. Venous thrombosis risk per type of administration was not evaluated.

Currently, no definitive conclusion on the risk of venous thrombosis associated with progestagen-only contraceptives can be drawn due to variation in the progestagen used, the dose and mode of administration, and because a small number of women use progestagen-only contraceptives. However, there is an indication that users of IUD containing levonorgestrel or users of oral levonorgestrel have the same risk of venous thrombosis as non-users, whereas users of injectable methoxyprogesterone acetate have an increased risk.

Hormone replacement therapy

Hormone replacement therapy (HRT) is mainly administered to relief hot flushes caused by diminishing estrogen levels as a result of failing ovaries (e.g., premature ovarian failure or surgically caused menopause) or physiological menopause[48]. Estrogen-only HRT is prescribed to women without a uterus. In women with a uterus, the estrogen compound is combined with a progestagen as progestagen is needed to shed the developed endometrium (when progestagen is sequentially administered) or to prevent endometrial hyperplasia caused by estrogen (when progestagen is continuously administered). Estrogens or the combination of estrogen and progestagen are administered orally or transdermally via a patch. An estrogen implant can be used as well, although it is not commonly applied. Besides the relief of symptoms of menopause, HRT was thought to prevent osteoporosis

and cardiovascular diseases. It was hypothesized that the risk of osteoporosis and cardiovascular diseases were due to the drop in estrogen levels caused by menopause and that the risks could be prevented by replenishing estrogen with HRT.

Combined and estrogen-only administration In 1895, it was suggested that ovarian secretions could be used to treat ovarian failure and in 1896 the first therapeutic interventions were reported[49]. Over the course of the next thirty years, the ovarian hormones were identified, i.e., estrone, estriol, estradiol and progesterone. In the 1930s estrogen was used as a therapy in women with premature menopause (onset of menopause before the age of 40)[50]. Use of estrogen-only HRT became widespread in the 1960s and 1970s. However, in the 1970s it was shown that women with an intact uterus using estrogen-only therapy were at an increased risk for endometrial cancer[51]. Thereafter, combined HRT was given to women with a uterus and estrogen-only to women without a uterus[52]. Since then many observational studies were conducted to establish whether HRT was protective against cardiovascular diseases. A meta-analysis of observational studies published in 1991 showed that the use of HRT was protective against cardiovascular diseases[53]. However, the hormones used and dose and the mode of administration was different across the studies making a comparison across studies difficult. Furthermore, women taking HRT may be different from women not taking HRT which can influence the results obtained in observational studies. These issues are reduced in trials where women are randomized to receive HRT or a placebo.

The HERS study[54] (Heart and Estrogen/progestin Replacement Study) was one of the first trials showing that HRT use did not reduce the rate of secondary coronary heart disease. In this RCT, women with established coronary disease were randomized to receive either combined HRT or a placebo. In a Cochrane review from 2005[55], the prevention of cardiovascular disease in postmenopausal women using HRT was assessed. A total of ten

RCTs were included of which two trials included healthy women and eight trials included women with heart disease. No protective effect of HRT was seen for any of the arterial outcomes assessed. One of the secondary outcomes evaluated was venous thrombosis. Combined oral HRT (i.e., conjugated equine estrogens (CEE) with MPA) was associated with a twofold increased risk of venous thrombosis. One trial used 17β-estradiol with norethisterone and also found an increase in risk[56]. Estrogen-only oral HRT (i.e., 17β-estradiol) was not associated with venous thrombosis; however, only two RCTs (i.e., SPRIT 2002[57] and WEST[58] trial) contained data on estrogen-only HRT. The Women's Health Initiative (WHI) group conducted the WHI Conjugated Equine Estrogen (CEE) trial published in 2006[59,60] (not included in the aforementioned Cochrane review) and evaluated the use of estrogen-only oral HRT on major disease incidence rates. The results from this WHI CEE trial[60] were that the use of estrogen-only oral HRT increased the risk of venous thrombosis although less pronounced than with the use of combined oral HRT (evaluated in the WHI E+P trial[61]). The results from the WHI CEE trial are in contrast to the results from the SPRIT 2002 and WEST trials which can be explained by the small number of events in the last two trials (a total of 9 and 7 events, respectively) compared with 179 events in the WHI CEE trial. Furthermore, both the SPRIT 2002 and WEST trial used 17β-estradiol as estrogen and included women with a first myocardial infarction (SPRIT 2002 trial) or women with an ischemic stroke or transient ischemic attack (WEST trial), whereas the WHI CEE trial used CEE as estrogen and included healthy women without a uterus. Besides data from RCTs, observational studies have also shown that HRT use was associated with venous thrombosis. A matched case-control study[62] showed that all types of HRT were associated with a 3.5-fold increased risk for venous thrombosis compared with non-users. A nested case-control study[63] confirmed these results showing that HRT use increased the risk of venous thrombosis 2.1-fold compared with non-users. Data col-

lected from observational studies and RCTs regarding HRT use and the risk of venous thrombosis were summarized in a meta-analysis. Both data from observational studies and RCTs showed a twofold increased risk for venous thrombosis when using oral HRT[64].

In addition to 17β-estradiol and CEE, esterified estrogens can also be used in HRT. Like CEE, esterified estrogens are a combination of naturally occurring estrogens and their conjugates, but in different relative amounts. In a case-control study[65], the risk of venous thrombosis with the use of esterified estrogens and CEE with or without MPA in HRT has been evaluated. Compared with non-users, the use of esterified estrogens with or without MPA did not increase the risk of venous thrombosis. Among hormone users, the use of CEE with or without MPA increased the risk of venous thrombosis in comparison to users of esterified estrogen without MPA.

No RCTs were conducted with HRT administered through a patch. In total four studies, i.e., three case-control studies including the ESTHER study[62,63,66,67] and one cohort study (the E3N cohort study)[68] evaluating the risk of venous thrombosis with HRT use provided data on the thrombotic risk of transdermal administered steroid hormones. Two case-control studies[62,63] reported that after adjustment for multiple risk factors the risk of venous thrombosis was increased with the use of transdermally administered HRT. However, no information was provided on the type of estrogen and whether in addition to estrogen, also a progestagen was supplied. The ESTHER study and the E3N cohort study, both conducted by the same research group, reported no increased risk for venous thrombosis in transdermal HRT users. In these studies, mostly 17β-estradiol was used. It is unclear whether this was estrogen only or combined with a progestagen. However, in an earlier publication of the ESTHER study[67] the addition of a progestagen to transdermal 17β-estradiol did not influence the risk of venous thrombosis.

In summary, the use of combined oral HRT is associated with

an increased risk of venous thrombosis. For estrogen-only oral HRT there is a strong indication that this is associated with an increased thrombotic risk as well. With regard to transdermal administered HRT, no firm conclusions can be drawn because only a small number of women used this type of administration.

Other sex hormone applications

Growth inhibition Tall stature is defined as the height of an individual two standard deviations above the corresponding mean height for a given age, sex and population group [69]. The strongest increase in height occurs when estradiol levels are low, although a direct relationship between estradiol and growth hormone levels is not yet established [69]. Sex steroids, estrogens for females and androgens for males, are used to limit the expected height in tall children. High doses of gonadal steroids, especially estrogens, accelerate bone maturation. In tall girls, 100-200 µg of ethinylestradiol is continuously orally administered and in the last 7-10 days of each month together with a progestagen to shed the developed endometrium. Since higher doses of the same hormones as in combined hormonal contraceptives are used, the question is whether the treatment for tall girls is also associated with an increase in risk of venous thrombosis.

Venous thrombosis during hormone treatment for tall stature has been reported only sporadically [70,71], and all such cases have occurred in a clinical situation involving an elevated risk for venous thrombosis, e.g., immobilisation or surgery. Venous thrombosis is much less common in children (i.e. 1 in 100,000 person per year) than in adults (i.e. 1 in 1,000 persons per year), and any venous thrombosis event in this young age group is usually due to a combination of multiple inherited and acquired risk factors [72].

Sex change Transsexuals denote individuals who desire to live permanently as a member of the opposite sex and who want to

undergo sex reassignment. To this end, transsexuals receive hormone therapy for life. Female-to-male transsexuals receive androgens to induce male body features, whereas male-to-female transsexuals receive either gonadotrophin-releasing hormone (GnRH) agonists or progestational compounds (e.g., CPA) to suppress the original male sex characteristics and subsequently ethinylestradiol (100 µg per day) to induce female body features. The dose of progestagen is about fifty-fold higher and the estrogen dose is about three times higher than in combined hormonal contraceptives. One of the adverse events of this hormone therapy is venous thrombosis. The occurrence of venous thrombosis in male-to-female transsexuals receiving oral progestagen and estrogen was 45-fold increased compared to the general population[73]. Because of the high incidence of venous thrombosis during this study, the administration route was changed from oral to transdermal in male-to-female transsexuals over the age of 40. The corresponding occurrence of venous thrombosis was still twenty-fold increased compared with the general population[74]. Of the 36 unprovoked cases, 21 transsexuals experienced a venous thrombosis in their first year of hormone therapy resembling the same risk pattern as in combined oral contraceptive users.

In conclusion, the use of orally administered hormones to induce female body characteristics in male-to-female transsexuals is associated with an increased risk of venous thrombosis.

Towards a mechanism of combined oral contraceptive induced venous thrombosis

Hypothesis

After reviewing the literature regarding steroid hormone use and venous thrombosis, we summarize several associations. The use of combined oral contraceptives is associated with an increased

risk of venous thrombosis. Compared with combined oral contraceptives containing a second generation progestagen (i.e., levonorgestrel), third generation contraceptives and contraceptives containing other non-classified progestagens induce a higher risk of venous thrombosis. Furthermore, the dose of ethinylestradiol in combined oral contraceptives is positively and monotonously gradedly associated with the risk of venous thrombosis. Regarding contraceptives containing solely progestagen, only associations concerning progestagens levonorgestrel and MPA can be summarized. Levonorgestrel administered orally or intrauterinely without ethinylestradiol is not associated with venous thrombosis, whereas the progestagen MPA administered via injection increases the risk. Finally, the use of combined and estrogen-only HRT administered orally increases the risk of venous thrombosis as well. In general, the use of orally administered synthetic sex steroid hormones (combined therapy either for contraceptive use or for HRT) is associated with an increased risk of venous thrombosis (Table 1.1).

Regarding the association between oral contraceptives and venous thrombosis, the progestagen levonorgestrel seems to have a unique role. Combined with ethinylestradiol, levonorgestrel users are at an increased risk of venous thrombosis, but not as much as users of a third generation progestagen or other non-classified progestagens, whereas the sole use of levonorgestrel (i.e., IUD or oral) does not increase the risk of venous thrombosis. Furthermore, levels of ethinylestradiol over 24 hours are lower in levonorgestrel users than in desogestrel users while receiving the same dose of ethinylestradiol (30 µg) [75]. Overall, levonorgestrel appears to be able to modify the effect caused by ethinylestradiol, whereas third generation progestagens seem to lack this ability.

Based on the literature it is difficult to determine whether estrogen, progestagen or combination of both is pivotal in the pathogenesis of venous thrombosis. However, based on the association between ethinylestradiol dose in combined oral contraceptives and venous thrombosis, it is likely that ethinylestradiol

Table 1.1: The risk of venous thrombosis for different applications of sex steroid hormones in users versus non-users

Application **Progestagen**	**Oral**	**Patch 3rd**	**Ring 3rd**	**Injectable MPA**	**IUD LNG**	**Implant 3rd**
Contraceptive use						
EE + P	+	+	?	NA	NA	NA
P	-/+*	NA	NA	+	-/+	?
HRT use						
E + P	+	?	NA	NA	NA	?
E	+	?	NA	NA	NA	?
Growth restriction						
EE + P	?	NA	NA	NA	NA	NA
Sex change (♂ → ♀)						
EE + P	+	?	NA	NA	NA	NA

EE, ethinylestradiol; E, estrogen; P, progestagen; NA, not applicable; +, increased risk of venous thrombosis; -/+ no association with venous thrombosis; -, decreased risk of venous thrombosis; ?, no data available

* No increased risk in levonorgestrel users compared with non-users

plays a role in the pathogenesis of venous thrombosis. The question is how ethinylestradiol leads to an increased risk of venous thrombosis. Because ethinylestradiol is a synthetic hormone and can be orally administered, the first-pass metabolism in the liver may play an important role. The first-pass metabolism in the liver is known to influence the bioavailability of many synthetic drugs. Several coagulation factors are produced in the liver making it likely that ethinylestradiol can influence the production of coagulation factors. However, the use of a transdermal patch or a vaginal ring that bypass this first-pass metabolism, is also associated with an increased risk of venous thrombosis suggesting that the first-pass metabolism of ethinylestradiol may not be the sole player in the pathogenesis of venous thrombosis. A study

reported that the area under the curve (AUC) of ethinylestradiol levels over 24 hours in vaginal ring and transdermal patch users are larger than with oral administration [76]. Therefore, total levels of ethinylestradiol may play a role as well.

In the Netherlands, the combined oral contraceptive is the most popular birth control method making the risk of venous thrombosis a realistic concern. Currently, it is still unclear how combined oral contraceptives, in particular ethinylestradiol, can cause venous thrombosis. The focus of this thesis is on the role of ethinylestradiol in the pathogenesis of venous thrombosis in premenopausal women.

Effects on coagulation and markers of coagulation or venous thrombosis

In 2005, the European Medicines Agency (EMA) provided an updated guideline on clinical investigation of newly developed steroid contraceptives in women to establish the contraceptive efficacy and to describe the risks and adverse events of the new contraceptive [77]. Regarding the safety of the contraceptive concerning venous thrombosis, the EMA suggested measuring the following haemostatic variables: prothrombin fragment 1+2 levels, D-dimer levels, factor VII, factor VIII, factor II, antithrombin, protein S and protein C. Besides these individual coagulation factors, measuring activated protein C (APC) resistance (ETP-based, APTT-based) was recommended by the EMA as well. APC resistance is the relative inability of protein C to cleave activated factor V or activated factor VIII leading to a prothrombotic state. Both APC resistance assays use different triggers, measure different endpoints and are influenced by different determinants; therefore, both assays provide insights into different mechanisms of APC resistance [78]. Further, EMA recommended to measure sex hormone binding globulin (SHBG) levels as an indicator of the hormonal activity of the contraceptives. These

individual coagulation factors, APC resistance and SHBG levels suggested by the EMA will be evaluated regarding the use of ethinylestradiol in contraception, restriction of tall stature and sex change. HRT use will not be assessed because ethinylestradiol is not used in HRT.

Individual coagulation factors The use of combined hormonal contraceptives influenced many coagulation factors (Table 1.2). Use of combined oral contraceptives increased factors involved in coagulation and fibrinolysis as well as some factors in anticoagulation, whereas other anticoagulation factors were decreased[79–81]. In third generation combined oral contraceptive users, the increase was more pronounced than in second generation users[79–81]. For instance, the increase in factor VII and the decrease in protein S concentrations were more pronounced in third generation oral contraceptive users.

The use of a vaginal ring containing a third generation progestagen showed the same effects on coagulation factors as combined oral contraceptives with third generation progestagens[82,83]. Compared with second generation combined oral contraceptive users, vaginal ring users showed a more pronounced increase in factor VII and a more pronounced decrease in functional protein S levels[83]. However, another study[82] reported that functional protein S levels were not influenced by the vaginal ring, although in this study women were able to choose their contraceptive, i.e., either a vaginal ring or a second generation combined oral contraceptive. As a consequence, the patient characteristics can influence these results because they may be different between users of a vaginal ring or a combined oral contraceptive.

The following coagulation factors were measured in transdermal patch (containing a third generation progestagen) users[84]: prothrombin fragment 1+2, antithrombin, and protein S concentrations. The effect on these coagulation factors were the same as in third generation combined oral contraceptive users with a more pronounced decrease in protein S concentrations. However,

Table 1.2: Effect on markers of venous thrombosis in sex steroid hormones versus non-users

Application **Progestagen**	**Oral**	**Patch** **3rd**	**Ring** **3rd**
Separate factors			
Coagulation			
F1+2	+	+	+
Factor II	+	?	?
Factor VII	+	?	+
Factor VIII	+	?	+
Anticoagulation			
Antithrombin	-	-	-/+
Protein C	+	?	+
Protein S	-	-	-/+
Fibrinolysis			
D-dimer	+	+	+
APC (ETP-based)			
APC resistance	+	+	+
Hormonal activity			
SHBG	+	+	+

APC, activated protein C; SHBG, sex hormone binding globulin; +, increased levels/activity; -/+ no change in levels/activity; -, decreased levels/activity; ?, no data available

no other studies evaluated the effect of transdermal patch use on coagulation factors.

Treatment for growth inhibition in tall girls (i.e., ethinylestradiol with a progestagen added in the last days of the cycle) lowered protein S concentrations[85] and functional antithrombin levels[85–87], and increased functional protein C levels[85] and prothrombin 1+2 concentrations[85]. Another study[88] showed that

factor II concentrations were increased in tall girls, whereas no effect was observed for factor VII, factor VIII and antithrombin concentrations. However, in this study only eight girls were included and compared to controls while the other studies compared the effect before treatment with the effect during treatment in a larger number of girls. Overall, treatment for growth inhibition had the same effects on coagulation factors as the use of combined oral contraceptives. Although the magnitude of the effects was difficult to assess because no comparison was made with a combined oral contraceptive.

Only functional protein C and protein S levels and factor II concentrations were measured in male-to-female transsexuals receiving ethinylestradiol and CPA. Male-to-female transsexuals had lower protein S concentrations and slightly higher functional levels of protein C than their baseline measurements[89]. No association between changes in coagulation and changes in hormone levels of 17β-estradiol, testosterone, LH and FSH was observed suggesting that the effect on coagulation is a sole effect of ethinylestradiol. No other studies evaluated the effect of this hormone therapy or any other coagulation factors.

The effects on coagulation factors in combined oral contraceptive users have been extensively researched showing that the use influenced levels of many coagulation factors. Few studies researched the effects of hormone use for contraception administered via a ring or patch, restriction of tall stature or sex change on coagulation factors, but from the studies that were conducted, it is likely that these applications can influence levels of coagulation factors.

Activated protein C (APC) resistance As mentioned before, activated protein C resistance can be measured in two ways; APTT-based or ETP-based. When the two methods were compared with regard to the effect of combined oral contraceptive use, the effect on the ETP-based APC resistance was more pronounced than on the APTT-based test[90]. Furthermore, third generation

combined oral contraceptives users showed a more pronounced increase in APC resistance (ETP-based) than second generation users providing a measure for the difference in venous thrombosis risk between these generations. For these reasons the ETP-based APC resistance test will only be taken into account.

The APC resistance predicts venous thrombosis risk in men and women, as well as in oral contraceptive users and non-users [91]. Several other studies confirmed that APC resistance was increased in combined oral contraceptive users [81,83,84] and that the effect was more pronounced in users of a third generation progestagen than with a second generation progestagen [81]. Both in users of a vaginal ring and a transdermal patch, the APC resistance was increased as well [83,84]. Compared with second generation combined oral contraceptive users, the increase in APC resistance was more pronounced in vaginal ring users [83]. The APC resistance increased appreciably with the use of a patch compared with a third generation combined oral contraceptive [84]. No data was available on girls receiving sex hormones for tall stature and the effect on APC resistance. In the male-to-female transsexuals receiving ethinylestradiol with CPA, the APC resistance was increased compared to baseline [89]. Overall, the use of ethinylestradiol with a progestagen led to the activation of coagulation in women using contraceptives administered via a pill, a vaginal ring or a transdermal patch, or in male-to-female transsexuals.

Sex hormone binding globulin (SHBG) Sex hormone binding globulin (SHBG) is a plasma glycoprotein, primarily produced in hepatocytes, that binds androgens and thereby regulates their bioavailability. SHBG levels vary due to multiple regulating factors such as age, body weight, sex steroids, or insulin. Estrogens like ethinylestradiol are able to increase the production of SHBG, whereas progestagens induce a decrease of SHBG levels depending on the type and dose [92,93]. Therefore, the effect of combined oral contraceptives on SHBG levels can be seen as the sum of

the stimulating effect of ethinylestradiol and the inhibiting effect of the progestagen resulting in the total estrogenicity of a contraceptive[93]. This estrogenicity of a contraceptive can influence the venous thrombosis risk[93]. A positive correlation was observed between SHBG levels and APC resistance supporting the hypothesis that SHBG levels can be seen as a marker for venous thrombosis[93,94]. However, SHBG levels have not yet been researched in association with venous thrombosis risk.

In general, combined oral contraceptive use was associated with an increase in SHBG levels and this increase was more pronounced in third generation progestagen users than in second generation users[95,96]. The use of a vaginal ring[83] or a transdermal patch[84] was also associated with an increase in SHBG levels. Compared with a second generation combined oral contraceptive, the SHBG levels were higher in both vaginal ring and transdermal patch users[97]. The SHBG increase was more pronounced in transdermal patch users compared to vaginal ring users[97]. No information was available on SHBG levels and hormone use for tall stature or sex change.

Data presented here confirmed that combined oral contraceptive users, vaginal ring users and transdermal patch users have an increased risk of venous thrombosis.

Experimental data on ethinylestradiol

Several epidemiological studies established that the use of combined oral contraceptives increased the risk of venous thrombosis. The use of contraceptives influenced the levels of coagulation factors and increased APC resistance and SHBG levels. Data from experimental research may provide further evidence on the mechanism how ethinylestradiol can influence the risk of venous thrombosis.

Ethinylestradiol on a cellular level Ethinylestradiol is able to bind to the estrogen receptor. This complex of ethinylestradiol

and estrogen receptor was able to function as a transcription factor, to enter the nucleus and to bind to estrogen response elements (ERE) in the DNA[98]. This resulted in transcriptional activation of nearby genes. Many coagulation factors genes contain a ERE suggesting that ethinylestradiol is able to influence coagulation factors levels directly.

17β-Estradiol and ethinylestradiol were compared with regard to their ability to translocate the estrogen receptor to the nucleus of hepatocytes[99]. 100-fold higher concentrations of 17β-estradiol was needed to lead to the same promotion of translocation as ethinylestradiol in parenchyma cells in the liver of rats. Furthermore, they also compared the metabolic pathways and found that 17β-estradiol was much more metabolized than ethinylestradiol. In the metabolism of 17β-estradiol, one major route[100] is the oxidation at the C-17 position which is blocked by the ethinyl group in ethinylestradiol at the same C-17 position (Figure 1.1). This potentially explains why 17β-estradiol was more metabolized than ethinylestradiol.

First-pass metabolism of ethinylestradiol An overview of the first-pass metabolism of ethinylestradiol is given in figure 1.2.

One of the enzymes involved in the ethinylestradiol first-pass metabolism in the liver is cytochrome p-450 3A4 (CYP3A4) which hydroxylates ethinylestradiol to hydroxy-ethinylestradiol. Several studies looked at the effect of ethinylestradiol and different progestagens on the content of cytochrome P-450 enzymes in the liver and the inhibition of CYP3A4[101–103]. Both ethinylestradiol and gestodene (third generation progestagen) were able to reduce the total content of this enzyme family in the liver with at least 30% and to inhibit the enzyme CYP3A4. However, a higher dose of ethinylestradiol and gestodene was used in this study than the dose used in combined oral contraceptives and the combination of ethinylestradiol and gestodene was not evaluated so the question remains whether the currently used dose of ethinylestradiol in combined oral contraceptives and the

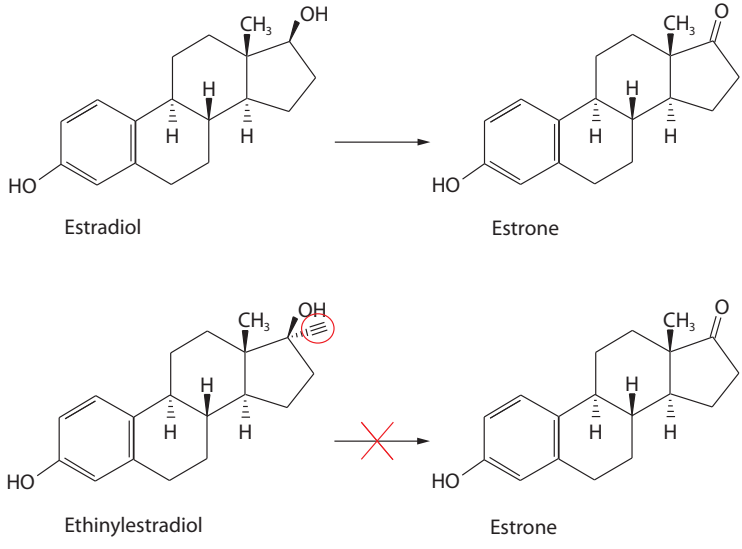

Figure 1.1: The oxidation at C-17 of estradiol to estrone is shown in the top part. This oxidation is blocked by the ethinyl-group in ethinylestradiol (indicated by circle) depicted in the bottom part

combination with gestodene is also able to inhibit the enzyme CYP3A4 in vivo.

Taken together the data from experimental research provides further insights into how ethinylestradiol can influence the venous thrombosis risk.

Research questions

Biochemical aspects

Ethinylestradiol levels vary according to the day in the cycle and the time since last oral contraceptive pill[104]. For an accurate

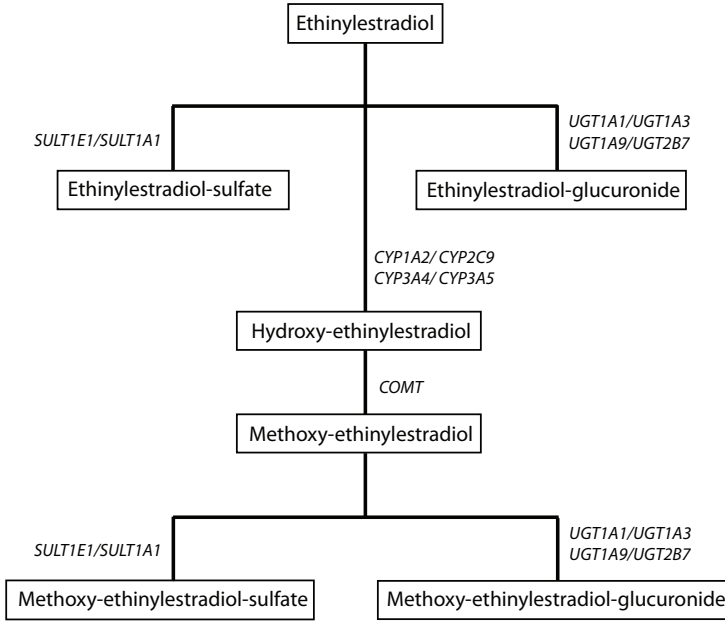

Figure 1.2: The first-pass metabolism of ethinylestradiol in the liver. Genes involved in this metabolism are depicted in italics

measurement, blood has to be drawn at the same moment for every woman included in a study. However, in current case-control studies of venous thrombosis, blood is taken at random in the menstrual cycle and after the thrombotic event when many women have stopped using combined oral contraceptives. In most research settings regarding venous thrombosis ethinylestradiol levels are not commonly measured. No study has assessed the association between the levels of ethinylestradiol and venous thrombosis risk.

Because SHBG levels are seen as a marker for the estrogenicity of combined oral contraceptives and venous thrombosis risk

and are not affected by daily fluctuations, SHBG levels will be measured instead of ethinylestradiol levels. To determine whether SHBG levels are a risk factor for venous thrombosis, the association between increased SHBG levels and the risk of venous thrombosis in women not using hormonal contraceptives will be discussed in **chapter 2**. The association between the dose of ethinylestradiol in combined oral contraceptives and SHBG levels will be discussed in **chapter 3**.

First-pass metabolism of ethinylestradiol To determine whether genetic variation in the first-pass metabolism of ethinylestradiol can at least in part explain the risk of venous thrombosis in oral contraceptive users, common genetic variation in enzymes in this metabolism will be investigated. Many genes and their enzymes are involved in the first-pass metabolism of ethinylestradiol (Figure 1.2). Genes are selected based on their ability to convert ethinylestradiol and on their expression in the liver. Genetic variation in the selected genes will be assessed through haplotypes. A haplotype is a combination of alleles on a chromosome that is not affected by recombination and consequently transmitted together. Because of this linkage, without actual measurement, a known single nucleotide polymorphism (SNP) can provide information about neighbouring SNPs.

Conjugation and hydroxylation are the first two steps in the first-pass metabolism of ethinylestradiol. Sulfonation and glucuronidation are both conjugation steps leading to inactive and water-soluble ethinylestradiol that can easily be excreted through the urine or bile. The genes *SULT1A1* and *SULT1E1* code for sulfotransferases that are involved in sulfonation[105–110] of ethinylestradiol and the genes *UGT1A1*, *UGT1A3*, *UGT1A9* and *UGT2B7* code for UDP-glucuronosyltransferases involved in the glucuronidation[110–113]. Hydroxylation and subsequent methylation, of the hydroxyl group, lead to hydroxy-ethinylestradiol and methoxy-ethinylestradiol, respectively. To inactivate these hormones, the aforementioned conjugation steps are repeated.

The genes *CYP1A2*, *CYP2C9*, *CYP3A4* and *CYP3A5* code for enzymes involved in the hydroxylation step[102,110,114,115] and the *COMT* gene codes for catechol O-methyltransferase involved in the methylation step[116]. Genetic variation in these genes, their effect on SHBG levels and the association with venous thrombosis will be discussed in **chapter 4**.

Clinical aspects

Current guidelines advice women to refrain from using combined hormonal contraceptives after a venous thrombosis. Progestagen-only contraceptives can be used. Adherence to these guidelines and potential explanations will be assessed in **chapter 5**.

Combined oral contraceptive use increases the risk of a first venous thrombosis, whether contraceptive use is also associated with a second event is unclear. To date, one study assessed the risk of a recurrence and hormonal contraceptive use[117]. A total of 14 recurrences were observed among premenopausal women exposed to hormonal risk factors (oral contraceptive use or pregnancy) of which 11 occurred in women using hormonal contraceptives. The incidence of a recurrence was 4.3 times higher in hormonal contraceptive users than in non-users (incidence rate ratio (IRR) 4.3, 95%CI: 1.7 to 11.1). We will analyse the incidence rate of recurrent venous thrombosis in premenopausal women and the effect of hormonal contraceptives used at the first or second event in **chapter 6**. The effect of oral and non-oral preparations on recurrent venous thrombosis will be discussed as well.

Although it has been shown that there is a difference in the risk of a first venous thrombosis per generation of progestagens, no clear overview of the associations between different combined oral contraceptives and the risk of venous thrombosis exists. We set out to provide an overview of the risk of a first venous thrombosis per combined oral contraceptive preparation. A network meta-analysis will be performed because combined oral

contraceptives are mostly compared to non-use or to a combined oral contraceptive containing levonorgestrel with 30 µg ethinylestradiol resulting in gaps in direct evidence. The results of the network meta-analysis will be discussed in **chapter 7**.

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Biochemical aspects

Chapter 2

Sex hormone binding globulin levels are not causally related with venous thrombosis risk in women not using hormonal contraceptives

Bernardine H. Stegeman
Frans M. Helmerhorst
Hans L. Vos
Frits R. Rosendaal
Astrid van Hylckama Vlieg

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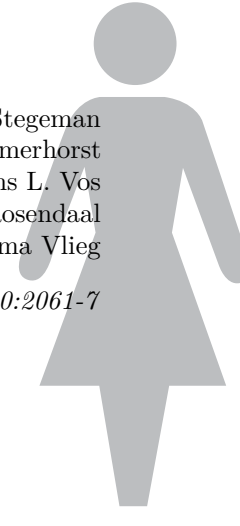

Abstract

Background: Oral contraceptives use increases the risk of venous thrombosis as well as sex hormone binding globulin (SHBG) levels. Furthermore, increased SHBG levels are positively associated with activated protein C (APC) resistance and thrombotic risk in oral contraceptive users.
Objectives: To determine whether increased SHBG levels are causally related to venous thrombosis in women not using hormonal contraceptives.
Methods: Premenopausal women were selected from a case-control study on venous thrombosis, the MEGA study (23 patients; 258 controls). Women using hormonal contraceptives were excluded. Firstly, the risk of venous thrombosis with SHBG levels above the normal reference range (70 nmol/L) was determined. Secondly, because multiple regulating factors affect SHBG levels and residual confounding may remain, we determined six single-nucleotide polymorphisms (SNPs) in the *SHBG* gene and assessed the risk of venous thrombosis in a different case-control study, the LETS (20 patients; 74 controls), and in MEGA study. Finally, the association between SHBG levels and the normalized activated partial thromboplastin time-based APC resistance (an intermediate endpoint for venous thrombosis) was determined.
Results: Elevated SHBG levels (>70.0 nmol/L) were associated with venous thrombosis (OR 1.92; 95%CI: 0.74-5.00). However, this finding can be explained by residual confounding. Two SNPs in the *SHBG* gene affected SHBG levels, but not venous thrombosis risk. Furthermore, SHBG levels in controls were not associated with APC resistance (SHBG level >70.0 versus ≤70.0 nmol/L: mean difference in normalized APC sensitivity ratio 0.03; 95%CI: -0.05 to 0.10). Exclusion of women with FV Leiden did not materially change these results.
Conclusions: Increased SHBG levels are not causally associated with the risk of venous thrombosis.

Introduction

Venous thrombosis is the formation of a blood clot in the veins, predominantly in the legs. The overall age-dependent incidence is 1-3 per 1000 persons per year [1], whereas the incidence in women of reproductive age is estimated to be 5-10 per 10,000 women-years [2]. Both genetic and acquired risk factors are known to influence the risk of venous thrombosis. An important acquired risk factor is the use of hormonal contraceptives in women [3]. The use of combined oral contraceptives (COCs) is associated with a four-fold to six-fold increased risk of venous thrombosis [4–7]. The risk of venous thrombosis is higher in so-called third generation progestagen (e.g., desogestrel, gestodene) COC users than second generation (e.g., levonorgestrel) COC users [8–11].

Results from several previous studies have suggested that the effect of a COC on sex hormone binding globulin (SHBG) levels reflects the risk of venous thrombosis. SHBG is a plasma glycoprotein that binds sex steroid hormones testosterone and 17 β -estradiol. Plasma SHBG is primarily produced in hepatocytes. In contraceptive users, estrogens such as ethinylestradiol increase the production of SHBG [12,13], whereas progestagens induce a decrease of SHBG levels depending on the type and dose used [14,15]. Therefore, the effect of COCs on SHBG levels can be seen as the sum of the stimulatory effect of ethinylestradiol and the inhibitory effect of the progestagen, resulting in the total estrogenicity of the pill [15,16]. This so-called total estrogenicity of a COC crudely correlates with the risk of venous thrombosis, in the sense that levenorgestrel-containing COCs have a lower associated risk and lower SHBG levels than third-generation pills containing desogestrel or gestodene [15,17–20]. Furthermore, SHBG levels in COC users were positively associated with thrombin generation-based activated protein C resistance (APC) resistance [16,21]. APC resistance is the relative inability of protein C to cleave factor Va or factor VIIIa, leading to a prothrombotic state. APC resistance has been shown to predict

venous thrombosis risk in both men and women[22,23].

In addition to COCs, many environmental risk factors affect SHBG levels, such as age[24], obesity[25,26], diabetes[27], liver diseases[28,29], and hyperthyroidism[30,31]. Regarding genetic variation in the *SHBG* gene, the single-nucleotide polymorphisms (SNPs) rs13894 (C/T) and rs727428 (G/A) decrease SHBG levels with an increasing number of minor alleles[32–34]. Minor alleles of SNP rs6259 (Asp356Asn) were associated with increasing SHBG levels. Furthermore, the combination of the SNPs rs6259 (Asp356Asn), rs858521 (C/G), and rs727428 (G/A) accounted for 24% of the variation in SHBG levels in postmenopausal women[33].

Although an increased SHBG level in oral contraceptive users is a marker for the risk of venous thrombosis, the question remains of whether increased SHBG levels are a risk factor for venous thrombosis in a causal sense. The aim of this study was threefold. First, the risk of venous thrombosis associated with SHBG levels was evaluated in non-contraceptive users. Second, to eliminate the influence of residual confounding, the effect on thrombotic risk of genetic variants in the *SHBG* gene that affect SHBG levels was assessed. A similar approach was used to study genetic variation in the *SHBG* gene and SHBG levels in association with diabetes[27]. Third, we investigated the association between SHBG levels and APC resistance, which is an established intermediate endpoint for venous thrombosis.

Methods

Participants Participants were selected from two large case-control studies, i.e., the Leiden Thrombophilia Study (LETS) and the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study. In the LETS, participants with a first episode of venous thrombosis in the leg, younger than 70 years and without a known malignant disorder, were enrolled between 1 January 1988 and 31 December 1992.

As controls, friends and partners of the patients were asked to participate. Details of the study have been described elsewhere[35]. In the MEGA study, participants with a first venous thrombosis in the leg or arm or pulmonary embolism were recruited between 1 March 1999 and 31 August 2004. Controls were either the partners of the patients or recruited through random digit dialing. Details of the study have been described elsewhere[36]. Both studies included objectively verified venous thrombotic events. In both the LETS and the MEGA study, participants were asked to fill in a questionnaire within a few weeks after the thrombotic event, and subsequently to provide a blood or buccal swab sample three months after discontinuation of anticoagulant therapy. The LETS and the MEGA study differed slightly in their inclusion and exclusion criteria. To make both studies comparable, patients with venous thrombosis in the arm or pulmonary embolism were excluded from the MEGA study.

The population of interest consisted of premenopausal women with or without a first event of venous thrombosis (N_{LETS}=337; N_{MEGA}=2657). For the current study, only idiopathic venous events were selected, so we excluded women who had any type of cancer (N_{MEGA}=63), were hospitalized (N_{LETS}=22; N_{MEGA}= 357), had undergone surgery (N_{LETS}=29; N_{MEGA}=277), had had bone fractures (N_{LETS}=2; N_{MEGA}=81) or injuries (N_{MEGA}=529) in the 12 months before the event. Furthermore, women who were pregnant (N_{LETS}=10; N_{MEGA}=65) or postpartum (N_{LETS}=4; N_{MEGA}=17), had a miscarriage (N_{LETS}=1; N_{MEGA}=10), used hormone replacement therapy (N_{MEGA}=14) or used hormonal contraceptives (N_{LETS}=213; N_{MEGA}=1013), in the 12 months before the event were excluded. Totals of 94 and 385 women were included from the LETS and MEGA study, respectively. For these women, DNA was available, through either a blood sample or buccal swab sample. Plasma was required for SHBG measurement; therefore, women with a buccal swab sample were excluded (N_{MEGA}=104). The amount of plasma left in the LETS was insufficient for measurement of SHBG levels.

Data regarding age and body mass index (BMI) were retrieved from the questionnaire. The BMI (kg/m^2) of these women was calculated from their reported weight and height. For the association with SHBG levels in controls, age and BMI were divided into three categories, i.e., for age into $\leq$30 years, 30-40 years and 40-50 years and for BMI into normal ($\leq$25 kg/m^2), overweight (25-30 kg/m^2) and obese (>30 kg/m^2).

DNA preparation and SNP typing Blood samples were taken at least 3 months after discontinuation of anticoagulant therapy. Blood was drawn after an overnight abstinence from intake of food, caffeine, and alcohol, and collected into vacuum tubes containing 0.106 mol/L trisodium citrate as anticoagulant. Blood was centrifuged to retrieve cell-free, citrated plasma. Processing of blood samples and subsequent DNA isolation have been described previously[35,36].

To determine the haplotypes in the *SHBG* gene, the Genome Variation Server (GVS)[37] was used. The GVS incorporates information from HapMap, and is sponsored by SeattleSNPs. The *SHBG* gene showed six haplotypes, hA to hF (frequencies in a European population of Northern and Western ancestry (CEU) according to HapMap data were 11%, 14%, 22%, 14%, 29%, and 10%, respectively). Only SNPs with a minor allele frequency of $\geq$5% were considered. The following haplotype-tagging SNPs were selected: rs13894, rs6259, rs8066665, rs2955617, rs858521 and rs727428. The combination of these six SNPs led to six haplotypes in the *SHBG* gene (Supplementary table). For four of these, an effect on SHBG levels was reported previously (we found no reports for SNPs rs8066665 and rs2955617). As the total number of known SNPs in the *SHBG* gene is relatively low, the selected SNPs were the only ones available to discriminate between the different haplotypes.

The SNPs were determined with the MassARRAY platform (Sequenom, San Diego, California, USA), according to manufacturer's protocols (Sequenom). Genotyping determination was

performed blinded to the case-control status. Five per cent of the samples were repeated for allele-calling consistency; no discrepancies were found.

Laboratory measurement As the amount of plasma left from the LETS samples was not sufficient for an SHBG measurement, SHBG levels were measured only from the MEGA study (N=281). SHBG levels (nmol/L) were measured with an immunometric assay (Immulite; Siemens Healthcare Diagnostics, Tarrytown, NY, USA). The sensitivity is 0.2 nmol/L and the assay has a log-term variation of 6% at levels of both 5 nmol/L and 80 nmol/L. The within-assay variation is 3-4% and the between-assay variation 3.5-6%. The samples were analyzed in a single series in random order. SHBG levels were measured without knowledge of any of the participant's characteristics.

APC resistance was determined in samples from the MEGA study. APC resistance was measured with Cephotest (Nycomed Pharma, Oslo, Norway). The normalized APC sensitivity ratio (nAPCsr) was defined as the activated partial thromboplastin time (APTT) in the presence of APC divided by the APTT in the absence of APC in participants divided by the same ratio determined in normal pool samples, i.e., (APTT+APC$_{participants}$ / APTT-APC$_{participants}$) / (APTT+APC$_{normalpool}$ / APTT-APC$_{normalpool}$).

Statistical analysis First, the association between SHBG levels and the risk of venous thrombosis was assessed in the MEGA study. SHBG levels were dichotomized with a cut-off value of 70 nmol/L SHBG, which is above the normal reference range. With logistic regression analysis with robust standard errors (SEs), the risk of venous thrombosis associated with SHBG levels of >70 nmol/L compared with ≤70 nmol/L was assessed by calculating odds ratios (OR) with 95% confidence intervals (CIs) with adjustment for age and BMI.

Second, the relationship between genetic variation in the *SHBG* gene and the risk of venous thrombosis was studied in the LETS and repeated in the MEGA study. For SNPs, Hardy-Weinberg Equilibrium was assessed with a chi-squared test in controls. Regarding haplotypes, the posterior probabilities of the individual haplotype combinations as estimated by PLINK (version 1.07)[38] were used as weights in the statistical analyses. No underlying genetic model was assumed for the SNPs or haplotypes (i.e., SNPs and haplotypes were defined categorically in the regression model). Linear regression analysis with robust SEs was used to determine the effect of a SNP or haplotype in the *SHBG* gene on SHBG levels in controls from the MEGA study. To determine the presence of a relevant effect of genetic variation in the *SHBG* gene on SHBG levels, we used a $\geq$20 nmol/L difference between carriers of two copies of a minor allele or haplotype and non-carriers of the given allele or haplotype. This value was based on the minimal difference in SHBG levels between users of a second generation oral contraceptive (which have the lowest levels of all pill-users) and non-users. Logistic regression with robust SEs was used to estimate the relative risk of venous thrombosis associated with different SNPs or haplotypes. The risks were determined in the LETS and replicated in the MEGA study.

Finally, the association between SHBG levels and nAPCsr was determined in the MEGA study. The association between nAPCsr and the risk of venous thrombosis was assessed by calculating the mean difference in nAPCsr between SHBG levels $>$70 nmol/L and $\leq$70 nmol/L. The 95% confidence interval was calculated with a robust SE. The analysis was repeated without women with FV Leiden which is known to lead to APC resistance.

Statistical analyses were performed with STATA, version 12.0 (StataCorp LP, College Station, TX, USA).

Results

From the LETS, 20 patients and 74 controls were included. A total of 23 patients and 258 controls were included from the MEGA study. Baseline characteristics of the participants from the LETS and MEGA study are depicted in table 2.1. In the MEGA study, the median SHBG level in the patients was 55.6 nmol/L (interquartile range (IQR) 37.0, range 21.2 to 458.2 nmol/L) and that in controls was 58.4 nmol/L (IQR 37.2, range 16.1 to 524.3 nmol/L). The influence of age and BMI on SHBG levels was assessed in the controls from the MEGA study. SHBG levels were higher in women aged 30-40 years than in women aged ≤30 years (mean difference: 20.6 nmol/L, 95%CI: -2.4 to 43.6). However, levels did not increase much with age after this: in women aged 40-50 years SHBG levels were only 6.9 nmol/L higher than in women aged ≤30 years (95%CI: -15.3 to 29.2). SHBG levels were lower in obese women (BMI of >30 kg/m^2) than in women with normal weight (BMI of ≤25 kg/m^2)(mean difference: 28.6 nmol/L, 95%CI: 9.1 to 48.1). There was no difference in SHBG level between women with a BMI of 25-30 kg/m^2 and those with a BMI of ≤25 kg/m^2 (mean difference: 2.5 nmol/L, 95%CI: -11.2 to 16.3).

Nine cases (39%) and 90 controls (35%) from the MEGA study had SHBG levels above normal reference range (>70 nmol/L) (Table 2.2). After adjustment for age and BMI, SHBG levels above 70 nmol/L were associated with a 1.9-fold increased risk of venous thrombosis (OR 1.92, 95%CI: 0.74 to 5.00).

To assess the association between SHBG levels and venous thrombosis, a total of six SNPs were selected in the *SHBG* gene, tagging six haplotypes (Supplementary table). In both the LETS and MEGA study, all SNPs were found to be in Hardy-Weinberg equilibrium (p>0.05) as measured in the controls. The effect of genetic variation in the *SHBG* gene on SHBG levels was substantial, making these SNPs informative for a 'Mendelian randomization' analysis. The predefined in- or decrease of 20

Table 2.1: Baseline characteristics from the LETS and MEGA study

	LETS study		MEGA study	
Variable	**Cases (N=20)**	**Controls (N=74)**	**Cases (N=29)**	**Controls (N=356)**
Age, mean(SD)	44(5)	41 (6)	40 (8)	39 (7)
Caucasian,[*] (%)	-	-	25 (89)	313 (90)
BMI,[†] mean(SD)	27.1 (6.7)	25.5 (4.4)	27.7 (5.3)	24.6 (4.3)
SHBG levels,[‡] median(IQR)	-	-	55.6 (37.0)	58.4 (37.2)

SD, standard deviation; IQR, interquartile range
[*] No data on ancestry was available in 9 women
[†] 18 women had no data on weight or height
[‡] Measured in 281 women (23 cases; 258 controls)

nmol/L is substantial relative to the SHBG level of the majority of the women in this study. In our population, 95% of women had SHBG levels of 128.5 nmol/L or lower.

Table 2.2: SHBG levels and the risk of venous thrombosis in the MEGA study

SHBG levels	**Cases (%)**	**Controls (%)**	**OR (95%CI)**	**Adjusted[*] (95%CI)**
≤70.0 nmol/L	14(61)	168 (65)	1	1
>70.0 nmol/L	9 (39)	90 (35)	1.20 (0.50 to 2.88)	1.92 (0.74 to 5.00)

[*] Adjusted for age and BMI

Homozygosity for the minor allele (genotype TT) of SNP rs13894 was associated with a decrease in SHBG levels of 50 nmol/L compared with homozygosity for the major allele (genotype CC). However, this was based on only one control with the TT genotype (Figure 2.1 & Table 2.3). The G allele of SNP

rs2955617 and the A allele of SNP rs727428 affected SHBG levels (Figure 2.1 & Table 2.3). Genotype GG of SNP rs2955617 decreased SHBG levels by 20.7 nmol/L (95%CI: -8.2 to 33.1) as compared with genotype TT. The same decrease was observed for genotype AA of SNP rs727428 (20.9 nmol/L, 95%CI: 4.9 to 36.8). For SNP rs727428, a linear association was observed; with each increase in the number of minor alleles, the SHBG level increases by 9.9 nmol/L (95%CI: 1.0 to 18.8).

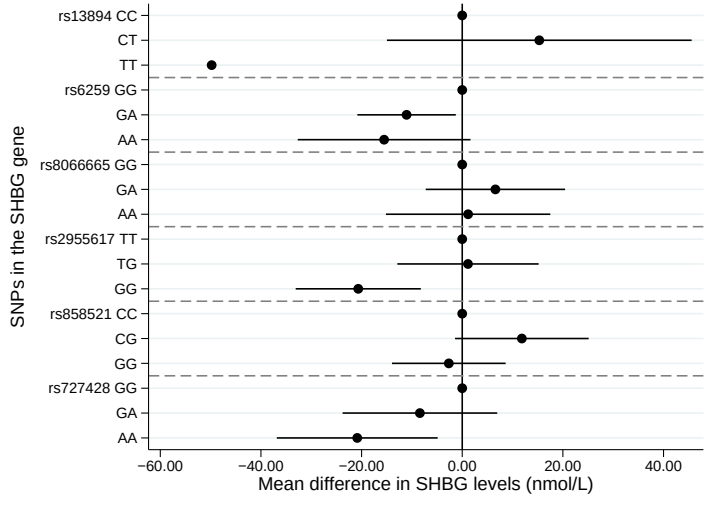

Figure 2.1: Mean difference with 95% confidence intervals (indicated with dots and lines). The common homozygotes were selected as reference (indicated with 0).

None of the cases in the LETS was homozygous for the minor allele of SNP rs2955617 (genotype GG); therefore, no risk could be calculated. In the MEGA study, genotype GG of SNP rs2955617 was not associated with risk of venous thrombosis (OR 1.32, 95%CI: 0.34 to 5.12). In the LETS, a decrease in the risk of

Table 2.3: Effect of SNPs in the *SHBG* gene on SHBG levels

SNP	N (%)	Mean SHBG levels (95%CI)	Mean difference (95%CI)
rs13894			
CC	227 (89)	66.7 (60.7 to 72.8)	Reference
CT	27 (11)	82.0 (52.0 to 112.1)	15.3 (-15.0 to 45.6)
TT	1 (0)	16.9	-49.8 (-55.9 to -43.7)
rs6259			
GG	204 (80)	70.5 (62.9 to 78.2)	Reference
GA	45 (18)	59.5 (53.4 to 65.5)	-11.1 (-20.8 to -1.3)
AA	5 (2)	55.0 (38.0 to 72.1)	-15.5 (-32.7 to 1.6)
rs8066665			
GG	91 (36)	64.8 (55.0 to 74.6)	Reference
GA	127 (50)	71.4 (61.7 to 81.1)	6.6 (-7.3 to 20.4)
AA	36 (14)	66.0 (52.8 to 79.1)	1.2 (-15.2 to 17.5)
rs2955617			
TT	123 (49)	70.3 (61.0 to 79.6)	Reference
TG	102 (40)	71.4 (60.9 to 81.9)	1.1 (-12.9 to 15.2)
GG	28 (11)	49.6 (41.3 to 58.0)	-20.7 (-33.1 to -8.2)
rs858521			
CC	76 (30)	62.5 (54.7 to 70.4)	Reference
CG	133 (52)	74.4 (63.7 to 85.1)	11.9 (-1.4 to 25.1)
GG	45 (18)	59.9 (51.7 to 68.1)	-2.7 (-14.0 to 8.6)
rs727428			
GG	85 (33)	76.0 (63.2 to 88.7)	Reference
GA	125 (49)	67.5 (58.9 to 76.1)	-8.4 (-23.8 to 7.0)
AA	45 (18)	55.1 (45.4 to 64.8)	-20.9 (-36.8 to -4.9)

venous thrombosis was observed in women with genotype AA of SNP rs727428 (OR 0.24, 95%CI: 0.03 to 2.19); however, this risk was not confirmed in the MEGA study (OR 1.05, 95%CI: 0.33 to 3.33). Although we observed a linear trend in SHBG levels with increasing number of minor alleles of this SNP, no association was observed with risk of venous thrombosis in either the LETS or MEGA study. Haplotype analysis gave the same results (data not shown).

Finally, the association between SHBG levels and APC resistance was evaluated in the MEGA study. SHBG levels of >70 nmol/L were associated with a 0.03 (95%CI: -0.05 to 0.102) increase in nAPCsr as compared with levels of ≤70 nmol/L. Adjustment for age and BMI did not alter the result (mean difference between SHBG levels >70 nmol/L versus ≤70 nmol/L in nAPCsr: 0.02, 95%CI: -0.06 to 0.10). Exclusion of women with FV Leiden did not materially change the results (mean difference: 0.03, 95%CI: -0.04 to 0.10 and adjusted for age and BMI: 0.03, 95%CI: -0.05 to 0.11).

Discussion

We set out to study whether a high SHBG level is causally related with the risk of venous thrombosis. First, we showed that there was a mild increase in risk associated with SHBG levels above normal (i.e., 70.0 nmol/L) after adjustment for age and BMI in non-users of hormonal contraceptives (OR 1.92, 95%CI: 0.74 to 5.00). However, as SHBG levels are affected by many regulating factors, residual confounding may remain. Therefore we performed a Mendelian randomization analysis: here, genetic variants are used that are associated with levels that, by definition, cannot have been affected by potential confounding factors. We showed that several SNPs were associated with SHBG levels, but not with thrombotic risk. Finally, no association could be found in non-users of hormonal contraceptives between SHBG levels and APC resistance, an established intermediate endpoint for venous thrombosis.

Our results in non-users are in contrast with the results observed in oral contraceptive users, where an increase in SHBG levels is associated with an increase in nAPCsr (endogenous thrombin potential-based) [21]. Apparently, both SHBG levels and the nAPCsr are affected by oral contraceptive use (Figure 2.2a). In non-users, there is no common factor that influences both SHBG levels and APC resistance (Figure 2.2b).

Table 2.4: Effect of SNPs in the *SHBG* gene on venous thrombosis risk

SNP	Cases (%)	Controls (%)	LETS OR (95%CI)	Cases (%)	Controls (%)	MEGA OR (95%CI)
rs13894						
CC	18 (90)	61 (85)	1	28 (97)	306 (87)	1
CT	2 (10)	10 (14)	0.68 (0.14 to 3.38)	1 (3)	44 (13)	0.25 (0.03 to 1.88)
TT	0 (0)	1 (1)	-	0 (0)	1 (0)	-
rs6259						
GG	16 (80)	57 (77)	1	18 (62)	278 (79)	1
GA	4 (20)	17 (23)	0.84 (0.25 to 2.85)	10 (34)	66 (19)	2.34 (1.03 to 5.31)
AA	0 (0)	0 (0)	-	1 (3)	6 (2)	2.57 (0.29 to 22.61)
rs8066665						
GG	8 (40)	24 (32)	1	8 (28)	129 (37)	1
GA	9 (45)	37 (50)	0.73 (0.25 to 2.15)	13 (45)	164 (47)	1.28 (0.51 to 3.18)
AA	3 (15)	13 (18)	0.69 (0.16 to 3.07)	8 (28)	57 (16)	2.26 (0.81 to 6.34)
rs2955617						
TT	11 (55)	32 (44)	1	13 (45)	165 (47)	1
TG	9 (45)	31 (42)	0.84 (0.31 to 2.32)	12 (41)	145 (42)	1.05 (0.46 to 2.38)
GG	0 (0)	10 (14)	-	4 (14)	39 (11)	1.30 (0.40 to 4.22)
rs858521						
CC	4 (21)	29 (39)	1	15 (52)	119 (34)	1
CG	10 (53)	34 (46)	2.13 (0.60 to 7.52)	12 (41)	168 (48)	0.57 (0.26 to 1.26)
GG	5 (26)	11 (15)	3.30 (0.75 to 14.57)	2 (7)	63 (18)	0.25 (0.06 to 1.14)
rs727428						
GG	7 (35)	22 (31)	1	11 (38)	118 (34)	1
GA	12 (60)	36 (51)	1.05 (0.36 to 3.06)	11 (38)	168 (48)	0.70 (0.29 to 1.68)
AA	1 (5)	13 (18)	0.24 (0.03 to 2.19)	7 (24)	65 (19)	1.16 (0.43 to 3.13)

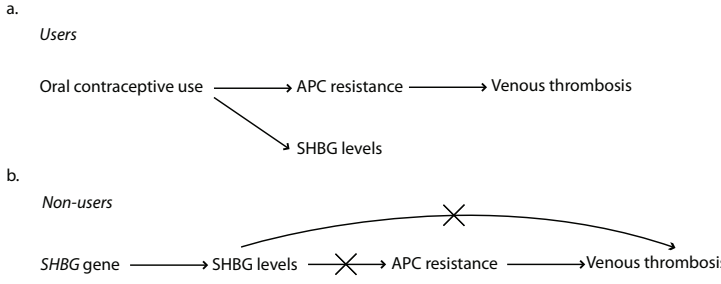

Figure 2.2: Overview of the relationship between SHBG levels, APC resistance and venous thrombosis in oral contraceptive users (a) and non-users (b)

Four of the SNPs described in our study had previously been associated with SHBG levels, i.e., SNPs rs13894, rs6259, rs858521 and rs727428. In contrast with the current results, SNPs rs6259 was previously associated with an increase in SHBG levels[32,33]. This difference may be explained by a difference in study population. We included premenopausal women, whereas previous studies included only women with hirsutism or postmenopausal women. As SNP rs13894 was present in only one woman, no effect on SHBG levels could be demonstrated. SNPs rs858521 and rs727428 were associated with a decrease in SHBG levels in our study, which was also reported in two other studies[33,34].

A limitation of our study is the small number of patients included. An explanation is that we restricted our study population to women who did not use hormonal contraceptives at the time of thrombosis. The strength of our study is that we used a three-pronged approach to evaluate a possible association between SHBG levels and venous thrombosis. All analyses consistently showed no association between SHBG levels and venous thrombosis, strengthening the conclusion that SHBG levels are not associated with the risk of venous thrombosis.

Although an association with venous thrombosis for the high-

est levels of SHBG cannot be excluded, SHBG levels within the range observed in this study are not causally related to an increased risk of venous thrombosis. This does not imply that SHBG level may not be a marker for venous thrombosis in oral contraceptive users, which in all likelihood it is, but that the level is only a marker, and not a cause. The situation is different for APC-resistance, which is an intermediate, i.e., both a marker and a cause. The explanation is that APC-resistance is a global read-out of the coagulation system, whereas SHBG level is not.

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Supplementary data

Supplementary table Haplotype structure in the SHBG gene based on six SNPs

	SNP[*]					
Haplotype	rs13894	rs6259	rs8066665	rs2955617	rs858521	rs727428
A	**T**	G	G	**G**	C	**A**
B	C	**A**	G	**G**	C	**A**
C	C	G	**A**	T	C	G
D	C	G	**A**	**G**	C	**A**
E	C	G	G	T	**G**	G
F	C	G	G	T	**G**	**A**

[*] Minor alleles are indicated in bold

Chapter 3

Effect of ethinylestradiol and progestagen in combined oral contraceptives on plasma sex hormone binding globulin levels in premenopausal women

Bernardine H. Stegeman
Marjolein Raps
Frans M. Helmerhorst
Hans L. Vos
Huib A.A.M. van Vliet
Frits R. Rosendaal
Astrid van Hylckama Vlieg

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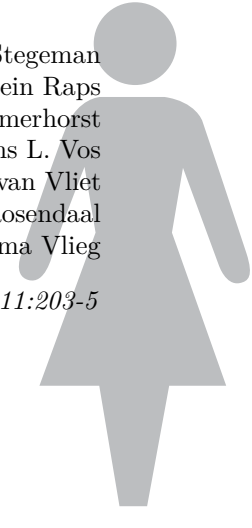

Abstract

Background: Sex hormone binding globulin (SHBG) levels may be a marker for the risk of venous thrombosis in oral contraceptive users. While the effects of different progestagen types on SHBG levels are well established, the association between the ethinylestradiol dose in combined oral contraceptives and SHBG levels remains to be studied.
Objectives: To determine the effect of the ethinylestradiol dose on SHBG levels.
Methods: Healthy premenopausal women using a combined oral contraceptive were included from a case-control study (MEGA study, N=181) and a cross-over study (DRSP study, N=101). Women exposed to risk factors for venous thrombosis (except for oral contraceptive use) were excluded. Mean differences with 95% confidence intervals were estimated, adjusted for confounders and depending on the analysis adjusted for the progestagen used.
Results: A total of 282 women were included from the MEGA and DRSP study. The mean SHBG level in these women was 139.5 nmol/L (95%CI: 131.2 to 147.8). After restriction to 30 µg ethinylestradiol, users of desogestrel, gestodene, or drospirenone had about 100 nmol/L higher SHBG levels than levonorgestrel users. SHBG levels were higher in users of ≥35 µg ethinylestradiol (mean difference: 136.4, 95%CI: 64.5 to 208.3) and in users of triphasic contraceptives (mean difference 50.9 nmol/L, 95%CI: 20.7 to 81.1) than in users of 20 µg ethinylestradiol. No difference was observed between users of 20 µg and 30 µg ethinylestradiol.
Conclusions: An increase in ethinylestradiol dose is associated with an increase in SHBG levels in combined oral contraceptive users.

Introduction

The use of combined oral contraceptives, containing an estrogen (i.e. ethinylestradiol) and a progestagen, is associated with an increased risk of venous thrombosis[1–5]. Because the estrogen compound in combined oral contraceptives was thought to cause the increased risk of venous thrombosis, the dose of ethinylestradiol has over time been reduced from ≥100 µg via 50 µg to 30 µg or 20 µg, indeed resulting in a lower risk of venous thrombosis[6–9]. The type of progestagen in combined oral contraceptives also affects the risk of venous thrombosis, e.g., the risk of venous thrombosis is higher in users of third generation combined oral contraceptives (containing desogestrel or gestodene) and in users of cyproterone acetate than in users of second generation combined oral contraceptives (containing levonorgestrel)[8–11]. Furthermore, in users of preparations containing ethinylestradiol and drospirenone (introduced in 2001) a sixfold increased risk of venous thrombosis compared with non-users was observed[8,9], which was later confirmed in two other studies[12,13].

Results from recent studies have suggested that the effect of a combined oral contraceptive on sex hormone binding globulin (SHBG) levels could be an indicator for the risk of venous thrombosis[14–16]. SHBG is a plasma glycoprotein that binds the sex steroid hormones testosterone and 17β-estradiol but not ethinylestradiol. SHBG is primarily produced in hepatocytes and variation in its plasma levels is due to multiple regulating factors such as age, body weight, sex steroids, and insulin. Users of combined oral contraceptives containing a third generation progestagen have higher SHBG levels than users of a second generation progestagen[14,15,17–19] reflecting the difference in venous thrombosis risk. In accordance with the hypothesis that SHBG levels are a marker of the risk of venous thrombosis, SHBG levels in combined oral contraceptives users are positively associated with thrombin generation-based activated protein C (APC) resistance[16]. APC resistance is the relative inability of protein C to

cleave activated factor V or activated factor VIII thereby leading to a more prothrombotic state. APC resistance has been shown to predict venous thrombosis risk in both men and women[20].

While SHBG levels have been shown to reflect the difference in venous thrombosis risk between different types of progestagen, the estrogen compound is thought to be the most important factor determining the venous thrombosis risk. If SHBG levels can be considered to be a marker for venous thrombosis and ethinylestradiol is the main compound in combined oral contraceptives causing venous thrombosis, then the ethinylestradiol dose in combined oral contraceptives should be reflected in SHBG levels. The aim of this study was to determine whether an increase in ethinylestradiol dose results in higher SHBG levels in healthy premenopausal women. Additionally, we assessed the effect of different progestagens on SHBG levels.

Methods

Participants Participants were selected from a large case-control study, i.e., the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study and from a crossover study, i.e., the DRSP (drospirenone/ethinylestradiol) study. In the MEGA study, participants with a first deep venous thrombosis in the leg or arm or pulmonary embolism were recruited between 1 March 1999 and 31 August 2004 (N=4930). Controls were either the partners of the patients or were recruited via random digit dialling (RDD) (N=6287). All participants were asked to fill in a questionnaire and to provide a blood or a buccal swab sample. Details of the study have been described elsewhere[21]. In the DRSP study, healthy women using the same type of combined oral contraceptive for at least four cycles were recruited between July and November 2002 (N=156). In this study, women were asked to switch from their current contraceptive to an oral contraceptive containing either levonorgestrel or drospirenone. All women were asked to fill in a questionnaire and

to provide a blood sample. Blood was drawn between days 18 and 21 of the pill-cycle. Only data on contraceptive use at baseline and blood samples collected before the switch were used for the current analysis. Women were excluded if there were contraindications for combined oral contraceptive use as stated by the World Health Organization, i.e., women with a history of deep venous thrombosis or pulmonary embolism, women with current deep venous thrombosis or pulmonary embolism or women undergoing major surgery and prolonged immobilization. Details of the study have been described elsewhere[16].

From the MEGA study, we selected premenopausal women without venous thrombosis (i.e., controls); both partner controls and controls recruited through RDD were included (N=1689). Women with known environmental thrombotic risk factors were excluded, i.e., women who had any type of cancer (N=26), had been hospitalized (N=154), underwent surgery (N=109), had bone fractures (N=28), or had injuries (N=274) in the twelve months before the index date. Because some women were exposed to one or more environmental risk factors for venous thrombosis, a total of 467 women were excluded . We also excluded women who were pregnant (N=65), were within four weeks postpartum (N=1) or were using hormone replacement therapy (N=13) at the index date or experienced a miscarriage (N=8) in the twelve months before the index date. Because we were interested in the effect of ethinylestradiol on SHBG levels, women who were using a progestagen-only contraceptive were also excluded (N=23). Blood samples were needed for the SHBG measurement; therefore women who did not provide a blood sample were excluded (N=661). We excluded women who did not use a combined oral contraceptive at the time of venipuncture (N=279). For the current analysis, this resulted in the inclusion of 181 healthy premenopausal women using combined oral contraceptives of which 73 women were partners of cases and 108 were recruited through RDD.

From the DRSP study, we excluded women exposed to known

environmental thrombotic risk factors, i.e., women who had any type of cancer (N=2), had been hospitalized (N=21), underwent surgery (N=10), had bone fractures (N=2), or had injuries (N=31) in the twelve months before the index date. All women were using a combined oral contraceptive at the index date and had given a blood sample. For the current analysis, this resulted in the inclusion of 101 healthy premenopausal women.

Laboratory measurements In the MEGA study, the day of a woman's four week cycle of pill use (3 weeks of pill use followed by a pill-free week) was not taken into account when inviting her to the clinic for a blood sample. Therefore, blood was drawn randomly during the four week cycle of pill use; however, whether the women were menstruating at venipuncture was recorded. In the DRSP study, blood was drawn between days 18 and 21 of the four week cycle of pill-use.

Collection and processing of blood samples have been described previously[16,21]. In short, for both studies, blood was drawn after an overnight fasting for food, caffeine and alcohol and collected in vacuum tubes containing 0.106 mol/L trisodium citrate as anticoagulant. Blood was centrifuged to retrieve cell-free, citrated plasma.

SHBG levels (nmol/L) were measured with an immunometric assay (Immulite; DPC, USA). The sensitivity is 0.2 nmol/L and has a long-term variation of 6% both at levels of 5 nmol/L and 80 nmol/L. The within-assay variation is 3 to 4% and the between-assay variation 3.5 to 6%. The samples were analysed in one series in random order. SHBG levels were measured without knowledge of the type of oral contraceptive used or any other of the participant's characteristics.

Statistical analysis The ethinylestradiol dose was categorised into four categories, i.e., 20 µg, 30 µg, ≥35 µg per pill and triphasic preparations. Triphasic contraceptives have varying ethinyl-

estradiol and progestagen doses per pill over 21 days. In the MEGA study, women were using triphasic contraceptives with the following regimen: 30 µg of ethinylestradiol in the first six days, 40 µg for five days and 30 µg for the last ten days. In the MEGA study, one woman used a biphasic contraceptive; during 21 days the dose of ethinylestradiol is 50 µg, the progestagen is only included in the preparation in the last fourteen days. Because the dose of ethinylestradiol does not change over 21 days, this woman was categorised in the ≥35 µg group. In the DRSP study, all women were using a monophasic contraceptive. For nine women, information on the ethinylestradiol dose in the combined oral contraceptive was not available, however information on progestagen used was available. For descriptive purposes, the progestagens used in the contraceptives were divided into second generation (i.e., levonorgestrel), third generation (i.e., gestodene, desogestrel, and norgestimate) and other progestagens (i.e., cyproterone acetate, drospirenone and first generation progestagens lynestrenol and norethisteron). For the calculation of mean differences in SHBG levels, the progestagens were not grouped by generation but separately evaluated.

The effect of the progestagen and dose of ethinylestradiol on SHBG levels was assessed using linear regression analysis. The analysis was adjusted for study and to ensure that the effect of the ethinylestradiol dose on SHBG levels is independent of the progestagen used, we adjusted this analysis for the progestagen used in the combined oral contraceptive. The analysis of the effect of the progestagen in combined oral contraceptives in association with SHBG levels was restricted to subjects taking 30 µg ethinylestradiol per contraceptive pill.

To reduce random variation in SHBG levels, the analyses were adjusted for multiple variables which can influence SHBG levels. The data were adjusted for whether women were menstruating at the time of venipuncture, and for age and BMI, which are known determinants of SHBG levels in non-users [22–24]. Results were expressed as the mean difference with 95% confi-

dence interval. Statistical analyses were performed with STATA, version 11.2 (Statacorp LP, College Station, TX, USA).

Results

Overall, 282 women using a combined oral contraceptive from the MEGA study (N=181) and the DRSP study (N=101) were included. The general characteristics of the combined population and separate per study are displayed in table 3.1. On average, women from the MEGA study were 8 years older than women from the DRSP study (mean difference: 8, 95%CI: 6 to 10). Women from both studies had a BMI of about 23 kg/m^2. In the MEGA study, 60% (N=109) of the women were using a second generation progestagen, while only 35% (N=35) of the women from the DRSP study were using this progestagen. The most frequently used dose of ethinylestradiol was 30 µg per pill in both studies (100 (58%) and 65 (64%) women in the MEGA and DRSP study, respectively). 32 women (19%) from the MEGA study were using a triphasic contraceptive. The mean SHBG plasma level was about the same in both studies (MEGA study: 143.5 nmol/L, 95%CI: 132.9 to 154.0, IQR 94.8, range 31.2 to 390.9 & DRSP study: 132.3 nmol/L, 95%CI: 118.9 to 145.7, IQR 106.0, range 28.0 to 284.0). The mean SHBG plasma level in women of both studies was 139.5 nmol/L (95%CI: 131.2 to 147.8, IQR 99.8, range 28.0 to 390.9). Both studies are combined in further analyses which were adjusted for study and potential confounders (i.e., age and BMI).

The results in table 3.2 show that not all combinations of ethinylestradiol dose and progestagen were present. A combined oral contraceptive containing 30 µg ethinylestradiol was most often combined with the second generation progestagen levonorgestrel, whereas a contraceptive with 20 µg ethinylestradiol with a third generation progestagen (i.e., desogestrel, gestodene, or norgestimate).

Menstruating at venipuncture may have affected the SHBG

Table 3.1: Baseline characteristics of study participants

Variables	MEGA study (N=181)	DRSP study (N=101)	Combined (N=282)
Age, mean(range)	36 (18-50)	27 (18-51)	33 (18-51)
BMI, mean(range)	23.4 (15.7-37.9)	23.3 (18.3-37.7)	23.3 (15.7-37.9)
Menstruating at venipuncture (%)[a]	11 (6)	-	11 (4)
Progestagen type (%)			
2nd,[b]	109 (60)	35 (35)	144 (51)
3rd,[c]	46 (25)	37 (37)	83 (29)
Other[d]	26 (14)	29 (29)	55 (20)
EE dose (%)[e]			
20 µg	14 (8)	17 (17)	31 (11)
30 µg	100 (58)	65 (64)	165 (60)
≥35 µg	26 (15)	19 (19)	45 (16)
Triphasic[f]	32 (19)	-	32 (12)

BMI, body mass index; EE, ethinylestradiol

[a] Data was available of 174 women from the MEGA study

[b] Second generation progestagen only includes levonorgestrel (N=144)

[c] Third generation progestagen include desogestrel (N=55), gestodene (N=24) and norgestimate (N=4)

[d] Other progestagen include lynestrenol (N=6), norethisteron (N=2), cyproterone acetate (N=30) and drospirenone (N=17)

[e] No information was available on the ethinylestradiol dose in nine women

[f] Triphasic contraceptives contain 30 µg in the first six days, followed by 40 µg for five days and ending with ten days of 30 µg ethinylestradiol

levels. In the MEGA study, SHBG levels were compared between menstruating women versus women taking a pill at venipuncture. 11 women were menstruating at time of venipuncture and the mean SHBG level was 102.1 nmol/L (95%CI: 59.1 to 145.0) whereas the mean SHBG level of the remaining women who were taking a pill (N=163) was 145.4 nmol/L (95%CI: 134.3 to 156.6). The mean difference was 43.4 nmol/L (95%CI: -1.0 to 87.7). Therefore, in addition to age, BMI and study, the linear regression analyses were adjusted for menstruating at venipuncture.

Table 3.3 shows the association of progestagen and ethinyles-

Table 3.2: Distribution of progestagen type and ethinylestradiol dose

Progestagen		**EE dose, n (%)**			
		20 µg	30 µg	≥ 35 µg	Triphasic‡
2nd,*		3 (10)	99 (60)	2 (5)	31 (97)
3rd,†		28 (90)	49 (30)	5 (14)	1 (3)
Other	Cyproterone acetate	0	0	30 (81)	0
	Drospirenone	0	17 (10)	0	0

EE, ethinylestradiol

* Second generation progestagen only includes levonorgestrel (N=135)

† Third generation progestagen include desogestrel (N=55), gestodene (N=24) and norgestimate (N=4)

‡ Triphasic contraceptives contain 30 µg in the first six days, followed by 40 µg for five days and ending with ten days of 30 µg ethinylestradiol

tradiol dose with SHBG levels. When we restricted our analysis to women receiving 30 µg of ethinylestradiol, users of desogestrel, gestodene, and drospirenone had higher SHBG levels than users of levonorgestrel (mean difference: 112.8 nmol/L, 95%CI: 97.3 to 128.2, 80.6 nmol/L, 95%CI: 57.3 to 104.0, and 111.1 nmol/L, 95%CI: 89.8 to 132.3 for desogestrel, gestodene, and drospirenone, respectively). Adjustment for factors influencing SHBG levels did not change these results.

Additional to the progestagens levonorgestrel, gestodene, desogestrel, and drospirenone, 30 women used cyproterone acetate. In contrast with these other progestogens, a contraceptive with cyproterone acetate contains 35 µg ethinylestradiol. The mean SHBG level in users of cyproterone acetate was high at 215.9 nmol/L (95%CI: 199.7 to 232.1); much higher than in users of oral contraceptives containing levonorgestrel with 30 µg ethinylestradiol (mean difference: 135.4 nmol/L, 95%CI: 116.9 to 153.9 adjusted for study and menstruating at venipuncture).

Users of ≥35 µg of ethinylestradiol had higher SHBG levels than users of 20 µg (mean difference: 145.4 nmol/L, 95%CI:

Table 3.3: Linear regression analysis

Variable	N (%)	Mean SHBG levels* (95%CI)	Adjusted difference* (95%CI)	Adjusted difference† (95%CI)
Progestagen‡				
Levonorgestrel	99 (60)	80.3 (72.3 to 88.2)	Reference	Reference
Desogestrel	36 (22)	193.0 (179.9 to 206.2)	112.8 (97.3 to 128.2)	116.9 (101.1 to 132.7)
Gestodene	13 (8)	160.9 (138.8 to 183.0)	80.6 (57.3 to 104.0)	81.5 (56.3 to 106.6)
Drospirenone	17 (10)	191.3 (171.8 to 210.9)	111.1 (89.8 to 132.3)	114.3 (93.1 to 135.5)
EE dose				
20 µg	31 (11)	101.6 (80.4 to 122.8)	Reference	Reference
30 µg	165 (60)	115.3 (103.9 to 126.8)	13.8 (-7.1 to 34.6)	13.9 (-8.3 to 36.2)
≥35 µg	45 (16)	247.0 (200.6 to 293.4)	145.4 (87.1 to 203.7)	136.4 (64.5 to 208.3)
Triphasic	32 (12)	152.5 (132.7 to 172.4)	51.0 (22.8 to 79.1)	50.9 (20.7 to 81.1)

CI, confidence interval; EE, ethinylestradiol
* Adjusted for progestagen in the case of ethinylestradiol dose and adjusted for study
† Further adjusted for age, BMI and menstruating at venipuncture
‡ Restricted to 30 µg ethinylestradiol

87.1 to 203.7). Also users of triphasic contraceptives had higher SHBG levels than users of 20 µg of ethinylestradiol (mean difference: 51.0 nmol/L, 95%CI: 22.8 to 79.1). The SHBG levels were only slightly higher in users of 30 µg compared with 20 µg of ethinylestradiol (mean difference: 13.8 nmol/L, 95%CI: -7.1 to 34.6). Adjustment for factors influencing SHBG levels did not change these results.

The same results were observed when the analysis was restricted to most commonly used progestagens (levonorgestrel, desogestrel and gestodene) or separately per these progestagens, although the number of women per category was very small (data not shown). Furthermore, similar results were observed when the analysis was performed per study (Supplementary table).

Discussion

When restricting to combined oral contraceptive preparations with 30 µg ethinylestradiol, users of combined oral contraceptives containing desogestrel, gestodene, and drospirenone had higher SHBG levels than users of levonorgestrel. Cyproterone acetate use was also associated with higher SHBG levels than levonorgestrel use, although we cannot exclude an effect caused by the difference in ethinylestradiol dose. Women using a combined oral contraceptive with ≥35 µg ethinylestradiol or women using a triphasic contraceptive had higher SHBG levels than women using a combined oral contraceptive with 20 µg. However, SHBG levels were only slightly higher in 30 µg ethinylestradiol users than in 20 µg users.

Estrogens such as ethinylestradiol increase the synthesis of SHBG[25], whereas progestagens induce a decrease in SHBG levels depending on the type and dose used[14,26]. In women receiving 15 µg of ethinylestradiol without a progestagen, the SHBG levels increased from 213.5 nmol/L on day 1 to 661.9 nmol/L on day 21 of the pill-cycle[27]. In contrast, women using 150 µg levonorgestrel without ethinylestradiol showed a decrease in the

SHBG levels over 23 days from 40.4 nmol/L to 15.5 nmol/L[28]. The effect of a combined oral contraceptive on SHBG levels may be seen as the result of the stimulating effect of ethinylestradiol and the inhibiting effect of the progestagen in the contraceptive[14]. The final net change is sometimes referred to as the total estrogenicity of the contraceptive. It has been suggested that this may reflect the magnitude of the risk of venous thrombosis[14].

In the literature, one paper reported on the effect of different oral contraceptives as well as the effect of the ethinylestradiol dose in combined oral contraceptives on SHBG levels; however, the difference in SHBG levels before and after a contraceptive was reported. No difference in SHBG levels between different contraceptives was stated[29]. No conclusions were drawn on whether the ethinylestradiol dose in different combined oral contraceptives was reflected in SHBG levels.

The positive association between ethinylestradiol dose and SHBG levels is in line with previous findings regarding the risk of venous thrombosis. Lidegaard et al reported that compared with users of oral contraceptive preparations containing 30-40 µg ethinylestradiol, the risk of venous thrombosis was higher in users of 50 µg ethinylestradiol (OR 1.6, 95%CI: 0.9 to 2.8) and lower in users of 20 µg (OR 0.6, 95%CI: 0.4 to 0.9)[30]. In the MEGA study, we also demonstrated that within users of oral contraceptives containing levonorgestrel, the risk of venous thrombosis adjusted for age was higher in users of 50 µg ethinylestradiol (OR 2.2, 95%CI: 1.3 to 3.7) than in users of 30 µg[9]. The risk of venous thrombosis was lower in users of 20 µg than in users of 30 µg; both in users of progestagens gestodene (OR 0.3, 95%CI: 0.2 to 0.7) and desogestrel (OR 0.7, 95%CI: 0.4 to 1.2).

Unfortunately, ethinylestradiol levels could not be measured directly because the blood was drawn at random during the four week cycle of pill use in the MEGA study and without considering the hours after a pill was taken, which both have a significant influence on ethinylestradiol levels[31]. Because of a half-life of SHBG of about 7 days[32], the hours after a pill was taken do

not influence the SHBG levels. Data were available on factors that were previously shown to influence SHBG levels and on whether women were menstruating at venipuncture. Regarding the analysis between ethinylestradiol dose and SHBG levels, we would have preferred to restrict our analysis to one progestagen; however, the number of women per category became very small leading to unreliable estimates. We combined two studies that differed in their design, which may have affected our results. However, sensitivity analyses showed that this did not influence our results. Finally, although we excluded women exposed to environmental risk factors, women with a positive family history were included. Nevertheless, we do not expect that having a positive family history influenced SHBG levels. Strengths of our study were that we included a relative large number of combined oral contraceptive users who were using many different types of prescriptions. Furthermore, SHBG levels as well as the difference in SHBG levels between different progestagens in combined oral contraceptive users were in the same range as observed in other studies[33–35].

In conclusion, users of the progestagens desogestrel, gestodene, and drospirenone had increased SHBG levels compared with levonorgestrel users. An increase in the ethinylestradiol dose in the combined oral contraceptive leads to an increase in the SHBG levels in premenopausal women using these combined oral contraceptives. This study demonstrates that SHBG levels reflect the ethinylestradiol dose used in combined oral contraceptives independent of the progestagen used. Because ethinylestradiol is important in the pathogenesis of venous thrombosis among combined oral contraceptive users, these findings strengthen the idea that SHBG levels in combined oral contraceptive users may be seen as a marker for the risk of venous thrombosis.

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Supplementary data

Supplementary table Results of sensitivity analysis per study

Variable	MEGA study Adjusted difference SHBG levels* (95%CI)	DRSP study Adjusted difference SHBG levels* (95%CI)
Progestagen†		
Levonorgestrel	Reference	Reference
Desogestrel	125.9 (103.4 to 148.4)	106.6 (84.7 to 128.4)
Gestodene	91.0 (61.4 to 120.7)	44.9 (-8.3 to 98.1)
Drospirenone	94.1 (55.9 to 132.4)	124.9 (99.8 to 150.0)
EE dose		
20 µg	Reference	Reference
30 µg	4.8 (-30.1 to 39.7)	12.5 (-15.8 to 40.8)
≥35 µg	130.8 (49.8 to 211.7)	141.2 (73.3 to 209.0)
Triphasic	45.2 (4.4 to 86.0)	-

CI, confidence interval; EE, ethinylestradiol

* Adjusted for progestagen in the case of ethinylestradiol dose, study, menstruating at venipuncture, age and BMI

† Restricted to 30 µg ethinylestradiol

Chapter 4

Genetic variation in ethinylestradiol metabolism and venous thrombosis

Bernardine H. Stegeman
Hans L. Vos
Frans M. Helmerhorst
Frits R. Rosendaal
Pieter H. Reitsma
Astrid van Hylckama Vlieg

Submitted for publication

Abstract

Background: Use of ethinylestradiol, one of the active ingredients in combined oral contraceptives, affects the incidence of venous thrombosis. To explain why some women develop thrombosis when using oral contraceptives and others do not, we hypothesized a role for the first-pass metabolism of ethinylestradiol in the liver.
Objectives: To determine the association between genetic variation in the first-pass metabolism of ethinylestradiol, venous thrombosis risk and the effect on SHBG levels, a marker for venous thrombosis risk in oral contraceptive users.
Methods: Premenopausal women with venous thrombosis and control subjects were included from two large case-control studies, i.e., the LETS and the MEGA study. Women exposed to acquired risk factors other than combined oral contraceptives were excluded. Haplotype-tagging SNPs were selected in 11 candidate genes; *COMT*, *CYP1A2*, *CYP2C9*, *CYP3A4*, *CYP3A5*, *SULT1A1*, *SULT1E1*, *UGT1A1*, *UGT1A3*, *UGT1A9*, and *UGT2B7*. Venous thrombosis risk was expressed as odds ratios (OR) with 95% confidence intervals (CI). For SHBG levels, mean differences with 95%CI were estimated in combined oral contraceptive-using control subjects from the MEGA study.
Results: 262 premenopausal women (103 cases; 159 controls) were included from the LETS and 1193 (397 cases; 796 controls) from the MEGA study. 74 SNPs in the 11 genes were determined. Two copies of haplotype D in the *UGT2B7* gene increased venous thrombosis risk (OR~3) as well as SHBG levels (mean difference 27.6 nmol/L, 95%CI: -61.7 to 116.9 compared with no copies) in oral contraceptive users and not in non-users. In oral contraceptive users, haplotype A and B in the *CYP3A4* gene were associated with the risk of venous thrombosis, but not in non-users; however, the effect on SHBG levels was not directional with the risk. None of the other haplotypes were associated with venous thrombosis.

Conclusion: Genetic variation in the *UGT2B7* gene can at least in part explain the risk of venous thrombosis in combined oral contraceptive users.

Introduction

Combined oral contraceptive use, containing an estrogen (i.e., ethinylestradiol) and a progestagen, increase venous thrombosis risk[1–4]. Over time the dose of ethinylestradiol was stepwise reduced from ≥100 µg to 50 µg and 30/20 µg, which resulted in the intended lowering in the risk of venous thrombosis[1,5,6]. The risk of venous thrombosis is the highest in the first three months of combined oral contraceptive use, i.e., about twelve-fold increased compared with non-users[7–9]. With extended use the risk remains approximately five-fold increased[9]. While some high-risk groups have been identified, i.e., women with prothrombotic genetic defects and women who are obese, it is largely unknown why oral contraceptive use leads to thrombosis in some women, and not in others.

Because they are taken orally, combined oral contraceptives are metabolised in the liver through the so-called first-pass metabolism. In the liver many coagulation factors are produced; therefore, we hypothesized that the first-pass metabolism of oral contraceptives, in particular of ethinylestradiol (Figure 4.1), influences the risk of venous thrombosis, and that genetic variation in involved genes explains the different susceptibility between women. In general, the first-pass metabolism of drugs mainly involves conjugation and hydroxylation. Sulfonation and glucuronidation are both conjugation steps leading to inactive and water-soluble compounds which are excreted by the kidneys or the intestinal tract (via bile). The genes *SULT1A1* and *SULT1E1* code for sulfotransferases that are involved in sulfonation of ethinylestradiol[10–15] and the genes *UGT1A1*, *UGT1A3* and *UGT1A9* code for UDP-glucuronosyltransferases involved in the glucuronidation of ethinylestradiol[15–18]. Hydroxylation and subsequent methylation of the hydroxyl group lead to hydroxy-ethinylestradiol and methoxy-ethinylestradiol, respectively. The genes *CYP1A2*, *CYP2C9*, *CYP3A4* and *CYP3A5* code for enzymes involved in the hydroxylation step[15,19–21] and the *COMT*

gene codes for catechol O-methyltransferase involved in the methylation step[22]. To inactivate hydroxyl-ethinylestradiol and methoxy-ethinylestradiol, the aforementioned conjugation steps are used.

Sex hormone binding globulin (SHBG) is a marker for the hormonal effects of combined oral contraceptives on venous thrombosis risk. SHBG is a hepatic plasma glycoprotein that binds the sex steroid hormones testosterone and 17β -estradiol, but not ethinylestradiol. Estrogens such as ethinylestradiol increase the synthesis of SHBG[23], while progestagens induce a decrease in SHBG levels depending on the type and dose[24,25]. The effect of a combined oral contraceptive on SHBG levels may be seen as the result of the stimulating effect of ethinylestradiol and the inhibiting effect of the progestagen in a contraceptive[25].

The aim of this study was to explain differences in susceptibility to the prothrombotic effect of oral contraceptives by assessing genetic variation in the first-pass metabolism of ethinylestradiol in premenopausal women. Because the investigated enzymes are also involved in other metabolic pathways, results from non-users were evaluated to assess the specificity of the association with risk. Any risk associations with venous thrombosis observed in non-users, in whom the first-pass metabolism of ethinylestradiol is not activated, will be a reflection of genetic variation in the other pathways. Furthermore, genetic variation in the first-pass metabolism of ethinylestradiol was linked to an intermediate variable, SHBG levels, in combined oral contraceptive users. A priori, three criteria were established to determine whether a haplotype was associated with venous thrombosis through changes in the first-pass metabolism of ethinylestradiol, i.e., a similar association with venous thrombosis in combined oral contraceptive users in two independent studies (LETS and MEGA study), no association in non-users, and a direction of the effect on SHBG levels in accordance with the association with venous thrombosis risk.

Methods

Participants Participants were selected from two case-control studies on venous thrombosis, i.e., the LETS (Leiden Thrombophilia Study) and the MEGA (Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis) study. In the LETS, participants with a first, symptomatic, objectively confirmed episode of deep venous thrombosis in the leg, younger than 70 years and without a known malignant disorder were enrolled between 1 January 1988 and 31 December 1992. As controls, acquaintances and partners of the patients were invited to participate[26]. In the MEGA study, participants with a first symptomatic deep venous thrombosis in the leg or arm or pulmonary embolism were recruited between 1 March 1999 and 31 August 2004. Controls were either the partners of the patients or recruited through random digit dialling (RDD). Details of the study have been described elsewhere[27]. Participants of both studies were asked to fill in a questionnaire and to provide a blood or buccal swab sample. The LETS and the MEGA study differed slightly in their in- and exclusion criteria. To increase homogeneity in the present analysis, only patients with a deep venous thrombosis of the leg were studied.

The population of interest consisted of premenopausal women younger than 50 years (N_{LETS}=347; N_{MEGA}=2657). Women who had any type of cancer (N_{MEGA}=63), were hospitalized (N_{LETS}=22; N_{MEGA}=357), had undergone surgery (N_{LETS}=29; N_{MEGA}=277), suffered bone fractures (N_{LETS}=2; N_{MEGA}=81) or had soft-tissue injuries (N_{LETS}=0; N_{MEGA}=529) in the twelve months before the index date were excluded. We also excluded women who were pregnant (N_{LETS}=11; N_{MEGA}=65), were within four weeks postpartum (N_{LETS}=4; N_{MEGA}=17) or were using hormone replacement therapy (N_{MEGA}=14) at the index date or had experienced a miscarriage (N_{LETS}=1; N_{MEGA}=10) in the twelve months before the index date. Oral contraceptive users were defined as users of a combined oral contraceptive at the

index date (i.e., a contraceptive containing ethinylestradiol and a progestagen). Hence, in both studies women using a contraceptive without ethinylestradiol, e.g., progestagen-only pills, were excluded (N_{LETS}=2; N_{MEGA}=26). Women without a blood or buccal samples were also excluded (N_{MEGA}=366). 500 patients and 955 controls were included in the current analysis (LETS: 103 cases and 159 controls; MEGA: 397 cases and 796 controls). In both studies, the sum of all exclusions does not add up to the total number of excluded participants because women could be exposed to one or more risk factors.

DNA preparation and SNP typing Collection and processing of blood samples and buccal swabs and subsequent DNA isolation have been described previously[26,27]. To determine the haplotypes in the selected genes, the Genome Variation Server (GWS)[28] was used. GVS incorporates information from HapMap and other sources and is sponsored by SeattleSNPs. Only SNPs with a minor allele frequency of 5% or more in Caucasians were considered.

A total of 11 genes involved in ethinylestradiol metabolism were selected prior to genotyping, i.e., *COMT*, *CYP1A2*, *CYP2C9*, *CYP3A4*, *CYP3A5*, *SULT1A1*, *SULT1E1*, *UGT1A1*, *UGT1A3*, *UGT1A9*, and *UGT2B7* in which a total of 74 SNPs were selected (Supplementary table 4.1). Care was taken to select SNPs from each haplotype that enabled a clear distinction between highly related genes. The SNPs were either determined with the MassARRAY platform (Sequenom, San Diego, California, USA) according to manufacturer's protocols (Sequenom) or determined with the 5' nuclease/Taqman assay (Assay-by-Design, Applied Biosystems, Foster City, California, USA). SNP rs28946889 was determined via restriction digest of the PCR product (details available on request). Genotyping determination was done blinded to the case/control status and study number. Five percent of the samples were repeated for allele-calling consistency, no discrepancies were found.

Laboratory measurements SHBG levels (nmol/L) were measured with an immunometric assay (Immulite 2000 XPi; Siemens Healthcare Diagnostics, Tarrytown, NY, USA) in combined oral contraceptive users of the control group of the MEGA (plasma of the LETS subjects was no longer available). The sensitivity of the assay is 0.2 nmol/L and the assay has a log-term variation of 6% at levels of 5 nmol/L and 80 nmol/L. The within-assay variation is 3-4% and the between-assay variation 3.5- 6%. The samples were analysed in a single series in random order. SHBG levels were measured without knowledge of any other of the participant's characteristics.

Statistical analysis Hardy-Weinberg Equilibrium was assessed with a Chi-squared test in controls from the LETS and MEGA study. Due to the large number of SNPs tested we used a Bonferroni correction for the Hardy-Weinberg Equilibrium tests. The posterior probabilities of correct haplotype assignment as estimated by PLINK (version 1.07) [29] were used as weights in all statistical analyses. Because women could be assigned two possible combinations of haplotypes, no number of women per copies of a haplotype was given in the tables. Odds ratios (OR) were calculated to determine the risk of venous thrombosis associated with the number of copies of a specific haplotype. To account for multiple testing, the False Discovery Rate q-value was calculated. First, the p-value of the association was derived from the univariate unconditional logistic regression analysis between a haplotype and venous thrombosis risk while assuming an additive model for the genetic relationship with venous thrombosis. Second, the p-values were ranked from smallest till largest, where rank order 1 was given to the smallest p-value. Third, the q-values were calculated as follows; the p-values were multiplied by the number of tests performed and then divided by the rank order of each p-value. For each test, the q-value is then defined as the minimum among tests with equal or higher rank. The threshold was set at 0.20 and can be interpreted as follows: among tests

with a q-value of 0.20 or lower, at most 20% or lower of the tests might be a false discovery. The analyses were restricted to combined oral contraceptives users or non-users. Linear regression analysis was used to determine the effect of a haplotype on SHBG levels in controls using combined oral contraceptives from the MEGA study. In this analysis, combined oral contraceptive use was defined at venipuncture and not at the index date. To determine whether a haplotype was associated with venous thrombosis, we used several criteria. An association with venous thrombosis in the same direction had to be present in the LETS and MEGA study, no association in non-users and a same directional effect on SHBG levels as expected based on the risk association with venous thrombosis. Statistical analyses were performed with STATA, version 12.0 (Statacorp LP, College Station, Texas, USA).

Results

General description of the population Baseline characteristics of the studies are given in Table 4.1. In the LETS, the mean age in cases and controls was 35 years (range cases: 16 to 49 and controls: 15 to 48). In the MEGA study, the mean age in the cases was 37 years (range: 18 to 49) and in the controls 38 years (range: 18 to 49). No information about ancestry was available in the LETS. In the MEGA study, about 90% of cases and controls reported to be from Western European ancestry. The majority of the cases (80% and 87%, respectively in the LETS and the MEGA study) were using combined oral contraceptives at the time of the event.

Hardy-Weinberg Equilibrium Hardy-Weinberg Equilibrium was assessed in the controls from the LETS and MEGA study. To control for multiple testing, we performed a Bonferroni correction. All SNPs were in Hardy-Weinberg Equilibrium ($p>0.0007$), except for SNP rs12445705 in the *SULT1A1* gene in the MEGA

Table 4.1: Baseline characteristics of the selected population from the LETS and the MEGA study

	LETS		MEGA study	
Variables	**Cases (N=103)**	**Controls (N=159)**	**Cases (N=397)**	**Controls (N=796)**
Mean age (SD)	35 (10)	35 (9)	37 (9)	38 (8)
Caucasian[a](%)	-	-	347 (90)	695 (89)
Pill use (%)	78 (80)	62 (42)	343 (87)	335 (42)

[a] No information about race was available in the LETS study

study. To exclude a measurement error, we confirmed this SNP with a Taqman assay. The results did not materially differ between the Sequenom and Taqman assay. Consensus among controls from the LETS was 95.7% and 97.1% in the MEGA study.

Results per gene family The results were grouped per gene family. In nine genes, no associations in the same direction were observed between the LETS and MEGA study (Supplementary tables 4.2-9). In *CYP3A4*, two of the five haplotypes were associated with the risk of venous thrombosis, i.e., the similar results were observed in both studies (Table 4.2 & 4.3), and in *UGT2B7*, two of the eight haplotypes were associated with venous thrombosis (Table 4.4 & 4.5).

Gene family *CYP* In combined oral contraceptive users, two haplotypes in *CYP3A4* were associated with venous thrombosis in both studies (Table 4.2). Carrying one copy of haplotype A was associated with a decreased risk of venous thrombosis (OR_{LETS}: 0.29, 95%CI: 0.11-0.76 and OR_{MEGA}: 0.57, 95%CI: 0.37-0.89). Similar results were observed for carriers of two copies of haplotype A (OR_{LETS}: 0.40, 95%CI: 0.14-1.13 and OR_{MEGA}: 0.59, 95%CI: 0.37-0.94). With an additive model, each copy of

Table 4.2: Results from carriers of haplotypes in the *CYP3A4* gene among oral contraceptive users

Haplotype		LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
A									
	0	1			1			125.5 (105.7-145.4)	Reference
	1	0.29 (0.11-0.76)			0.57 (0.37-0.89)			141.3 (126.0-156.7)	15.8 (-8.5 to 40.1)
	2	0.40 (0.14-1.13)			0.59 (0.37-0.94)			152.1 (134.2-170.1)	26.6 (-0.3 to 53.5)
	Additive	0.72 (0.45-1.17)	0.18	0.88	0.83 (0.66-1.03)	0.09	0.98		
B									
	0	1			1			145.5 (134.2-156.8)	Reference
	1	2.14 (0.76-6.05)			1.86 (1.17-2.94)			116.2 (89.4-142.9)	-29.4 (-58.0 to -0.7)
	2	-			0.34 (0.04-3.31)			161.9 (135.4-188.3)	16.3 (-12.6 to 45.3)
	Additive	1.41 (0.52-3.83)	0.50	0.88	1.49 (0.95-2.33)	0.79	0.98		
C									
	0	1			1			142.1 (131.5-152.7)	Reference
	1	0.42 (0.10-1.78)			1.46 (0.65-3.30)			177.7 (109.1-246.3)	35.6 (-34.0 to 105.3)
	2	-			-			-	-
	Additive	0.42 (0.10-1.78)	0.24	0.88	1.46 (0.65-3.30)	0.36	0.98		
D									
	0	1			1			149.3 (134.8-163.9)	Reference
	1	0.92 (0.45-1.90)			1.06 (0.78-1.46)			137.3 (121.4-153.2)	-12.1 (-33.4 to 9.3)
	2	3.81 (0.75-19.29)			0.75 (0.32-1.75)			115.8 (85.4-146.2)	-33.5 (-67.4 to 0.4)
	Additive	1.36 (0.80-2.29)	0.26	0.88	0.99 (0.76-1.29)	0.93	0.98		
E									
	0	1			1			144.1 (133.4-154.9)	Reference
	1	-			1.46 (0.65-3.31)			128.1 (74.2-181.9)	-16.1 (-71.2 to 39.0)
	2	-			-			-	-
	Additive	-	-	-	1.46 (0.65-3.31)	0.36	0.98		

the allele reduced the risk by 20-30% (OR_{LETS}: 0.72, 95%CI: 0.45-1.17 and OR_{MEGA}: 0.83, 95%CI: 0.66-1.03). Carrying one copy of haplotype B increased venous thrombosis risk (OR_{LETS}: 2.14, 95%CI: 0.76-6.05; OR_{MEGA}: 1.86, 95%CI: 1.17-2.94). None of the cases in the LETS study were carriers of two copies of haplotype B, whereas only one case in the MEGA study carried two copies of haplotype B. With an additive model, a ~1.5 fold increase in risk was observed per extra allele (OR_{LETS}: 1.41, 95%CI: 0.52-3.83 and OR_{MEGA}: 1.49, 95%CI: 0.95-2.33). None of the other haplotypes in *CYP3A4* were associated with venous thrombosis risk, i.e., associations in the same direction in both the LETS and MEGA study were not observed.

Both haplotypes A and B in *CYP3A4* were associated with changes in SHBG levels in combined oral contraceptive users among the controls of the MEGA study. Carrying haplotype A increased SHBG levels in controls of the MEGA study (mean difference one copy versus no copies: 15.8 nmol/L, 95%CI: -8.5 to 40.1 and mean difference two copies versus no copies: 26.6 nmol/L, 95%CI: -0.3 to 53.5). We observed a decrease in SHBG levels among controls of the MEGA study carrying one copy of haplotype B (mean difference: -29.4 nmol/L, 95%CI: -58.0 to -0.7). For both haplotyoes, the effect on SHBG levels was in the opposite direction as expected based on the effect on the risk of venous thrombosis; the protective haplotype A is associated with increased SHBG levels, whereas the reverse was true for the pro-thrombotic haplotype B.

In table 4.3, the effect of haplotypes in *CYP3A4* on risk of venous thrombosis in non-users is given. None of the haplotypes were associated with venous thrombosis in non-users, i.e., no associations in the same direction were observed.

Gene family *UGT* Carrying one copy of haplotype D of *UGT2B7* was not associated with venous thrombosis (OR_{LETS}: 1.02, 96%CI: 0.49-2.12 and OR_{MEGA}: 0.93, 95%CI: 0.67-1.30). Carriers of two copies of haplotype D in *UGT2B7* had an in-

Table 4.3: Results from carriers of haplotypes in the emphCYP3A4 gene among non-users

Haplotype	LETS (N=104) OR (95%CI)	p	FDR q	MEGA (N=513) OR (95%CI)	p	FDR q
A						
0	1			1		
1	0.94 (0.23-3.89)			0.71 (0.33-1.52)		
2	1.13 (0.25-5.07)			0.90 (0.40-2.02)		
Additive	1.10 (0.52-2.32)	0.80	0.98	1.00 (0.64-1.55)	0.99	0.99
B						
0	1			1		
1	0.83 (0.17-4.14)			1.33 (0.60-2.95)		
2	-			-		
Additive	0.83 (0.17-4.14)	0.82	0.98	1.11 (0.55-2.23)	0.77	0.94
C						
0	1			1		
1	4.75 (0.87-25.93)			1.18 (0.26-5.31)		
2	-			-		
Additive	4.75 (0.87-25.93)	0.07	0.98	1.18 (0.26-5.31)	0.83	0.95
D						
0	1			1		
1	0.59 (0.20-1.68)			1.45 (0.81-2.60)		
2	1.16 (0.11-12.23)			0.93 (0.21-4.21)		
Additive	0.74 (0.28-1.94)	0.54	0.98	1.22 (0.78-1.91)	0.38	0.91
E						
0	1			1		
1	2.14 (0.18-25.22)			0.54 (0.07-4.16)		
2	-			-		
Additive	2.14 (0.18-25.22)	0.55	0.98	0.54 (0.07-4.16)	0.55	0.94

creased risk of venous thrombosis in both LETS and MEGA study (OR_{LETS}: 3.78, 95%CI: 0.40-35.84 and OR_{MEGA}: 2.61, 95%CI: 1.07-6.34) (Table 4.4). Because the effect seemed clearly recessive, we did not interpret the results from an additive model. None of the other haplotypes in *UGT2B7* were consistently associated with venous thrombosis in both studies. Fully in line with the effect of this haplotype on the risk of venous thrombosis, no effect on SHBG levels in controls of the MEGA study was observed in carriers of one copy of haplotype D in *UGT2B7* (mean difference one copy versus no copies: -5.5 nmol/L, 95%CI:

-28.5 to 17.6), and an increase in SHBG levels was observed with carriers of two copies of haplotype D (mean difference two copies versus no copies of haplotype D: 27.6 nmol/L, 95%CI: -61.7 to 116.9).

In table 4.5, the results for the *UGT2B7* gene are given in non-users. Carriership of one copy of haplotype E was associated with an increase in risk of venous thrombosis (OR_{LETS}: 1.58, 95%CI: 0.15-16.42 and OR_{MEGA}: 1.47, 95%CI: 0.32-6.77). Carriers of two copies of haplotype E were not present in either the LETS or MEGA study and therefore the odds ratio assuming an additive model did not change.

Multiple testing Overall, eight tests of a total of 321 tests were significant at a 0.05 level. Given the large number of tests, we calculated FDR q-values which were all above 0.20.

Discussion

In a search of explanations for ethinylestradiol-associated venous thrombosis, we studied haplotypes of 11 genes involved in ethinylestradiol metabolism in two case-control studies on venous thrombosis with a total of 500 patients and 955 control women. For nine genes we found no association of the haplotypes with risk, while two genes (*CYP3A4* and *UGT2B7*) were associated with venous thrombosis risk. Haplotpe D in the *UGT2B7* gene showed a consistent effect on SHBG levels as well. Homozygous carriers of haplotype D in *UGT2B7* had a three-fold increased risk of thrombosis and a substantial increase in SHBG levels. For all genotypes the FDR was above the threshold of 0.20. However, the likelihood that the association is the result of chance variation is unlikely given the large odds ratios in two separate studies (3.87 and 2.61) in combined oral contraceptive users, the absence of an effect in non-users meaning the result is specific for the first-pass metabolism of ethinylestradiol and the substantial chance in SHBG levels in combined oral contraceptive users

Table 4.4: Results from carriers of haplotypes in the *UGT2B7* gene among non-users

Haplotype		LETS (N=104) OR (95%CI)	p	FDR q	MEGA (N=513) OR (95%CI)	p	FDR q
A							
	0	1			1		
	1	1.58 (0.15-16.42)			-		
	2	-			-		
	Additive	1.58 (0.15-16.42)	0.70	0.98	-	-	-
B							
	0	1			1		
	1	1.21 (0.38-3.88)			0.49 (0.21-1.13)		
	2	-			0.84 (0.10-6.83)		
	Additive	1.07 (0.37-3.09)	0.90	0.98	0.59 (0.28-1.26)	0.17	0.89
C							
	0	1			1		
	1	0.92 (0.27-3.20)			1.46 (0.76-2.79)		
	2	-			1.07 (0.13-8.71)		
	Additive	0.72 (0.26-1.98)	0.53	0.98	1.31 (0.77-2.25)	0.32	0.91
D							
	0	1			1		
	1	2.10 (0.71-6.15)			0.90 (0.48-1.71)		
	2	-			2.06 (0.65-6.53)		
	Additive	1.25 (0.59-2.66)	0.56	0.98	1.14 (0.67-1.93)	0.63	0.94
E							
	0	1			1		
	1	1.58 (0.15-16.42)			1.47 (0.32-6.77)		
	2	-			-		
	Additive	1.58 (0.15-16.42)	0.70	0.98	1.47 (0.32-6.77)	0.62	0.94
F							
	0	1			1		
	1	2.91 (0.98-8.71)			1.19 (0.64-2.20)		
	2	-			-		
	Additive	1.96 (0.78-4.92)	0.15	0.98	0.87 (0.55-1.40)	0.58	0.94
G							
	0	1			1		
	1	0.43 (0.09-2.08)			0.97 (0.49-1.89)		
	2	-			0.96 (0.12-7.79)		
	Additive	0.38 (0.09-1.49)	0.17	0.98	0.97 (0.54-1.74)	0.92	0.99
H							
	0	1			1		
	1	0.98 (0.25-3.90)			1.43 (0.74-2.76)		
	2	-			-		
	Additive	0.86 (0.25-2.96)	0.81	0.98	1.24 (0.69-2.22)	0.48	0.94

Table 4.5: Results from carriers of haplotypes in the *UGT2B7* gene among combined oral contraceptive users

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
A								
0	1			1			144.3 (133.3-155.2)	Reference
1	-			2.62 (0.69-10.05)			108.7	-35.6 (-46.5 to -24.6)
2	-			-			-	-
Additive	-	-	-	2.91 (0.88-9.66)	0.08	0.98		
B								
0	1			1			140.6 (127.6-153.7)	Reference
1	1.14 (0.50-2.58)			0.74 (0.52-1.06)			150.4 (130.8-170.0)	9.8 (-13.9 to 33.5)
2	-			0.65 (0.22-1.91)			185.0 (81.2-288.7)	44.3 (-60.9 to 149.5)
Additive	1.27 (0.59-2.73)	0.53	0.88	0.76 (0.56-1.03)	0.08	0.98		
C								
0	1			1			145.3 (132.2-158.5)	Reference
1	1.25 (0.56-2.75)			1.07 (0.74-1.55)			141.4 (122.1-160.7)	-3.9 (-27.5 to 19.6)
2	-			2.28 (0.58-8.92)			113.2	-32.1 (-45.4 to -18.9)
Additive	1.07 (0.50-2.27)	0.86	0.97	1.16 (0.84-1.61)	0.37	0.98		
D								
0	1			1			145.1 (131.7-158.4)	Reference
1	1.02 (0.49-2.12)			0.93 (0.67-1.30)			139.6 (120.9-158.2)	-5.5 (-28.5 to 17.6)
2	3.78 (0.40-35.84)			2.61 (1.07-6.34)			172.7 (84.9-260.4)	27.6 (-61.7 to 116.9)
Additive	1.26 (0.69-2.30)	0.46	0.88	1.15 (0.87-1.50)	0.33	0.98		
E								
0	1			1			143.5 (132.3-154.7)	Reference
1	-			0.59 (0.32-1.12)			151.9 (107.8-196.1)	8.4 (-37.3 to 54.1)
2	-			-			-	-
Additive	-	-	-	0.59 (0.32-1.12)	0.11	0.98		

Continued on next page

Table 4.5 – continued from previous page

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
F								
0	1			1			149.1 (135.2-163.1)	Reference
1	0.59 (0.26-1.34)			1.17 (0.83-1.65)			138.1 (119.9-156.4)	-11.0 (-34.1 to 12.1)
2	0.51 (0.08-3.21)			1.08 (0.48-2.42)			99.7 (86.6-112.7)	-49.5 (-68.7 to -30.3)
Additive	0.64 (0.33-1.24)	0.19	0.88	1.12 (0.84-1.48)	0.44	0.98		
G								
0	1			1			146.1 (132.8-159.3)	Reference
1	0.47 (0.20-1.12)			0.90 (0.62-1.31)			135.0 (118.8-151.1)	-11.1 (-32.3 to 10.1)
2	-			1.04 (0.40-2.75)			190.7 (50.5-330.8)	44.6 (-97.0 to 186.2)
Additive	0.41 (0.19-0.88)	0.02	0.88	0.94 (0.69-1.29)	0.71	0.98		
H								
0	1			1			141.0 (129.1-153.0)	Reference
1	1.21 (0.55-2.69)			0.90 (0.62-1.32)			150.0 (124.3-175.2)	8.7 (-19.7 to 37.2)
2	-			1.08 (0.36-3.27)			200.2 (129.7-270.8)	59.2 (-12.8 to 131.2)
Additive	1.34 (0.64-2.82)	0.44	0.88	0.94 (0.68-1.30)	0.72	0.98		

consistent with an increased risk. *UGT2B7* is therefore a strong candidate for futher investigation, and may prove clinically relevant given its high odds ratio specific for oral contraceptive use.

To bypass the first-pass metabolism of ethinylestradiol in the liver, other types of contraception with hormones were developed that deliver the hormones directly to the systemic circulation, i.e., the transdermal patch and the vaginal ring. Two case reports concerning mesenteric vein thrombosis[30] and cerebral venous sinus thrombosis[31] and two serious adverse event in two trials[32,33] were reported in users of the vaginal ring. This potential association with venous thrombosis was confirmed in a large cohort study showing that the use of vaginal ring increased the risk of venous thrombosis compared to non-users[34]. The risk of venous thrombosis in transdermal patch users has been assessed in two observational studies using health insurance databases. Transdermal patch users have about the same risk of venous thrombosis compared to third generation users or higher[35–38], i.e., a risk that is substantially higher than that conferred by the safest oral contraceptives (containing second generation progestagens). These results were confirmed by a large cohort study[34].

To our knowledge, no studies regarding the genetics of the first-pass metabolism of ethinylestradiol in association with venous thrombosis have been published before. Earlier studies of the first-pass metabolism of estrogens showed that genetic variation in this metabolism was associated with esophageal cancer[39] and prostate cancer[40]. However, either the *CYP3A4* or *UGT2B7* genes were not studied or no association was found with these genes.

A limitation of our study is that only common genetic variation (i.e., SNPs with a frequency above 5%) was taken into account. However, rare haplotypes were captured since these common SNPs could code for haplotypes with a frequency below 5%. A strength of our study is that a highly selected population was used to ensure that venous thrombosis events were only re-

lated to oral contraceptive use. Furthermore, haplotypes were used to look at the entire gene of interest in contrast to individual SNPs.

In conclusion, genetic variation in the *UGT2B7* gene explains part of the risk of venous thrombosis in combined oral contraceptive users.

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Supplementary data

Supplementary table 4.1 Overview of the selected SNPs in the 11 genes from ethinylestradiol metabolism

Gen	SNP	Allel change	MAF	Assay
COMT	rs4333017	C/T	0.175	Sequenom
COMT	rs9605030	C/T	0.117	Sequenom
COMT	rs9617850	G/A	0.169	Sequenom
COMT	rs5746846	G/C	0.500	Sequenom
COMT	rs174675	C/T	0.258	Sequenom
COMT	rs5746847	C/T	0.433	Sequenom
COMT	rs4680	G/A	0.483	Sequenom
COMT	rs887199	G/A	0.125	Sequenom
COMT	rs887204	A/G	0.292	Sequenom
COMT	rs165849	A/G	0.308	Taqman
COMT	rs2240713	G/A	0.068	Sequenom
CYP1A2	rs1378942	T/G	0.339	Sequenom
CYP1A2	rs762551	A/C	0.308	Sequenom
CYP1A2	rs8033381	A/G	0.263	Sequenom
CYP1A2	rs2071501	T/G	0.054	Sequenom
CYP2C9	rs9332174	A/G	0.225	Sequenom
CYP2C9	rs10509679	G/A	0.110	Sequenom
CYP2C9	rs2475377	G/A	0.059	Sequenom
CYP2C9	rs2253635	A/G	0.331	Sequenom
CYP2C9	rs4917636	A/G	0.158	Sequenom
CYP2C9	rs9332245	G/A	0.058	Taqman
CYP3A4	rs2242480	C/T	0.080	Sequenom
CYP3A4	rs6945984	T/C	0.117	Sequenom
CYP3A4	rs2246709	A/G	0.308	Sequenom
CYP3A4	rs4646437	C/T	0.133	Taqman
CYP3A5	rs4646450	C/T	0.175	Sequenom
CYP3A5	rs2740565	T/A	0.067	Sequenom
CYP3A5	rs11734	C/G	0.052	Sequenom
CYP3A5	rs7792939	T/C	0.125	Sequenom
CYP3A5	rs2687134	C/A	0.050	Taqman
SULT1A1	rs17707300	A/G	0.333	Taqman
SULT1A1	rs9924471	C/T	0.136	Sequenom
SULT1A1	rs12445705	G/A	0.121	Sequenom
SULT1A1	rs10521145	C/T	0.119	Taqman

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Supplementary table 4.1 – continued from previous page

Gen	SNP	Allel change	MAF	Assay
SULT1E1	rs1590128	A/G	0.292	Taqman
SULT1E1	rs13112570	G/A	0.360	Taqman
SULT1E1	rs12506209	C/T	0.350	Taqman
SULT1E1	rs1220833	G/A	0.017	Taqman
SULT1E1	rs3736599	G/A	0.110	Taqman
SULT1E1	rs4149526	G/T	0.292	Taqman
SULT1E1	rs3775770	G/A	0.283	Taqman
SULT1E1	rs1220724	C/T	0.142	Taqman
SULT1E1	rs1220702	C/G	0.167	Taqman
SULT1E1	rs3822173	C/T	0.108	Taqman
UGT1A	rs2602376	C/T	0.220	Sequenom
UGT1A	rs4553819	A/G	0.375	Sequenom
UGT1A	rs10179094	T/A	0.342	Sequenom
UGT1A	rs17864689	G/T	0.075	Sequenom
UGT1A	rs6761246	C/A	0.373	Sequenom
UGT1A	rs12466747	G/C	0.242	Sequenom
UGT1A	rs7583278	C/T	0.325	Sequenom
UGT1A	rs6744284	C/T	0.250	Sequenom
UGT1A	rs28898590	G/T	0.058	Sequenom
UGT1A	rs4663326	A/G	0.133	Taqman
UGT1A	rs904855	G/C	0.092	Sequenom
UGT1A	rs871514	A/G	0.424	Sequenom
UGT1A	rs10179091	T/C	0.451	Taqman
UGT1A	rs6742078	G/T	0.283	Sequenom
UGT1A	rs11891311	G/A	0.314	Sequenom
UGT1A	rs28898621	C/T	0.092	Sequenom
UGT1A	rs28946889	G/T	0.306	Restriction
UGT1A	rs4663972	T/C	0.225	Sequenom
UGT1A	rs10203853	T/A	0.492	Sequenom
UGT1A	rs12468017	T/C	0.150	Sequenom
UGT1A	rs17868346	A/G	0.258	Sequenom
UGT1A	rs6719561	C/T	0.383	Sequenom
UGT2B7	rs4587017	G/T	0.483	Sequenom
UGT2B7	rs3924194	C/G	0.169	Sequenom
UGT2B7	rs6600898	G/C	0.450	Sequenom
UGT2B7	rs10028494	A/C	0.133	Sequenom
UGT2B7	rs4385139	G/C	0.474	Sequenom
UGT2B7	rs6600894	G/A	0.217	Taqman
UGT2B7	rs7662029	G/A	0.500	Sequenom
UGT2B7	rs7680341	A/G	0.175	Sequenom

Supplementary table 4.2 Results from carriers of haplotypes in the *COMT* gene among OC users

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
COMT block 1								
A								
0	1			1			144.4 (131.1-157.7)	Reference
1	0.45 (0.20-1.02)			1.22 (0.84-1.76)			140.1 (119.9-160.3)	-4.3 (-28.7 to 20.1)
2	-			0.39 (0.08-2.05)			105.7 (40.3-171.2)	-38.6 (-105.9 to 28.6)
Additive	0.55 (0.24-1.24)	0.15	0.88	1.09 (0.77-1.53)	0.63	0.98		
B								
0	1			1			139.7 (127.8-151.6)	Reference
1	1.18 (0.45-3.07)			0.82 (0.54-1.24)			154.2 (125.1-183.3)	14.5 (-17.1 to 46.1)
2	-			1.38 (0.23-8.32)			162.2	22.5 (10.6 to 34.5)
Additive	1.36 (0.57-3.22)	0.49	0.88	0.87 (0.59-1.27)	0.47	0.98		
C								
0	1			1			146.4 (133.5-159.4)	Reference
1	0.92 (0.40-2.14)			0.93 (0.66-1.32)			134.9 (112.7-157.1)	-11.5 (-37.3 to 14.3)
2	0.54 (0.08-3.42)			2.05 (0.77-5.51)			102.1 (38.5-165.6)	-44.4 (-109.6 to 20.8)
Additive	0.83 (0.44-1.59)	0.58	0.88	1.08 (0.81-1.44)	0.62	0.98		
D								
0	1			1			143.4 (131.9-155.0)	Reference
1	1.54 (0.42-5.59)			1.33 (0.74-2.41)			126.8 (97.8-155.8)	-16.7 (-48.0 to 14.6)
2	-			-			-	-
Additive	1.54 (0.42-5.59)	0.51	0.88	1.41 (0.80-2.49)	0.23	0.98		
E								
0	1			1			145.4 (133.5-157.3)	Reference
1	0.81 (0.25-2.70)			1.15 (0.69-1.94)			115.4 (91.9-139.9)	-29.5 (-56.3 to -2.7)
2	-			-			-	-
Additive	0.64 (0.23-1.75)	0.39	0.88	1.22 (0.74-2.01)	0.44	0.98		

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Supplementary table 4.2 – continued from previous page

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
F								
0	1			1			138.3 (115.3-161.3)	Reference
1	1.40 (0.60-3.27)			0.97 (0.67-1.40)			142.9 (127.9-158.0)	4.6 (-23.0 to 32.3)
2	1.68 (0.63-4.49)			0.93 (0.60-1.43)			146.4 (123.4-169.5)	8.1 (-24.7 to 40.9)
Additive	1.30 (0.79-2.13)	0.30	0.88	0.96 (0.78-1.20)	0.73	0.98		
SNP rs4680								
GG	1			1			137.7 (117.4-157.9)	Reference
GA	1.62 (0.74-3.55)			1.20 (0.84-1.71)			153.9 (137.6-170.3)	16.3 (-8.2 to 40.7)
AA	1.71 (0.70-4.19)			1.18 (0.75-1.86)			127.4 (112.7-142.2)	-10.3 (-40.8 to 20.3)
Additive	1.37 (0.91-2.06)	0.14	0.88	1.10 (0.88-1.37)	0.42	0.98		
COMT block 2								
A								
0	1			1			118.2 (93.7-142.7)	Reference
1	0.71 (0.21-2.46)			0.97 (0.57-1.66)			151.6 (134.0-169.1)	33.3 (2.9 to 63.7)
2	0.54 (0.16-1.79)			0.87 (0.51-1.48)			141.5 (125.3-157.8)	23.3 (-6.4 to 52.9)
Additive	0.74 (0.44-1.25)	0.26	0.88	0.92 (0.73-1.16)	0.47	0.98		
B								
0	1			1			141.8 (130.9-152.6)	Reference
1	0.94 (0.13-6.95)			1.09 (0.48-2.47)			173.3 (102.5-244.0)	31.5 (-40.3 to 103.3)
2	-			-			-	-
Additive	0.94 (0.13-6.95)	0.95	0.98	1.09 (0.48-2.47)	0.84	0.98		
C								
0	1			1			144.0 (132.0 to 156.1)	Reference
1	2.03 (0.72-5.66)			1.13 (0.72-1.77)			148.1 (118.5-177.6)	4.0 (-28.0 to 36.1)
2	-			0.57 (0.13-2.39)			95.1 (25.6-164.6)	-48.9 (-119.9 to 22.0)
Additive	2.03 (0.73-5.66)	0.18	0.88	1.01 (0.69-1.48)	0.96	0.98		

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Supplementary table 4.2 – continued from previous page

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
D								
0	1			1			144.2 (130.2-158.3)	Reference
1	0.91 (0.41-2.01)			1.15 (0.81-1.64)			142.7 (123.4-161.9)	-1.6 (-25.6 to 22.4)
2	0.85 (0.05-14.09)			0.85 (0.30-2.37)			139.1 (100.3=177.9)	-5.2 (-46.7 to 36.4)
Additive	0.91 (0.45-1.86)	0.80	0.95	1.08 (0.80-1.46)	0.63	0.98		
E								
0	1			1			146.1 (134.3-157.9)	Reference
1	2.37 (0.70-8.04)			1.02 (0.58-1.80)			109.9 (84.6=135.3)	-36.1 (-64.3 to -8.0)
2	-			0.93 (0.06-15.04)			236.5	90.4 (78.6 to 102.3)
Additive	2.37 (0.70-8.04)	0.17	0.88	1.02 (0.60-1.72)	0.96	0.98		

Supplementary table 4.3 Results from carriers of haplotypes in the *COMT* gene among non-users

Haplotype	LETS (N=104) OR (95%CI)	p	FDR q	MEGA (N=513) OR (95%CI)	p	FDR q
COMT block 1						
A						
0	1			1		
1	0.95 (0.30-2.99)			1.12 (0.58-2.16)		
2	-			-		
Additive	0.95 (0.30-2.99)	0.93	0.98	0.92 (0.53-1.59)	0.76	0.94
B						
0	1			1		
1	2.25 (0.72-7.07)			1.31 (0.65-2.61)		
2	-			-		
Additive	2.83 (1.06-7.51)	0.04	0.98	1.13 (0.61-2.09)	0.70	0.94
C						
0	1			1		
1	0.98 (0.33-2.93)			0.51 (0.25-1.06)		
2	-			0.37 (0.05-2.85)		
Additive	0.70 (0.31-1.61)	0.40	0.98	0.54 (0.29-1.02)	0.06	0.86
D						
0	1			1		
1	0.34 (0.04-2.89)			1.82 (0.64-5.21)		
2	-			-		
Additive	0.34 (0.04-2.89)	0.33	0.98	1.59 (0.60-4.20)	0.35	0.91
E						
0	1			1		
1	1.57 (0.41-6.05)			0.78 (0.26-2.29)		
2	-			-		
Additive	1.57 (0.41-6.05)	0.51	0.98	0.68 (0.26-1.76)	0.43	0.92
F						
0	1			1		
1	0.68 (0.22-2.05)			1.77 (0.85-3.66)		
2	0.63 (0.14-2.75)			1.44 (0.59-3.54)		
Additive	0.76 (0.36-1.61)	0.48	0.98	1.22 (0.83-1.78)	0.31	0.91
SNP rs4680						
GG	1			1		
GA	2.06 (0.52-8.20)			0.66 (0.33-1.31)		
AA	4.04 (0.91-18.02)			1.09 (0.51-2.31)		
Additive	1.24 (0.80-1.93)	0.33	0.98	1.02 (0.68-1.53)	0.93	0.99

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Supplementary table 4.3 – continued from previous page

Haplotype		LETS (N=104) OR (95%CI)	p	FDR q	MEGA (N=513)) OR (95%CI)	p	FDR q
COMT block 2							
A							
	0	1			1		
	1	0.35 (0.08-1.49)			0.47 (0.19-1.13)		
	2	0.44 (0.10-1.88)			0.63 (0.28-1.44)		
	Additive	0.77 (0.34-1.71)	0.52	0.98	0.90 (0.57-1.42)	0.65	0.94
B							
	0	1			1		
	1	-			1.51 (0.50-4.55)		
	2	-			-		
	Additive	-	-	-	1.51 (0.50-4.55)	0.46	0.94
C							
	0	1			1		
	1	1.42 (0.44-4.55)			1.05 (0.44-2.51)		
	2	-			1.25 (0.15-10.39)		
	Additive	1.42 (0.44-4.55)	0.55	0.98	1.07 (0.53-2.17)	0.84	0.95
D							
	0	1			1		
	1	1.20 (0.40-3.56)			0.90 (0.46-1.75)		
	2	-			1.12 (0.25-5.12)		
	Additive	1.64 (0.60-4.44)	0.34	0.98	0.96 (0.55-1.67)	0.88	0.97
E							
	0	1			1		
	1	0.36 (0.17-0.46)			1.26 (0.42-3.76)		
	2	-			-		
	Additive	0.36 (0.04-3.05)	0.35	0.98	1.26 (0.42-3.76)	0.68	0.94

Supplementary table 4.4 Results from carriers of haplotypes in the *CYP* genes among OC users

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
CYP1A2								
A								
0	1			1			138.1 (110.1-166.2)	Reference
1	0.94 (0.30-2.94)			1.00 (0.62-1.63)			140.6 (125.2-156.0)	2.5 (-29.8 to 34.7)
2	1.20 (0.37-3.85)			1.15 (0.70-1.89)			150.5 (132.2-168.8)	12.3 (-21.5 to 46.1)
Additive	1.15 (0.67-1.97)	0.62	0.88	1.09 (0.86-1.38)	0.47	0.98		
B								
0	1			1			144.4 (133.0-155.9)	Reference
1	0.98 (0.35-2.76)			1.43 (0.89-2.30)			144.2 (106.0-182.3)	-0.3 (-40.3 to 39.8)
2	-			2.53 (0.49-13.19)			106.3 (98.3-114.3)	-38.1 (-52.2 to -24.1)
Additive	1.40 (0.62-3.19)	0.42	0.88	1.47 (0.97-2.22)	0.07	0.98		
C								
0	1			1			144.4 (133.3-155.6)	Reference
1	1.94 (0.38-9.84)			1.35 (0.63-2.88)			133.2 (81.3-185.1)	-11.2 (-64.4 to 42.0)
2	-			-			-	-
Additive	1.94 (0.38-9.84)	0.43	0.88	1.13 (0.55-2.32)	0.73	0.98		
D								
0	1			1			144.3 (130.1-158.6)	Reference
1	0.45 (0.19-1.06)			0.79 (0.56-1.11)			144.7 (127.4-162.1)	0.4 (-22.1 to 23.0)
2	1.01 (0.21-4.83)			0.43 (0.17-1.09)			132.0 (66.1-197.9)	-12.3 (-80.1 to 55.5)
Additive	0.70 (0.37-1.32)	0.27	0.88	0.74 (0.55-0.99)	0.04	0.98		
E								
0	1			1			144.9 (133.2-156.5)	Reference
1	0.69 (0.15-3.23)			0.95 (0.54-1.65)			135.4 (107.2-163.7)	-9.4 (-40.1 to 21.2)
2	-			-			-	-
Additive	0.69 (0.15-3.23)	0.63	0.89	0.95 (0.54-1.65)	0.85	0.98		

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Supplementary table 4.4 – continued from previous page

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
CYP2C9								
A								
0	1			1			144.2 (129.5-159.0)	Reference
1	1.12 (0.51-2.45)			1.00 (0.72-1.40)			138.7 (123.7-153.8)	-5.5 (-26.8 to 15.8)
2	3.00 (0.57-15.83)			0.58 (0.24-1.44)			170.3 (110.0-230.5)	26.0 (-36.4 to 88.4)
Additive	1.39 (0.78-2.47)	0.26	0.88	0.92 (0.69-1.21)	0.53	0.98		
B								
0	1			1			146.4 (133.6-159.3)	Reference
1	2.26 (0.89-5.77)			0.92 (0.65-1.30)			142.1 (120.7-163.4)	-4.4 (-29.4 to 20.8)
2	1.07 (0.25-4.54)			0.65 (0.24-1.74)			84.4 (70.6-98.1)	-62.1 (-81.0 to -43.2)
Additive	1.39 (0.73-2.64)	0.31	0.88	0.88 (0.66-1.19)	0.41	0.98		
C								
0	1			1			141.6 (130.5 to 152.7)	Reference
1	0.24 (0.05-1.20)			1.01 (0.55-1.85)			166.3 (122.6 to 210.0)	24.7 (-20.5 to 70.0)
2	-			-			-	-
Additive	0.24 (0.05-1.20)	0.08	0.88	1.01 (0.55-1.85)	0.98	0.99		
D								
0	1			1			157.9 (139.0-176.9)	Reference
1	0.58 (0.27-1.24)			0.90 (0.64-1.26)			130.8 (116.8-144.9)	-27.1 (-50.8 to -3.4)
2	0.66 (0.22-1.96)			1.10 (0.68-1.76)			150.0 (120.5-179.4)	-8.0 (-43.2 to 27.2)
Additive	0.75 (0.44-1.25)	0.27	0.88	1.01 (0.81-1.27)	0.92	0.98		
E								
0	1			1			146.5 (133.6-159.4)	Reference
1	0.89 (0.37-2.11)			1.27 (0.87-1.85)			130.0 (112.0-148.1)	-16.5 (-38.8 to 5.9)
2	0.89 (0.05-14.74)			2.40 (0.61-9.40)			234.2 (105.9-362.4)	87.7 (-42.0 to 217.3)
Additive	0.90 (0.42-1.92)	0.79	0.95	1.33 (0.95-1.85)	0.09	0.98		

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Supplementary table 4.4 – continued from previous page

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
F								
0	1			1			138.0 (127.0-149.0)	Reference
1	0.82 (0.33-2.02)			0.88 (0.56-1.39)			174.6 (143.4-205.7)	36.6 (3.4 to 69.8)
2	-			1.90 (0.17-21.13)			390.9	252.9 (241.9 to 264.0)
Additive	0.98 (0.43-2.27)	0.97	0.98	0.94 (0.91-1.44)	0.76	0.98		
CYP3A5								
A								
0	1			1			138.8 (104.6-173.0)	Reference
1	2.25 (0.66-7.64)			0.66 (0.39-1.13)			138.3 (122.5-154.0)	-0.5 (-38.3 to 37.3)
2	3.37 (0.98-11.59)			0.67 (0.39-1.14)			151.0 (134.7-167.3)	12.2 (-26.0 to 50.3)
Additive	1.72 (1.00-2.95)	0.05	0.88	0.88 (0.70-1.11)	0.28	0.98		
B								
0	1			1			144.7 (133.4-156.1)	Reference
1	1.19 (0.30-4.71)			1.48 (0.86-2.55)			135.3 (101.3-169.4)	-9.4 (-45.4 to 26.6)
2	-			-			-	-
Additive	1.19 (0.30-4.71)	0.80	0.95	1.48 (0.86-2.55)	0.15	0.98		
C								
0	1			1			143.5 (132.5-154.5)	Reference
1	0.62 (0.10-3.86)			1.73 (0.75-3.97)			154.8 (92.6-217.1)	11.3 (-52.3 to 74.9)
2	-			-			180.8	37.3 (26.2 to 48.4)
Additive	0.62 (0.10-3.86)	0.61	0.88	1.37 (0.62-3.03)	0.43	0.98		
D								
0	1			1			144.8 (133.3-156.3)	Reference
1	0.33 (0.12-0.93)			0.99 (0.65-1.51)			139.5 (109.2-169.8)	-5.3 (-37.8 to 27.1)
2	-			1.91 (0.17-21.23)			-	-
Additive	0.31 (0.12-0.82)	0.18	0.88	1.03 (0.69-1.54)	0.90	0.98		

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Supplementary table 4.4 – continued from previous page

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
E								
0	1			1			144.0 (133.2-154.8)	Reference
1	-			6.92 (0.51-93.52)			-	-
2	-			-			-	-
Additive	-	-	-	6.92 (0.51-93.52)	0.15	0.98		
F								
0	1			1			142.5 (131.7-153.2)	Reference
1	0.93 (0.13-6.91)			1.30 (0.54-3.14)			190.5 (107.6-273.4)	48.0 (-35.8 to 131.8)
2	-			-			-	-
Additive	0.93 (0.13-6.91)	0.95	0.98	1.30 (0.54-3.14)	0.56	0.98		
G								
0	1			1			147.7 (134.5-160.9)	Reference
1	0.70 (0.30-1.62)			0.92 (0.63-1.33)			131.0 (113.6-148.4)	-16.7 (-38.7 to 5.3)
2	0.42 (0.04-4.84)			0.78 (0.23-2.58)			162.9 (86.5-239.2)	15.2 (-62.8 to 93.1)
Additive	0.68 (0.33-1.39)	0.29	0.88	0.91 (0.66-1.26)	0.56	0.98		

Supplementary table 4.5 Results from carriers of haplotypes in the *CYP* genes among non-users

Haplotype	LETS (N=104) OR (95%CI)	p	FDR q	MEGA (N=513) OR (95%CI)	p	FDR q
CYP1A2						
A						
0	1			1		
1	0.38 (0.05-2.81)			1.05 (0.46-2.40)		
2	0.77 (0.13-4.45)			0.90 (0.38-2.16)		
Additive	1.14 (0.44-2.97)	0.79	0.98	0.93 (0.62-1.41)	0.74	0.94
B						
0	1			1		
1	2.63 (0.57-12.08)			0.62 (0.25-1.57)		
2	-			-		
Additive	2.63 (0.57-12.08)	0.22	0.98	0.57 (0.24-1.33)	0.19	0.89
C						
0	1			1		
1	-			0.99 (0.26-3.80)		
2	-			-		
Additive	-	-		0.99 (0.26-3.80)	0.98	0.99
D						
0	1			1		
1	0.95 (0.23-3.85)			1.14 (0.61-2.14)		
2	-			0.51 (0.07-3.95)		
Additive	0.76 (0.24-2.41)	0.64	0.98	0.98 (0.59-1.62)	0.94	0.99
E						
0	1			1		
1	0.63 (0.07-5.64)			1.56 (0.65-3.79)		
2	-			4.50 (0.40-50.82)		
Additive	0.63 (0.07-5.64)	0.68	0.98	1.73 (0.82-3.65)	0.15	0.89
CYP2C9						
A						
0	1			1		
1	0.68 (0.22-2.14)			1.37 (0.74-2.52)		
2	1.42 (0.24-8.23)			2.79 (0.96-8.12)		
Additive	0.96 (0.41-2.22)	0.92	0.98	1.53 (0.95-2.46)	0.08	0.86
B						
0	1			1		
1	0.64 (0.19-2.16)			0.63 (0.30-1.29)		
2	-			0.60 (0.08-4.69)		
Additive	0.57 (0.19-1.66)	0.30	0.98	0.66 (0.35-1.26)	0.21	0.89
C						
0	1			1		
1	0.53 (0.06-4.60)			0.71 (0.21-2.40)		
2	-			-		
Additive	0.53 (0.06-4.60)	0.56	0.98	0.71 (0.21-2.40)	0.58	0.94

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Supplementary table 4.5 – continued from previous page

Haplotype		LETS (N=104) OR (95%CI)	p	FDR q	MEGA (N=513)) OR (95%CI)	p	FDR q
D							
	0	1			1		
	1	1.16 (0.38-3.55)			0.54 (0.29-1.02)		
	2	1.30 (0.27-6.28)			0.72 (0.30-1.74)		
	Additive	1.14 (0.54-2.43)	0.73	0.98	0.74 (0.46-1.19)	0.21	0.89
E							
	0	1			1		
	1	0.84 (0.21-3.32)			1.77 (0.90-3.50)		
	2	-			-		
	Additive	1.43 (0.45-4.50)	0.55	0.98	1.33 (0.76-2.31)	0.32	0.91
F							
	0	1			1		
	1	1.18 (0.29-4.81)			1.32 (0.59-2.96)		
	2	-			-		
	Additive	1.18 (0.29-4.81)	0.82	0.98	1.21 (0.57-2.55)	0.62	0.94
CYP3A5							
A							
	0	1			1		
	1	0.92 (0.22-3.88)			1.14 (0.41-3.14)		
	2	0.57 (0.12-2.78)			1.28 (0.46-3.54)		
	Additive	0.68 (0.32-1.44)	0.32	0.98	1.13 (0.72-1.77)	0.60	0.94
B							
	0	1			1		
	1	1.03 (0.11-9.88)			0.82 (0.24-2.81)		
	2	-			-		
	Additive	1.03 (0.11-9.88)	0.98	0.99	0.69 (0.24-1.99)	0.50	0.94
C							
	0	1			1		
	1	0.02 (0.00-0.21)			2.01 (0.65-6.22)		
	2	-			-		
	Additive	0.02 (0.00-0.21)	0.00	0.08	1.71 (0.61-4.79)	0.31	0.91
D							
	0	1			1		
	1	1.09 (0.27-4.38)			1.03 (0.46-2.30)		
	2	-			-		
	Additive	0.92 (0.27-3.13)	0.90	0.98	0.95 (0.45-1.98)	0.88	0.97
E							
	0	1			1		
	1	0.95 (0.09-9.77)			-		
	2	-			-		
	Additive	0.95 (0.09-9.77)	0.96	0.99	-	-	-

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Supplementary table 4.5 – continued from previous page

Haplotype	LETS (N=104) OR (95%CI)	p	FDR q	MEGA (N=513)) OR (95%CI)	p	FDR q
F						
0	1			1		
1	-			0.78 (0.10-6.12)		
2	-			-		
Additive	-	-	-	0.69 (0.11-4.39)	0.69	94
G						
0	1			1		
1	0.94 (0.30-2.93)			1.04 (0.53-2.03)		
2	2.08 (0.17-24.94)			0.88 (0.11-7.07)		
Additive	1.14 (0.44-2.91)	0.79	0.98	1.01 (0.57-1.79)	0.98	0.99

Supplementary table 4.6 Results from carriers of haplotypes in the *SULT* genes among OC users

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
SULT1A1								
A								
0	1			1			145.4 (128.5-162.3)	Reference
1	0.75 (0.36-1.59)			0.93 (0.67-1.30)			145.2 (128.2-162.1)	-0.2 (-24.3 to 23.9)
2	0.91 (0.31-2.70)			1.40 (0.86-2.27)			129.3 (112.3-146.4)	-16.1 (-40.2 to 8.1)
Additive	0.90 (0.54-1.49)	0.68	0.91	1.11 (0.89-1.39)	0.35	0.98		
B								
0	1			1			137.9 (123.2-152.5)	Reference
1	0.82 (0.39-1.73)			1.07 (0.76-1.49)			149.9 (133.2-166.7)	12.1 (-10.3 to 34.4)
2	1.40 (0.48-4.08)			1.25 (0.77-2.01)			137.3 (102.3-172.3)	-0.6 (-38.8 to 37.6)
Additive	1.08 (0.67-1.74)	0.76	0.95	1.10 (0.88-1.38)	0.39	0.98		
C								
0	1			1			145.4 (134.0-156.7)	Reference
1	0.99 (0.28-3.43)			0.78 (0.45-1.38)			121.9 (93.9-150.0)	-23.4 (-53.8 to 7.0)
2	-			-			-	-
Additive	0.99 (0.28-3.43)	0.98	0.98	0.86 (0.50-1.48)	0.58	0.98		
D								
0	1			1			141.0 (129.5-152.4)	Reference
1	1.53 (0.66-3.58)			0.85 (0.58-1.27)			153.8 (126.9-180.8)	12.9 (-16.5 to 42.3)
2	-			3.77 (0.42-34.05)			59.4	-81.6 (-93.1 to -70.1)
Additive	1.67 (0.76-3.67)	0.20	0.88	0.96 (0.66-1.38)	0.82	0.98		
E								
0	1			1			142.5 (129.5-155.4)	Reference
1	0.47 (0.19-1.20)			0.71 (0.49-1.04)			147.4 (128.8-165.9)	4.9 (-17.9 to 27.7)
2	0.74 (0.04-12.24)			-			86.9 (55.6 to 118.1)	-55.6 (-89.6 to -21.6)
Additive	0.56 (0.24-1.29)	0.17	0.88	0.67 (0.46-0.95)	0.03	0.98		

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Supplementary table 4.6 – continued from previous page

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
SULT1E1 promoter region								
A								
0	1			1			148.8 (132.7-164.9)	Reference
1	0.81 (0.40-1.61)			1.09 (0.80-1.50)			137.4 (123.4-151.3)	-11.4 (-32.8 to 10.0)
2	0.98 (0.20-4.71)			1.57 (0.80-3.06)			140.3 (98.7-181.9)	-8.5 (-53.4 to 36.4)
Additive	0.88 (0.50-1.55)	0.66	0.91	1.17 (0.91-1.50)	0.23	0.98		
B								
0	1			1			141.7 (126.9-156.5)	Reference
1	1.25 (0.62-2.53)			0.94 (0.69-1.30)			139.8 (124.3-155.3)	-1.9 (-23.5 to 19.6)
2	0.30 (0.07-1.25)			0.60 (0.35-1.04)			161.9 (124.3-199.5)	20.2 (-20.4 to 60.8)
Additive	0.80 (0.47-1.37)	0.42	0.88	0.84 (0.66-1.06)	0.13	0.98		
C								
0	1			1			145.3 (128.9-161.7)	Reference
1	1.66 (0.79-3.47)			1.33 (0.96-1.84)			140.2 (124.8-155.7)	-5.1 (-27.7 to 17.6)
2	1.14 (0.41-3.15)			0.80 (0.49-1.32)			147.1 (118.3-175.9)	1.8 (-31.5 to 35.2)
Additive	1.18 (0.72-1.94)	0.51	0.88	1.00 (0.80-1.26)	0.99	0.99		
D								
0	1			1			143.0 (132.1-153.9)	Reference
1	1.45 (0.40-5.22)			0.75 (0.40-1.42)			153.6 (117.6-189.6)	10.6 (-27.1 to 48.3)
2	-			-			-	-
Additive	1.45 (0.40-5.22)	0.57	0.88	0.75 (0.40-1.42)	0.38	0.98		
SNP rs3736599								
GG	1			1			141.8 (130.1-153.4)	Reference
GA	1.02 (0.47-2.21)			1.08 (0.72-1.62)			148.3 (123.0-173.5)	6.5 (-21.5 to 34.5)
AA	1.64 (0.14-18.80)			0.99 (0.28-3.46)			248.3	106.5 (-35.9 to 248.9)
Additive	1.07 (0.55-2.08)	0.84	0.97	1.01 (0.88-1.16)	0.91	0.98		

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Supplementary table 4.6 – continued from previous page

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
SULT1E1 gene region								
A								
0	1			1			148.5 (133.3-163.7)	Reference
1	0.96 (0.47-1.95)			1.02 (0.74-1.41)			141.5 (124.1-158.9)	-7.0 (-30.3 to 16.2)
2	1.12 (0.32-3.88)			0.70 (0.41-1.20)			128.6 (104.1-153.1)	-19.9 (-48.9 to 9.1)
Additive	1.02 (0.61-1.71)	0.95	0.98	0.90 (0.72-1.14)	0.39	0.98		
B								
0	1			1			151.0 (136.3-165.8)	Reference
1	1.05 (0.51-2.15)			1.24 (0.90-1.71)			131.0 (116.4-145.7)	-20.0 (-40.9 to 0.9)
2	5.09 (1.05-24.63)			1.07 (0.57-2.01)			154.0 (99.9-208.1)	3.0 (-53.4 to 59.4)
Additive	1.56 (0.95-2.57)	0.08	0.88	1.13 (0.88-1.45)	0.33	0.98		
C								
0	1			1			141.1 (129.2-153.0)	Reference
1	1.06 (0.49-2.29)			1.07 (0.75-1.53)			148.3 (126.1-170.6)	7.2 (-18.3 to 32.7)
2	0.81 (0.19-3.44)			0.62 (0.20-1.91)			248.3	107.2 (95.2 to 119.2)
Additive	0.97 (0.55-1.71)	0.92	0.97	0.98 (0.72-1.34)	0.91	0.98		
D								
0	1			1			137.7 (125.7-149.8)	Reference
1	0.53 (0.24-1.18)			0.87 (0.61-1.25)			152.6 (131.1-174.2)	14.9 (-10.0 to 39.8)
2	0.34 (0.03-3.89)			0.67 (0.21-2.15)			200.1 (163.4-236.8)	62.4 (23.5 to 101.2)
Additive	0.54 (0.27-1.09)	0.09	0.88	0.86 (0.63-1.17)	0.34	0.98		
E								
0	1			1			143.7 (132.5-155.0)	Reference
1	1.08 (0.35-3.32)			1.11 (0.66-1.84)			145.8 (112.6-178.9)	2.1 (-33.1 to 37.3)
2	-			-			103.9 (71.3 to 136.5)	-39.8 (-74.5 to -5.2)
Additive	1.08 (0.35-3.32)	0.89	0.98	0.92 (0.58-1.47)	0.74	0.98		

Supplementary table 4.7 Results from carriers of haplotypes in the *SULT* genes among non-users

Haplotype	LETS (N=104) OR (95%CI)	p	FDR q	MEGA (N=513) OR (95%CI)	p	FDR q
SULT1A1						
A						
0	1			1		
1	2.30 (0.79-6.66)			0.92 (0.48-1.76)		
2	-			1.26 (0.52-3.05)		
Additive	1.05 (0.57-1.92)	0.89	0.98	1.08 (0.68-1.69)	0.75	0.94
B						
0	1			1		
1	1.83 (0.53-6.27)			0.80 (0.43-1.48)		
2	2.27 (0.48-10.71)			0.48 (0.16-1.44)		
Additive	1.52 (0.74-3.14)	0.26	0.98	0.73 (0.47-1.14)	0.16	0.89
C						
0	1			1		
1	0.50 (0.06-4.29)			2.53 (1.14-5.63)		
2	-			-		
Additive	0.50 (0.06-4.29)	0.53	0.98	2.08 (1.00-4.33)	0.05	0.86
D						
0	1			1		
1	0.57 (0.15-2.18)			0.83 (0.36-1.92)		
2	-			-		
Additive	0.57 (0.15-2.18)	0.42	0.98	0.73 (0.35-1.54)	0.41	0.91
E						
0	1			1		
1	1.04 (0.20-5.37)			1.46 (0.73-2.92)		
2	-			3.31 (0.34-32.78)		
Additive	0.85 (0.21-3.41)	0.82	0.98	1.53 (0.82-2.88)	0.18	0.89
SULT1E1 promoter region						
A						
0	1			1		
1	0.94 (0.35-2.53)			0.82 (0.45-1.50)		
2	-			1.09 (0.40-3.02)		
Additive	0.64 (0.31-1.33)	0.23	0.98	0.95 (0.59-1.52)	0.82	0.95
B						
0	1			1		
1	0.84 (0.29-2.41)			1.75 (0.94-3.27)		
2	0.85 (0.16-4.54)			1.46 (0.55-3.86)		
Additive	0.89 (0.42-1.88)	0.76	0.98	1.34 (0.90-1.97)	0.15	0.89
C						
0	1			1		
1	0.98 (0.32-2.98)			0.93 (0.52-1.68)		
2	2.71 (0.71-10.33)			0.55 (0.16-1.91)		
Additive	1.52 (0.74-3.13)	0.26	0.98	0.83 (0.54-1.29)	0.41	0.91

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Supplementary table 4.7 – continued from previous page

Haplotype	LETS (N=104) OR (95%CI)	p	FDR q	MEGA (N=513)) OR (95%CI)	p	FDR q
D						
0	1			1		
1	0.91 (0.18-4.64)			-		
2	-			-		
Additive	0.91 (0.18-4.64)	0.91	0.98	-	-	-
SNP rs3736599						
GG	1			1		
GA	0.92 (0.23-3.63)			1.47(0.75-2.89)		
AA	-			1.06 (0.13-8.58)		
Additive	0.70 (0.30-1.66)	0.42	0.98	0.96 (0.68-1.34)	0.80	0.94
SULT1E1 gene region						
A						
0	1			1		
1	0.67 (0.24-1.86)			1.17 (0.64-2.15)		
2	-			1.00 (0.36-2.77)		
Additive	0.51 (0.23-1.15)	0.11	0.98	1.06 (0.69-1.61)	0.80	0.94
B						
0	1			1		
1	2.32 (0.73-7.38)			1.02 (0.55-1.90)		
2	3.38 (0.75-15.28)			1.92 (0.73-5.05)		
Additive	1.89 (0.94-3.81)	0.07	0.98	1.24 (0.78-1.97)	0.37	0.91
C						
0	1			1		
1	0.94 (0.32-2.75)			0.89 (0.46-1.73)		
2	-			0.49 (0.06-377)		
Additive	0.76 (0.31-1.87)	0.56	0.98	0.82 (0.48-1.42)	0.49	0.94
D						
0	1			1		
1	0.25 (0.03-2.03)			0.82 (0.37-1.80)		
2	3.72 (0.22-63.33)			-		
Additive	0.73 (0.16-3.27)	0.69	0.98	0.71 (0.35-1.41)	0.33	0.91
E						
0	1			1		
1	1.29 (0.32-5.23)			0.73 (0.25-2.12)		
2	-			4.30 (0.38-48.42)		
Additive	1.29 (0.32-5.23)	0.72	0.98	1.03 (0.40-2.60)	0.96	0.99

Supplementary table 4.8 Results from carriers of haplotypes in the *UGT* genes among OC users

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
UGT1A Block 1								
A								
0	1			1			148.0 (132.5-163.5)	Reference
1	0.86 (0.40-1.86)			1.07 (0.77-1.48)			142.8 (124.8-160.7)	-5.2 (-29.1 to 18.6)
2	1.83 (0.54-6.19)			0.61 (0.34-1.07)			127.3 (101.4-153.2)	-20.7 (-51.1 to 9.7)
Additive	1.18 (0.69-2.02)	0.54	0.88	0.88 (0.70-1.12)	0.31	0.98		
B								
0	1			1			144.1 (133.0-155.3)	Reference
1	1.14 (0.24-5.37)			1.18 (0.59-2.38)			128.4 (93.7-163.0)	-15.8 (-52.3 to 20.7)
2	-			0.95 (0.13-6.80)			-	-
Additive	1.57 (0.47-5.29)	0.46	0.88	1.11 (0.62-1.98)	0.72	0.98		
C								
0	1			1			144.1 (132.9-155.2)	Reference
1	1.72 (0.30-9.81)			0.86 (0.37-1.97)			142.8 (97.4-188.1)	-1.3 (-48.3 to 45.7)
2	-			-			58.1	-85.9 (-97.2 to -74.7)
Additive	1.72 (0.30-9.81)	0.54	0.88	0.75 (0.35-1.60)	0.46	0.98		
D								
0	1			1			139.2 (128.2-150.2)	Reference
1	0.82 (0.25-2.72)			0.91 (0.55-1.49)			172.8 (132.5-213.2)	33.6 (-8.4 o 75.7)
2	-			0.93 (0.13-6.67)			225.6	86.4 (75.4 to 97.4)
Additive	0.82 (0.25-2.72)	0.75	0.95	0.92 (0.58-1.44)	0.71	0.98		
E								
0	1			1			147.4 (129.1-165.6)	Reference
1	0.69 (0.33-1.44)			1.27 (0.91-1.78)			135.8 (121.8-149.8)	-11.6 (-34.7 to 11.6)
2	0.69 (0.18-2.64)			1.21 (0.75-1.96)			157.9 (125.2-190.6)	10.5 (-27.2 to 48.2)
Additive	0.76 (0.43-1.34)	0.35	0.88	1.14 (0.91-1.43)	0.26	0.98		

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Supplementary table 4.8 – continued from previous page

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
F								
0	1			1			140.6 (127.3-153.9)	Reference
1	0.82 (0.38-1.76)			0.99 (0.71-1.39)			151.6 (131.9-171.3)	11.0 (-12.9 to 34.9)
2	-			1.12 (0.49-2.55)			112.8 (73.0-152.6)	-27.8 (-70.1 to 14.4)
Additive	1.12 (0.58-2.15)	0.74	0.95	1.02 (0.77-1.34)	0.91	0.98		
UGT1A Block 2								
A								
0	1			1			153.4 (130.2-176.7)	Reference
1	0.56 (0.24-1.31)			1.07 (0.72-1.59)			134.3 (118.1-150.6)	-19.1 (-47.6 to 9.5)
2	0.94 (0.32-2.72)			1.16 (0.75-1.78)			145.1 (128.0-162.3)	-8.3 (-37.4 to 20.8)
Additive	0.94 (0.56-1.56)	0.80	0.95	1.08 (0.87-1.33)	0.50	0.98		
B								
0	1			1			138.0 (127.3-148.7)	Reference
1	0.73 (0.22-2.43)			0.83 (0.50-1.38)			172.8 (132.5-213.2)	34.8 (-7.1 to 76.8)
2	-			0.91 (0.13-6.50)			225.6	87.6 (76.9 to 98.4)
Additive	0.73 (0.22-2.43)	0.61	0.88	0.85 (0.54-1.35)	0.49	0.98		
C								
0	1			1			142.2 (127.8-156.7)	Reference
1	0.98 (0.46-2.07)			1.13 (0.81-1.57)			143.6 (124.4-162.8)	1.4 (-22.8 to 25.6)
2	1.57 (0.50-4.91)			0.75 (0.43-1.30)			140.6 (114.1-167.1)	-1.7 (-32.0 to 28.7)
Additive	1.17 (0.70-1.94)	0.55	0.88	0.95 (0.75-1.21)	0.70	0.98		
D								
0	1			1			144.3 (133.1-155.4)	Reference
1	0.66 (0.19-2.28)			1.10 (0.56-2.19)			116.3 (85.2-147.4)	-28.0 (-61.1 to 5.2)
2	-			0.46 (0.04-5.16)			-	-
Additive	0.66 (0.19-2.28)	0.51	0.88	0.98 (0.54-1.76)	0.93	0.98		

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Supplementary table 4.8 – continued from previous page

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
E								
0	1			1			142.5 (131.5-153.6)	Reference
1	1.74 (0.56-5.43)			0.94 (0.61-1.46)			144.7 (109.8-179.5)	2.1 (-34.6 to 38.9)
2	-			1.84 (0.17-20.48)			81.5	-61.1 (-72.2 to -49.9)
Additive	1.74 (0.56-5.43)	0.34	0.88	0.98 (0.65-1.48)	0.93	0.98		
UGT1A Block 3								
A								
0	1			1			153.9 (128.5-179.3)	Reference
1	0.46 (0.19-1.12)			1.09 (0.72-1.64)			137.3 (121.4-153.1)	-16.7 (-46.8 to 13.4)
2	0.73 (0.25-2.08)			1.18 (0.76-1.82)			145.4 (128.0-162.8)	-8.5 (-39.5 to 22.4)
Additive	0.87 (0.53-1.44)	0.59	0.88	1.08 (0.88-1.34)	0.46	0.98		
B								
0	1			1			144.5 (133.4-155.5)	Reference
1	3.64 (0.39-33.73)			1.04 (0.61-1.79)			134.5 (93.5-175.4)	-10.0 (-52.5 to 32.5)
2	-			-		0.98	-	-
Additive	3.64 (0.39-33.73)	0.26	0.88	1.04 (0.61-1.79)	0.88			
C								
0	1			1			140.1 (124.4-155.8)	Reference
1	0.62 (0.27-1.43)			1.01 (0.72-1.42)			144.6 (126.5-162.6)	4.5 (-19.5 to 28.5)
2	1.19 (0.42-3.37)			0.79 (0.50-1.25)			148.9 (124.9-173.0)	8.9 (-20.0 to 37.8)
Additive	1.05 (0.64-1.73)	0.85	0.97	0.91 (0.73-1.14)	0.41	0.98		

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Supplementary table 4.8 – continued from previous page

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
UGT1A Block 4								
A								
0	1			1			137.9 (124.5-151.4)	Reference
1	0.68 (0.33-1.37)			1.30 (0.94-1.80)			153.1 (133.4-172.8)	15.2 (-8.7 to 39.1)
2	0.96 (0.15-6.18)			1.08 (0.61-1.93)			141.0 (109.0-173.1)	3.1 (-31.9 to 38.2)
Additive	0.77 (0.42-1.41)	0.40	0.88	1.14 (0.90-1.46)	0.28	0.98		
B								
0	1			1			144.0 (129.7-158.3)	Reference
1	1.00 (0.47-2.11)			1.06 (0.77-1.47)			147.9 (128.0-167.7)	3.9 (-20.7 to 28.5)
2	1.10 (0.35-3.39)			0.75 (0.42-1.33)			128.3 (105.5-151.1)	-15.7 (-42.8 to 11.4)
Additive	1.03 (0.61-1.74)	0.91	0.98	0.94 (0.74-1.19)	0.62	0.98		
C								
0	1			1			145.6 (134.4-156.8)	Reference
1	0.55 (0.12-2.59)			0.82 (0.43-1.57)			116.2 (87.7-144.7)	-29.4 (-60.2 to 1.3)
2	-			0.95 (0.06-15.37)			-	-
Additive	0.55 (0.12-2.59)	0.45	0.88	0.84 (0.46-1.53)	0.58	0.98		
D								
0	1			1			139.7 (129.0-150.5)	Reference
1	0.75 (0.21-2.73)			0.94 (0.57-1.55)			171.6 (129.2-213.9)	31.9 (-12.1 to 75.8)
2	-			0.48 (0.04-5.32)			225.6	85.9 (75.0 to 96.7)
Additive	0.75 (0.21-2.73)	0.67	0.91	0.90 (0.57-1.43)	0.65	0.98		
E								
0	1			1			144.1 (133.1-155.1)	Reference
1	4.00 (0.45-35.52)			1.08 (0.63-1.86)			139.1 (97.3-181.0)	-4.9 (-48.4 to 38.5)
2	-			-			-	-
Additive	4.00 (0.45-35.52)	0.21	0.88	1.08 (0.63-1.86)	0.78	0.98		

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Supplementary table 4.8 – continued from previous page

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
F								
0	1			1			151.9 (135.7-168.1)	Reference
1	1.26 (0.62-2.57)			0.98 (0.71-1.36)			134.6 (118.1-151.0)	-17.3 (-40.6 to 5.9)
2	1.12 (0.23-5.38)			0.88 (0.50-1.56)			142.8 (123.6-161.9)	-9.2 (-34.4 to 16.1)
Additive	1.17 (0.65-2.09)	0.61	0.88	0.96 (0.75-1.21)	0.71	0.98		
UGT1A Block 5								
A								
0	1			1			153.4 (130.2-176.7)	Reference
1	1.29 (0.62-2.71)			1.03 (0.73-1.46)			134.3 (118.1-150.6)	-19.1 (-47.6 to 9.5)
2	0.48 (0.04-5.57)			1.03 (0.48-2.19)			145.1 (128.0-162.3)	-8.3 (-37.4 to 20.8)
Additive	1.10 (0.57-2.12)	0.78	0.95	1.02 (0.78-1.34)	0.86	0.98		
B								
0	1			1			138.0 (127.3-148.7)	Reference
1	1.39 (0.65-2.97)			1.09 (0.78-1.51)			172.8 (132.5- 213.2)	34.8 (-7.1 to 76.8)
2	2.13 (0.68-6.74)			0.85 (0.50-1.47)			225.6	87.6 (76.9 to 98.4)
Additive	1.44 (0.85-2.44)	0.18	0.88	0.98 (0.77-1.24)	0.87	0.98		
C								
0	1			1			142.2 (127.8-156.7)	Reference
1	0.97 (0.39-2.41)			0.98 (0.67-1.44)			143.6 (124.4-162.8)	1.4 (-22.8 to 25.6)
2	-			1.27 (0.43-3.72)			140.6 (114.1-167.1)	-1.7 (-32.0 to 28.7)
Additive	0.97 (0.39-2.41)	0.95	0.98	1.03 (0.74-1.42)	0.87	0.98		
D								
0	1			1			144.3 (133.1-155.4)	Reference
1	0.71 (0.33-1.51)			0.98 (0.69-1.38)			116.3 (85.2-147.4)	-28.0 (-61.1 to 5.2)
2	1.63 (0.14-18.81)			0.78 (0.33-1.85)			-	-
Additive	0.83 (0.42-1.63)	0.59	0.88	0.94 (0.71-1.25)	0.67	0.98		

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Supplementary table 4.8 – continued from previous page

Haplotype	LETS (N=140) OR (95%CI)	p	FDR q	MEGA (N=678) OR (95%CI)	p	FDR q	SHBG levels (95%CI)	Mean difference (95%CI)
E								
0	1			1			142.5 (131.5-153.6)	Reference
1	0.39 (0.16-0.92)			1.05 (0.74-1.49)			144.7 (109.8-179.5)	2.1 (-34.6 to 38.9)
2	-			1.79 (0.65-4.94)			81.5	-61.1 (-72.2 to -49.9)
Additive	0.61 (0.27-1.37)	0.23	0.88	1.13 (0.84-1.52)	0.41	0.98		

Supplementary table 4.9 Results from carriers of haplotypes in the *UGT* genes among non-users

Haplotype	LETS (N=104) OR (95%CI)	p	FDR q	MEGA (N=513) OR (95%CI)	p	FDR q
UGT1A Block 1						
A						
0	1			1		
1	0.84 (0.26-2.68)			2.00 (1.08-3.70)		
2	1.58 (0.14-17.48)			0.89 (0.25-3.16)		
Additive	1.00 (0.36-2.74)	1.00	1.00	1.28 (0.87-1.89)	0.20	0.89
B						
0	1			1		
1	1.67 (0.16-17.44)			1.55 (0.51-4.68)		
2	-			-		
Additive	0.79 (0.18-3.51)	0.75	0.98	1.55 (0.51-4.68)	0.44	0.92
C						
0	1			1		
1	-			1.60 (0.53-4.85)		
2	-			-		
Additive	-	-	-	1.30 (0.50-3.39)	0.59	0.94
D						
0	1			1		
1	2.09 (0.48-9.11)			1.62 (0.64-4.07)		
2	-			-		
Additive	2.09 (0.48-9.11)	0.33	0.98	1.62 (0.64-4.07)	0.31	0.91
E						
0	1			1		
1	0.82 (0.24-2.80)			1.12 (0.59-2.13)		
2	1.31 (0.21-8.41)			1.20 (0.47-3.04)		
Additive	1.04 (0.39-2.75)	0.94	0.98	1.10 (0.71-1.70)	0.67	0.94
F						
0	1			1		
1	0.54 (0.15-1.90)			0.45 (0.22-0.93)		
2	2.10 (0.17-25.88)			0.40 (0.05-3.10)		
Additive	0.80 (0.25-2.55)	0.71	0.98	0.49 (0.26-0.95)	0.04	0.86
UGT1A Block 2						
A						
0	1			1		
1	0.87 (0.20-3.71)			1.17 (0.53-2.61)		
2	1.20 (0.24-6.01)			0.73 (0.29-1.84)		
Additive	1.13 (0.48-2.68)	0.78	0.98	0.84 (0.56-1.26)	0.40	0.91
B						
0	1			1		
1	1.41 (0.34-5.86)			1.24 (0.42-3.70)		
2	-			-		
Additive	1.41 (0.34-5.86)	0.63	0.98	1.24 (0.42-3.70)	0.70	0.94

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Supplementary table 4.9 – continued from previous page

Haplotype	LETS (N=104) OR (95%CI)	p	FDR q	MEGA (N=513)) OR (95%CI)	p	FDR q
C						
0	1			1		
1	0.83 (0.27-2.60)			2.06 (1.08-3.93)		
2	2.22 (0.35-14.20)			1.08 (0.30-3.89)		
Additive	1.17 (0.48-2.89)	0.73	0.98	1.37 (0.91-2.05)	0.13	0.89
D						
0	1			1		
1	0.89 (0.10-8.22)			0.82 (0.19-3.63)		
2	-			-		
Additive	0.89 (0.10-8.22)	0.92	0.98	0.82 (0.19-3.63)	0.80	0.94
E						
0	1			1		
1	0.30 (0.04-2.48)			0.66 (0.25-1.75)		
2	-			-		
Additive	0.28 (0.04-2.14)	0.22	0.98	0.64 (0.25-1.61)	0.34	0.91
UGT1A Block 3						
A						
0	1			1		
1	0.89 (0.21-3.79)			1.31 (0.58-2.99)		
2	0.46 (0.09-2.46)			0.78 (0.31-1.97)		
Additive	0.66 (0.31-1.40)	0.28	0.98	0.84 (0.57-1.23)	0.37	0.91
B						
0	1			1		
1	1.64 (0.29-9.25)			0.19 (0.03-1.40)		
2	-			-		
Additive	1.64 (0.29-9.25)	0.57	0.98	0.19 (0.03-1.33)	0.09	0.86
C						
0	1			1		
1	1.24 (0.41-3.74)			2.16 (1.10-4.26)		
2	2.07 (0.41-10.48)			1.74 (0.66-4.56)		
Additive	1.39 (0.62-3.09)	0.43	0.98	1.43 (0.98-2.10)	0.06	0.86
UGT1A Block 4						
A						
0	1			1		
1	0.52 (0.17-1.55)			0.73 (0.39-1.34)		
2	0.23 (0.03-1.99)			0.32 (0.07-1.38)		
Additive	0.50 (0.22-1.13)	0.10	0.98	0.65 (0.40-1.04)	0.07	0.86
B						
0	1			1		
1	1.71 (0.59-4.91)			2.65 (1.39-5.04)		
2	1.96 (0.18-21.48)			1.25 (0.35-4.53)		
Additive	1.57 (0.67-3.64)	0.30	0.98	1.55 (1.06-2.27)	0.02	0.86

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Supplementary table 4.9 – continued from previous page

Haplotype	LETS (N=104) OR (95%CI)	p	FDR q	MEGA (N=513)) OR (95%CI)	p	FDR q
C						
0	1			1		
1	1.32 (0.25-7.02)			0.69 (0.16-3.02)		
2	-			-		
Additive	1.32 (0.25-7.02)	0.74	0.98	0.69 (0.16-3.02)	0.63	0.94
D						
0	1			1		
1	2.01 (0.55-7.36)			1.16 (0.39-3.43)		
2	-			-		
Additive	2.01 (0.55-7.36)	0.29	0.98	1.16 (0.39-3.43)	0.79	0.94
E						
0	1			1		
1	1.88 (0.33-10.63)			0.19 (0.03-1.40)		
2	-			-		
Additive	1.25 (0.31-5.00)	0.75	0.98	0.19 (0.03-1.36)	0.10	0.86
F						
0	1			1		
1	1.25 (0.44-3.54)			1.83 (0.99-3.39)		
2	-			0.92 (0.26-3.26)		
Additive	0.93 (0.41-2.09)	0.85	0.98	1.26 (0.85-1.87)	0.26	0.91
UGT1A Block 5						
A						
0	1			1		
1	0.41 (0.11-1.58)			0.93 (0.50-1.75)		
2	0.89 (0.09-8.74)			-		
Additive	0.62 (0.20-1.87)	0.39	0.98	0.74 (0.45-1.23)	0.25	0.91
B						
0	1			1		
1	0.89 (0.31-2.57)			1.67 (0.89-3.14)		
2	0.69 (0.07-6.50)			0.99 (0.32-3.08)		
Additive	0.86 (0.37-1.99)	0.72	0.98	1.20 (0.80-1.79)	0.38	0.91
C						
0	1			1		
1	1.15 (0.36-3.68)			1.01 (0.49-2.12)		
2	4.83 (0.28-84.00)			1.76 (0.20-15.53)		
Additive	1.45 (0.54-3.92)	0.46	0.98	1.09 (0.56-2.10)	0.80	0.94
D						
0	1			1		
1	2.71 (0.94-7.79)			1.11 (0.59-2.11)		
2	-			1.06 (0.23-4.82)		
Additive	1.68 (0.73-3.86)	0.22	0.98	1.08 (0.65-1.80)	0.78	0.94

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Supplementary table 4.9 – continued from previous page

Haplotype	LETS (N=104) OR (95%CI)	p	FDR q	MEGA (N=513)) OR (95%CI)	p	FDR q
E						
0	1			1		
1	0.74 (0.19-2.90)			0.98 (0.49-1.96)		
2	1.42 (0.14-14.90)			0.66 (0.08-5.21)		
Additive	0.96 (0.34-2.65)	0.93	0.98	0.92 (0.52-1.64)	0.79	0.94

Clinical aspects

Chapter 5

Hormonal contraceptive use after venous thrombosis: practice from 1999-2004

Bernardine H. Stegeman
Hetty Jolink
Frans M. Helmerhorst
Frits R. Rosendaal
Astrid van Hylckama Vlieg

Submitted for publication

Abstract

Background: Current guidelines state that women using combined hormonal contraceptives at the time of a venous thrombosis event should stop using these combined preparations. Progestagen-only contraceptives are advised to be used in these women. The aim of this study is to determine to what extent the guidelines concerning hormonal contraceptive use after a venous thrombosis are followed in daily practice.
Methods: Women younger than 50 years who experienced a hormonal contraceptive-associated venous thrombosis were selected from a case-control study, the MEGA study. Data on hormonal contraceptive use after a venous thrombosis was available through an interview at venipuncture. In addition, participants from the follow-up study provided data on advice, given by a physician, to stop hormonal contraceptive use. Risk differences with 95% confidence interval (CI) were calculated.
Results: 703 women were included with a mean follow-up at interview of 10.8 months (range: 2.1 to 35.4 months). 521 (74%) women stopped hormonal contraceptive use, 63 (9%) changed to a different contraceptive method, and 119 (17%) continued the same method. 143 (21%) of 682 women who used a combined oral contraceptive at the thrombotic event were still using a combined preparation after the event contrasting the guidelines. Women who continued their contraceptive use had less often a positive family history of venous thrombosis and more often surgery (RD 19%, 95%CI: 9 to 30%) or a plaster cast (RD 17%, 95%CI: 3 to 34%) at the time of venous thrombosis than women who stopped. 39 (12%) of 332 women using a combined preparation and who received advice to stop from a physician, continued their combined oral contraceptive.
Conclusions: The finding that women continue their combined oral contraceptive use after receiving advice to stop is a real concern, in particular since there are indications that this use could increase the risk of a recurrence.

Introduction

Combined oral contraceptive use (containing ethinylestradiol and a progestagen) is associated with an increased risk of venous thrombosis. Regarding the use of combined oral contraceptives after a hormonal contraceptive-associated venous thrombosis, the WHO guideline from 2004 recommends that women with a history of venous thromboembolic disease should refrain from using combined hormonal contraceptives[1]. The same recommendation is stated in national guidelines from the Netherlands[2,3]. In addition, package insertions of combined oral contraceptives advise women to refrain from the use of these contraceptives in case of a (experienced) thromboembolic event. By law, the manufacturer is required to list contra-indications on package insertions in the Netherlands. Both national and WHO guidelines state that progestagen-only contraceptives such as progestagen-only pills or intra-uterine devices may be used in case of a history of venous thrombosis[1–3].

Little is known about hormonal contraceptive use after venous thrombosis in clinical practice. Only one study assessed whether continuing hormonal contraceptives influenced the risk of a recurrence. In the Leiden Thrombophilia Study (LETS) it was shown that almost 30% of women continued oral contraceptive use after an oral contraceptive-associated venous thrombosis[4] and after a mean follow-up of 7.3 years approximately 45% either continued or restarted oral contraceptives[5]. The recurrence rate in women who continued or restarted the use of a hormonal contraceptive was much higher (48.8 per 1000 patient-years) than in women who stopped hormonal contraceptive use (10.5 per 1000 patient-years) (age-adjusted incidence rate ratio 4.3, 95%CI: 1.7 to 11.1)[6].

There is limited knowledge regarding daily medical practice after a hormonal contraceptive-associated venous thrombotic event. To assess the adherence to the recommendations in the guidelines, short term follow-up data on hormonal contracep-

tive use was analyzed from the cases enrolled in the MEGA study (a case-control study of venous thrombosis). We focused in particular on what proportion of patients with a hormonal contraceptive-associated venous thrombosis stopped, changed or continued their hormonal contraceptive use. We determined what changes were made when women changed to a different hormonal contraceptive method after a thrombotic event. Secondly, we assessed whether acquired risk factors for venous thrombosis were associated with the decision to stop, change or continue oral contraceptive use. In a proportion of these women, long term follow-up data was available with information about the advice received from the physician. In these women, we assessed the proportion of women who did receive advice to stop their oral contraceptive use and how many followed this advice.

Methods

Study design For this study, we included patients with a first episode of deep venous thrombosis or a pulmonary embolism, who were enrolled in a large case-control study on risk factors for venous thrombosis, i.e., the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study[7]. Consecutive patients younger than 70 years with a first symptomatic deep vein thrombosis in the leg or arm or pulmonary embolism were recruited from six anticoagulation clinics in the Netherlands between 1 March 1999 and 31 August 2004 (N=5183). Control subjects were partners or recruited via random digit dialling. Only the case group is included in the current analysis.

All patients completed a detailed questionnaire on risk factors for venous thrombosis, including the use of oral contraceptives. The questions covered a period of one year before the thrombotic event. Three months after discontinuation of the anticoagulation therapy, patients were invited to the anticoagulation clinic for a blood sample. During this visit participants were interviewed

regarding the period from the venous thrombotic event until the venipuncture. This interview included items on the change of hormonal contraceptive methods since the diagnosis of venous thrombosis. Patients included in the MEGA case-control study subsequently took part in a follow-up study. In the follow-up questionnaire, a question was asked whether or not advice from the treating physician was received regarding contraceptive use after the first event. Because not all patients participated in this follow-up study, data was available in a proportion of the included women for the current analysis.

Of 5183 cases included in the MEGA study, 2799 were women of whom 1703 were younger than 50 years. Women not taking hormonal contraceptives at the time of a venous thrombosis were excluded (N=569). Of 431 women no data was available on hormonal contraceptive use after the thrombotic event, i.e., at the time of venipuncture. In total, 703 women were included in the current analysis of which 414 women also filled in the follow-up questionnaire and thus had data on advice received from a physician.

Analysis We first determined what proportion of women with venous thrombosis during hormonal contraceptive use stopped, continued, or changed their hormonal contraceptive use after the event. Baseline characteristics (i.e., age, body mass index (BMI), current smoking, positive family history, and type of contraception) were studied separately for women who stopped, changed, or continued their contraception. BMI was analyzed as a categorical variable with three categories: BMI $\leq$25 kg/m^2, BMI 25-30 kg/m^2, and BMI >30 kg/m^2. Smoking behaviour was represented as current, past, or never smokers. A positive family history was defined as having one or more first-degree relatives with a history of venous thrombosis. Contraception was divided into combined hormonal contraceptives (i.e., containing ethinylestradiol and a progestagen) and progestagen-only contraceptives. The most commonly used administration route for combined

formulations is orally but occasionally it is administered transdermally or transvaginally. Progestagen-only formulations are administered orally (mini pill) as well as subcutaneously (via implant or injection) or intrauterinely (intrauterine device). In our study, none of the women used a combined transdermal patch or combined vaginal ring. All other applications of hormones were used. Combined oral contraceptives were further subdivided according to the type of progestagen used; first generation (i.e., lynestrenol and norethisteron), second generation (i.e., levonorgestrel), and third generation combined oral contraceptives (i.e., gestodene, desogestrel and norgestimate). The progestagens introduced after the third generation progestagens, i.e., cyproterone acetate and drospirenone, were analysed separately. Progestagen-only contraceptives were subdivided into oral progestagen-only contraceptives (the so-called progestagen-only pills (POP)), hormonal intra-uterine device, implants, and injectables.

Secondly, we assessed which type of hormonal contraceptive women started to use when they changed their hormonal contraceptive use after the thrombotic event.

Thirdly, we determined whether exposure to an acquired risk factor for venous thrombosis was associated with stopping, changing, or continuing to use hormonal contraceptives. The following acquired risk factors were taken into account: surgery, plaster cast, immobilization, injury, and travel. A thrombotic event was classified as secondary to surgery, plaster cast, immobilization, or injury when one or more of these risk situations had occurred within 3 months prior to the thrombotic event. A thrombotic event was classified as secondary to travel when a patient had travelled for more than 4 hours in the period of eight weeks prior to the thrombotic event.

Finally, in women with data about advice received from a physician, we determined what proportion of women stopped, changed, or continued their contraceptive use when they were advised to stop.

Risk differences (RD) with 95% confidence intervals (CI) were calculated between different proportions. The Newcombe method 10 was used to calculate these risk differences as this method performed better with low sample sizes[8]. All statistical analyses were performed with STATA, version 12.0 (Statacorp LP, College Station, TX, USA).

Results

A total of 703 women with a hormonal contraceptive-associated venous thrombosis provided information on contraceptive use after the event. At an average of 10.8 months (range: 2.1 to 35.4 months) after venous thrombosis, 521 (74%) women had stopped the use of hormonal contraceptives, 63 (9%) changed to another hormonal contraceptive and 119 (17%) had continued with the same contraceptive.

General characteristics of the study population are presented in table 5.1 for women who stopped, changed or continued hormonal contraceptives. On average, women were about the same age (stop: 36 years (range 18 - 49), change: 34 years (range 20 - 48) and continue: 36 years (range 18 - 49)). The proportion of women stopping, changing or continuing their hormonal contraceptive did not markedly differ between BMI categories or smoking behaviour. Women with a positive family history of venous thrombosis were more likely to stop and less likely to change or continued their hormonal contraceptive than women without a positive family history. The vast majority was using a combined preparation at the time of venous thrombosis and the most commonly used progestagen was a second or third generation progestagen. Women using a combined oral contraceptive were more likely to stop and less likely to change or continue their contraceptive than women using a progestagen-only contraceptive. While no difference in age was observed, users of a first generation progestagen in their combined oral contraceptives were more likely to stop their contraceptive use and users

of cyproterone acetate and drospirenone were more likely to continue their contraceptive than users of other progestagens.

Table 5.1: Baseline chacteristics

Women who	**Stop N=521**	**Change N=63**	**Continue N=119**
Age, mean (range), years	36 (18-49)	34 (20-48)	36 (18-49)
BMI[a], kg/m^2			
>30 (%)	126 (76)	11 (7)	29 (17)
25-30 (%)	156 (75)	20 (10)	33 (16)
<25 (%)	210 (73)	29 (10)	50 (17)
Smoking[a]			
Current (%)	213 (76)	22 (8)	45 (16)
Past (%)	89 (77)	10 (9)	17 (15)
Never (%)	203 (71)	30 (11)	52 (18)
Positive family history[a]			
Yes (%)	115 (82)	7 (5)	18 (13)
No (%)	300 (72)	40 (10)	75 (18)
Contraception			
Combined oral contraceptives (%)	508 (75)	60 (9)	114 (17)
First generation (%)	32 (86)	1 (3)	4 (11)
Second generation (%)	212 (76)	13 (5)	53 (19)
Third generation (%)	213 (74)	37 (13)	38 (13)
Cyproterone acetate (%)	45 (64)	8 (11)	17 (24)
Drospirenone (%)	6 (67)	1 (11)	2 (22)
Progestagen-only contraceptives (%)	12 (60)	3 (15)	5 (25)
Oral progestagen-only[b](%)	0 (0)	1 (100)	0 (0)
Intra-uterine device (%)	0 (0)	0 (0)	1 (100)
Implanon (%)	1 (100)	0 (0)	0 (0)
Injectables (%)	11 (65)	2 (12)	4 (24)
Unknown (%)	1 (100)	0 (0)	0 (0)

[a] Data on BMI, smoking, and family history was available in 665, 682, and 556 women, respectively.
[b] User of lynestrenol, a progestagen belonging to the first generation

An overview of the contraceptive used at and after the event among women who changed their contraceptive is given in table 5.2. The majority of these women changed to either a second gen-

eration combined oral contraceptive (41%) or to an intra-uterine device (40%). 54% of third generation combined oral contraceptive users switched to a second generation contraceptives, whereas 54% of second generation combined oral contraceptive users changed to an intra-uterine device (Table 5.2).

Regarding adherence to the guidelines, of 682 women who were using a combined oral contraceptive at the venous thrombosis event, 508 (74%) women discontinued use of combined oral contraceptives. However, 143 (21%) either changed to another combined oral contraceptive or continued to use their combined preparation, while 31 (5%) women changed to progestagen-only preparations.

At the time of interview not all women had stopped anticoagulant therapy. Hormonal contraceptive use might be continued to prevent pregnancy during anticoagulant therapy. 64 (10%) women were using anticoagulant therapy at time of the interview. After excluding these women, the distribution of women who stopped, changed or continued their contraceptive did not change; 421 (76%) women stopped, 46 (8%) changed and 85 (15%) continued their contraceptive use.

Risk differences (RD) in acquired risk factors - surgery, plaster cast, immobilization, injury and travel - for the women who stopped, continued or changed their contraceptive method are presented in table 5.3. Of the 349 women not exposed to any risk factor, 267 (77%) stopped, 31 (9%) changed and 51 (15%) continued their contraceptive use. 337 women were exposed to one or more acquired risk factors, of which 240 (71%) stopped, 31 (9%) changed and 66 (20%) continued their contraceptive use. Women, who had undergone surgery within 3 months prior to the diagnosis of thrombosis, were less likely to stop their contraceptive use than unexposed women (58% versus 77%, RD -19%, 95%CI: -30 to -8%) and more likely to continue their contraceptive use (34% versus 15%, RD 19%, 95%CI: 9 to 30%). Women, who had a plaster cast within 3 months prior to the diagnosis of thrombosis, were less likely to stop their contraceptive use than

Table 5.2: Pattern of changes after an hormonal contraceptive-associated venous thrombosis event

	Contraceptive method after venous thrombosis							
Contraceptive at event	**1st gen**	**2nd gen**	**3rd gen**	**DRSP**	**POP**	**IUD**	**Implanon**	**Injectables**
Combined oral contraceptives								
1st gen (%)	-	-	-	-	-	1 (100)	-	-
2nd gen (%)	-	3 (23)	1 (8)	-	-	7 (54)	-	2 (15)
3rd gen (%)	-	20 (54)	-	-	2 (5)	10 (27)	2 (5)	3 (8)
CPA (%)	-	3 (38)	1(13)	1(13)	-	3 (38)	-	-
DRSP (%)	-	-	-	-	-	1 (100)	-	-
Progestagen-only contraceptives								
POP (%)	-	-	-	-	-	1 (100)	-	-
Injectables (%)	-	-	-	-	-	2 (100)	-	-
Total	0	26 (41)	2 (3)	1 (2)	2 (3)	25 (40)	2 (3)	5 (8)

1st gen, first generation progestagen; 2nd gen, second generation progestagen; 3rd gen, third generation progestagen; CPA, cyproterone acetate; DRSP, drospirenone; POP, progestagen-only pills; IUD, intra-uterine device

unexposed women (59% versus 77%, RD -17%, 95%CI: -35 to -1%) and more likely to continue their contraceptive use (31% versus 15%, RD 17%, 95%CI: 3 to 34%). Regarding women exposed to immobilization, injure or travel, no differences were observed between exposed and unexposed women.

Finally, a total of 414 women had information on whether they received advice to stop using hormonal contraceptives. 341 (82%) were advised to stop using hormonal contraceptives, while 67 (16%) women did not receive this advice. Six women stated that they did not know whether they received any advice. Women who received advice regarding hormonal contraceptive use were more likely to stop their contraceptive use than women who did not receive any advice (83% versus 34%, RD 49%, 95%CI: 36 to 60%) (Table 5.4). Of 332 women using a combined oral contraceptive who were advised to stop, 277 (83%) did stop their preparation, 16 (5%) changed to progestagen-only contraceptives and 39 (12%) either continued or changed to another combined preparation.

Discussion

In this study we found that the majority of women with a first episode of venous thrombosis while using a hormonal contraceptive method had discontinued the use of hormonal contraceptives after the thrombotic event. However, about 25% of these women either changed or continued their contraceptive. Anticoagulant therapy at the time of interview did not explain why women continued their contraceptive use. Women who had had a thrombosis following surgery or plaster cast continued their hormonal contraceptive method more often than unexposed women. The majority of the women who received advice to stop using their hormonal contraceptive, did follow this advice.

The majority of women changing oral contraceptives, change to a combined preparation with a second generation progestagen or an intra-uterine device. The reason behind changing to a

Table 5.3: Transient risk factors in women who stop, continue or change their hormonal contraceptive method

	Stop N=521	**RD (95%CI)**	**Change N=69**	**RD (95%CI)**	**Continue N=119**	**RD (95%CI)**
Risk factor						
None (%)	267 (77)	Reference	31 (9)	Reference	51 (15)	Reference
Any (%)	240 (71)	-5 (-12 to 1)	31 (9)	0 (-4 to 5)	66 (20)	5 (-1 to 11)
Surgery (%)	48 (58)	-19 (-30 to -8)	7 (8)	0 (-6 to 8)	28 (34)	19 (9 to 30)
Immobilization (%)	70 (70)	-7 (-17 to 3)	14 (14)	5 (-1 to 14)	16 (16)	1 (-6 to 11)
Plaster cast (%)	19 (59)	-17 (-35 to -1)	3 (9)	1 (-7 to 16)	10 (31)	17 (3 to 34)
Injury (%)	73 (74)	-3 (-13 to 6)	8 (8)	-1 (-6 to 7)	18 (18)	4 (-4 to 13)
Travel (%)	108 (82)	6 (-3 to 13)	8 (6)	-3 (-7 to 3)	15 (11)	-3 (-9 to 4)
Advice to stop						
Do not know	3 (50)		1 (17)		2 (33)	
No	23 (34)	Reference	9 (13)	Reference	35 (52)	Reference
Yes	284 (83)	49 (36 to 60)	27 (8)	-6 (-16 to 2)	30 (9)	-43 (-55 to -31)

second generation combined oral contraceptive may be the lower risk for venous thrombosis in these users compared with either third generation or cyproterone acetate users [9–12]. However, these risk estimates are for a first event and whether these estimates can also be applied to a second event is unclear.

The association between the presence of certain acquired risk factors (surgery or a plaster cast) and the continuation of oral contraceptive use suggests that physicians think it is safe to continue oral contraceptives when a strong acquired risk factor for thrombosis has been established as an (additional) cause of the thrombotic event. Venous thrombosis is a multicausal disease, i.e., for the occurrence of deep vein thrombosis a combination of risk factors needs to be present [13]. The recurrence rate in surgically provoked events is low in patients without other clinical risk factors [14,15], whether this is also the case in women with a surgically provoked thrombotic event who use and continue to use oral contraceptives is unknown. It is therefore unclear whether the continuation of combined oral contraceptives is justified in this subgroup of patients.

Little is known about hormonal contraceptive use after venous thrombosis in clinical practice and about the risk of a recurrence in hormonal contraceptive users. The recommendations in the WHO guideline are based on studies which only included first events. No data on second events were available before publication of the WHO guideline in 2004. Only one study, which was published in 2010, analyzed the recurrence rate of thromboembolic disease in women who had actually continued or stopped oral contraceptive use after their first episode of oral contraceptive-associated venous thrombosis. They found that an increased rate for venous thrombosis in hormonal contraceptive users compared to non-users [6]. However, only 11 recurrences were found among hormonal contraceptive users. The number became too low per type of contraceptive to infer any other conclusions.

The statements regarding progestagen-only contraceptive use in the WHO guideline were based on one observational study

from the WHO[16]. Because of different types of progestagen and different doses for different applications, it is difficult to come to a conclusion about progestagen-only preparations and the risk of a first event. Even less is known about progestagen-only preparations and the risk of a second event. The previous study also reported on recurrences in progestagen-only preparations[6]; however, only 2 recurrences were observed in injectable users among a few progestagen-only preparation users.

This is one of the few studies to provide information on contraceptive use after a hormonal contraceptive-associated venous thrombosis. We had detailed information on contraceptive use before and after venous thrombosis, as well as on acquired risk factors. Therefore we were able to examine whether the presence of these risk factors was associated with the decision to stop or continue oral contraceptives, although we do not know whether this was actually taken into account with the decision making. Although we know whether these women were advised to stop their contraceptive use, we do not know why women stopped, changed or continued their contraceptive use. In particular, in women who received advice to stop using their contraceptive, it would be beneficial to know their reason to continue their contraceptive. In the Netherlands, the WHO guideline is implemented in national guidelines and we observed that about 20% do not follow the advice from the physician to stop their contraceptive use. The percentage of women receiving no advice may be different per country depending on implementation of guidelines. However, the problem that women continue their combined oral contraceptive use after receiving advice to stop is a real concern.

In conclusion, the fact that women seemed to disregard the advice from a physician is a real concern, emphasized by the potential increased risk of a recurrence when continuing hormonal contraceptives.

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Chapter 6

Recurrent venous thrombosis in premenopausal women: effect of continuing or starting hormonal contraceptive use

Bernardine H. Stegeman
Jasmijn Timp
Linda E. Flinterman
Suzanne C. Cannegieter
Frans M. Helmerhorst
Frits R. Rosendaal
A. van Hylckama Vlieg

Manuscript in preparation

Abstract

Background: There is a large body of literature available on hormonal contraceptive use and the risk of a first venous thrombotic event. Despite guideline recommendations to discontinue hormonal contraceptive use after a thrombotic event, still a sizeable proportion of women continue or start using hormonal contraceptives after a venous thrombosis. The aim of this study was to evaluate the effect of this use on the recurrence risk in premenopausal women.
Methods: Patients with a first venous thrombosis included in the MEGA case-control study between 1999 and 2004, were followed for a recurrent venous thrombotic event up to 2009. Included in the current analyses were premenopausal female patients with a first venous thrombosis and for whom detailed information was available on hormonal contraceptive use during follow-up. Time-dependent Cox-proportional hazards models were used to estimate hazard ratios (HR) with 95% confidence intervals (CI), adjusted for age and BMI at baseline
Results: 702 premenopausal women with a first venous thrombosis were followed for a total of 4673 woman-years (median 7.0 years; range, 12 days to 9.9 years) during which 74 recurrent events occurred resulting in a recurrence rate of 15.8 (95%CI: 12.6 to 19.8) per 1000 woman-years. 210 women used hormonal contraceptives, mainly orally administered, during follow-up with a total follow-up of 545 woman-years. They experienced 21 recurrent thrombotic events indicating a rate of 38.5 per 1000 woman-years, which was triple the rate of non-use during follow-up (HR 2.8, 95%CI: 1.7 to 4.7). None of the women (N=20, 70 woman-years of follow-up) using a levonorgestrel-releasing intra-uterine device developed a recurrent venous thrombosis.
Discussion: Hormonal contraceptive use after a first venous thrombosis increases the risk of a recurrent venous thrombotic event. A levonorgestrel-releasing intra-uterine device may be a safe contraceptive to use after a venous thrombosis.

Introduction

Combined oral contraceptives (containing ethinylestradiol and a progestagen) increase the risk of a first venous thrombosis two- to fourfold[1–4]. Several studies have assessed the risk of venous thrombosis associated with oral progestagen-only pills (POP)[5–9]; however, because these may be prescribed to a subset of women who are already at increased risk of venous thrombosis, no definite conclusions can be drawn.

Oral contraceptive use is the most common route of administration. However, steroid hormones for contraceptive use can also be administered via other administration routes or applications, i.e., intrauterinely (intrauterine device (IUD)), transdermally (patch), subcutaneously (injectable or implant), or transvaginally (ring). Regarding non-oral contraceptives, the use of combined preparations (i.e., a vaginal ring or a transdermal patch) is associated with an increased risk of a first venous thrombosis[10–12]. Progestagen-only non-oral preparations, i.e., the levonorgestrel-releasing IUD or the injectable preparation containing methoxyprogesterone acetate, appear to affect the risk of venous thrombosis differently. Whereas the risk of a first venous thrombosis is not increased in intra-uterine device (IUD) users[12,13], there is an association with a mildly elevated risk of venous thrombosis for the use of an injectable containing methoxyprogesterone acetate[8,13,14]. Overall, there is a large body of literature available about hormonal contraceptive use and the risk of a first venous thrombosis.

National[15,16] and international[17] guidelines advise to discontinue hormonal contraceptive use after a venous thrombotic event, in particular combined preparations (oral contraceptive, transdermal patch and vaginal ring). The guidelines are based on the assumption that risk factors for a first event also increase the risk of a recurrence. However, this is not necessarily so[18]. The absolute risk of recurrent venous thrombosis is higher than of a first venous thrombosis. The populations at risk differ and

therefore, possibly also the risk profiles.

Despite these guidelines, still a large proportion of women either continues or starts hormonal contraceptive use after a first venous thrombosis. One study found that 39% of women using hormonal contraceptives at the first event either continued or restarted after the event [19]. Furthermore, we previously reported that in the participants of our study, 143 (21%) of 682 combined oral contraceptive users at the first event continued combined oral contraceptive use after the event [20].

Currently, there is only one report on recurrent venous thrombosis risk associated with hormonal contraceptive use [21]. The aim of the present study was therefore to evaluate the effect of hormonal contraceptive use, administration route and type of combined oral contraceptive (dose of ethinylestradiol and type of progestagen) on the recurrence risk of venous thrombosis in premenopausal women.

Methods

Participants Participants were cases from a population-based case-control study; the Multiple Environmental and Genetic Assessment of venous thrombosis (MEGA) study. Details of the study have been described elsewhere [22]. Between 1 March 1999 and 31 August 2004, 4956 consecutive patients with an objectively diagnosed first deep vein thrombosis of the leg or pulmonary embolism were included. Patients were aged 18-70 years and were enrolled from six anticoagulation clinics in the Netherlands. Anticoagulation clinics monitor all patients taking vitamin K antagonists in a well-defined geographical area. All patients filled in a questionnaire on risk factors for venous thrombosis. About three months after discontinuation of the anticoagulation therapy, patients were invited to the anticoagulation clinic for a blood sample. During this visit participants were interviewed regarding the period from the venous thrombotic event until the venipuncture. This interview included items on the change of

hormonal contraceptive methods since the diagnosis of venous thrombosis.

Of 4956 eligible patients, 4731 gave informed consent for follow-up. During follow-up, patients received a short questionnaire containing questions on recurrence of venous thrombosis and the use of oral anticoagulation therapy. Patients willing to fill in a detailed questionnaire on risk factors for venous thrombosis during follow-up received this questionnaire by mail or internet. This detailed questionnaire contained questions about hormonal contraceptive use after the first venous thrombosis, type of contraceptive used and when applicable starting date and date of discontinuation of hormonal contraceptives. Patients participating in a pilot study received only a detailed questionnaire which also included the question on recurrence.

Questionnaires were sent by mail to patients between January 2008 and December 2009. Questions were asked by telephone interview when questionnaires were not returned. During the same period information about recurrences was retrieved from the anticoagulation clinics where patients were initially included for their first event and, in case they moved house, at the clinic nearest to their new address. Deaths due to recurrent venous thrombosis were obtained at the Central Bureau of Statistics (CBS) and were included. Discharge letters, to obtain information on diagnostic procedures, were requested from the clinician who diagnosed the recurrence according to the patient or the anticoagulation clinic. Patients were followed from the date of their first episode of venous thrombosis until a second thrombotic event or until the end-of-study, defined as the date of filling in the short questionnaire. In case patients did not fill in the short questionnaire, they were followed until the last known visit to the anticoagulation clinic, death, or emigration. Details of the follow-up study have been described elsewhere[23]. This study was approved by the Medial Ethics Committee of the Leiden University Medical Center.

For the current analyses, we focussed on premenopausal wo-

men with venous thrombosis younger than 50 years (N=1526). Women who were pregnant (N=42), were postpartum (defined as four weeks after delivery) (N=18), or were users of hormone replacement therapy (N=16) at the time of the first event were excluded. Women with a diagnosis of cancer in the five years prior to the first event were also excluded (N=51). Of the remaining 1399 premenopausal women, 845 (60%) women provided information on hormonal contraceptive use by filling in the detailed questionnaire. Data on hormonal contraceptive use provided in the detailed questionnaire was crosschecked with data retrieved at the time of the first venous thrombosis and at the time of venipuncture in the case-control study. 143 (17%) women were inconclusive about their contraceptive use and were excluded from further analyses. A total of 702 women were included in the present analysis. 13 women only provided data regarding duration of use but not the type of contraceptive. These women were only included in the overall analysis of hormonal contraceptive use during follow-up.

Recurrent venous thrombosis A recurrent event was defined by information provided by patients through the short questionnaire, anticoagulation clinics, or discharge letters. A decision rule regarding certainty of the diagnosis was made according to the information collected per patient. In short, reported recurrences were classified into certain recurrences when there was a discharge letter stating a diagnosis of a recurrent event based on clinical and radiological data, or when both the anticoagulation clinic and the patient reported a recurrent event at either a clearly different location than the first event or more than one year has passed since the first event, or when a registered death from a recurrent event at least six months after the first event was found. Details of this decision rule have been described previously [23]. In this study, certain recurrences were used as endpoint and patients with an uncertain recurrence were censored at time of their uncertain recurrence.

Hormonal contraceptives Hormonal contraceptive use was defined as use of a contraceptive that contains steroid hormones. Users of a copper-IUD were considered non-users. Hormonal contraceptive use was categorised according to the route of administration route, i.e., into oral and non-oral preparations. Oral preparations were stratified into combined and progestagen-only preparations. Because many different preparations of combined oral contraceptives are available, these contraceptives were categorised according to the dose of ethinylestradiol and type of progestagen. Non-oral preparations were further stratified according to the specific application (vaginal ring, transdermal patch, implant, injectable, and levonorgestrel-releasing intrauterine device (IUD)).

Statistical analysis In this analysis premenopausal women with information on hormonal contraceptive use after a first venous thrombosis from a detailed questionnaire were included. The start of follow-up was defined as the date of the first venous thrombosis. The end of follow-up was defined as the date of a recurrent event or the date of filling in the short questionnaire or the detailed questionnaire, in case of participation in the pilot, when no recurrent event developed. Observation time was calculated as the time at risk from the first thrombotic event to the end of follow-up.

Hormonal contraceptive use was taken as a time-dependent exposure to allow for women switching from use to non-use and vice versa during follow-up. Consequently, one woman could contribute follow-up time for hormonal contraceptive use as well as for non-use. The risk of recurrent venous thrombosis was estimated for hormonal contraceptive use at the first event and for hormonal contraceptive use during follow-up. The effect of oral and non-oral preparations on the risk of recurrent venous thrombosis was assessed and compared with non-use. All analyses were adjusted for the confounders' age and BMI (at baseline). Time-dependent Cox-proportional hazards models were used to

calculate hazard ratios (HR). All statistical analyses were performed with STATA, version 12.0 (Statacorp LP, College Station, TX, USA).

Results

702 women with a first venous thrombotic event were followed for a total of 4673 woman-years (median 7.0 woman-years; range, 12 days to 9.9 woman-years). Baseline characteristics of the study population are given in table 6.1. On average, women were 36 years of age (range 18 to 49 years) at baseline. 290 (43%) women had a BMI at baseline <25 kg/m^2. 533 (76%) women used hormonal contraceptives at the first venous thrombotic event, whereas 169 (24%) were non-users at the first event.

Table 6.1: Baseline characteristics of premenopausal women with venous thrombosis

Variables	**All N=702**
Age at 1st event, mean(range), yrs	36 (18-49)
BMI at 1st event	
<25 kg/m2	290 (43)
25-30 kg/m2	214 (32)
>30 kg/m2	163 (24)
Hormonal contraceptive use at 1^{st} event	533 (76)

BMI, body mass index

Among 702 women, 74 recurrences occurred, of which 21 recurrences were during hormonal contraceptive use. Overall, the rate of recurrent venous thrombosis was 15.8 (95%CI: 12.6 to 19.8) per 1000 woman-years. The recurrence rate in hormonal contraceptive users at the first event was similar (15.1, 95%CI:

11.6-19.8 per 1000 woman-years) as in non-users at the first event (18.0, 95%CI: 11.6-27.9) (Table 6.2). This was evident from the hazard ratio for users at the first event compared to non-users (HR 0.9, 95%CI: 0.5 to 1.5, adjusted for age and BMI) (Table 6.2).

Among women using hormonal contraceptives during follow-up, we observed a recurrence rate of 38.5 (95%CI: 25.1 to 59.1) per 1000 woman-years and among non-users during follow-up, a rate of 12.8 (95%CI: 9.8 to 16.8). This implied that hormonal contraceptive use after a first venous thrombosis tripled the risk of recurrent thrombosis (HR 3.0, 95%CI: 1.8 to 5.0). After adjustment for age and BMI at the first event, the hazard ratio did not change (HR 2.8, 95%CI: 1.7 to 4.7) (Table 6.2). Restricted to women who were using hormonal contraceptives at the first event, a similar risk increase was found associated with hormonal use during follow-up, with those who discontinued use as reference group (HR 3.4, 95%CI: 1.9-5.9, and after adjustment for age and BMI: HR 3.1, 95%CI: 1.7-5.4) (Table 6.2).

Hormonal contraceptive preparations were classified into oral and non-oral (Table 6.2). 16 recurrences occurred during oral contraceptive use (all combined oral contraceptives). The recurrence rate for combined oral contraceptive use during follow-up was 41.1 (95%CI: 25.1-67.1) per 1000 woman-years, again threefold higher than for non-use during follow-up (HR 3.2, 95%CI: 1.8 to 5.7; HR 2.9 after adjustment for age and BMI, 95%CI: 1.6 to 5.2). Only two women used a vaginal ring during follow-up of whom 1 had a recurrence, suggestive of a high recurrence risk. Because only one recurrence occurred among 31 non-oral preparation users, vaginal ring, levonorgestrel-releasing IUD, injectable and implanon users, no reliable risk estimates could be inferred. However, notable was that out of 20 women using a levonorgestrel-releasing IUD (70 woman-years of follow-up), none had a recurrence.

The recurrence rate per type (dose and content) combined oral contraceptive is reported in Table 6.3. Both dose of ethinyles-

Table 6.2: Recurrence rate in premenopausal women and the influence of hormonal contraceptive use

	N*	FU	IR (per 1000) (95% CI)	HR (95% CI)	HR‡ (95% CI)	HR§ (95% CI)
Non-use at 1^{st} event	20	1109	18.0 (11.6-27.9)	1	1	1
HC use at 1^{st} event	54	3564	15.1 (11.6-19.8)	0.8 (0.5-1.4)	0.8 (0.5-1.4)	0.9 (0.5-1.5)
Non-use during FU	53	4129	12.8 (9.8-16.8)	1	1	1
HC use during FU†	21	545	38.5 (25.1-59.9)	3.0 (1.8-5.0)	3.0 (1.8-5.0)	2.8 (1.7-4.7)
Oral preparation						
COC	16	389	41.1 (25.2-67.1)	3.2 (1.8-5.6)	3.2 (1.8-5.6)	2.9 (1.6-5.2)
POP	0	12	-	-	-	-
Non-oral preparation						
Vaginal ring	1	4	269.0 (37.9-1909.4)	-	-	-
IUD	0	70	-	-	-	-
Injectable	0	32	-	-	-	-
Implanon	0	1	-	-	-	-
HC use at 1^{st} event	54	3046	11.2 (8.0-15.6)	1	1	1
HC use at 1^{st} event and during FU	20	518	38.6 (24.9-59.8)	3.4 (1.9-5.9)	3.4 (2.0-6.0)	3.1 (1.7-5.4)

COC, combined oral contraceptive; FU, follow-up time in woman-years; HC, hormonal contraceptive; HR, hazard ratio; IR, incidence rate; POP, progestagen-only pill

* Number of recurrences

† No data on the type of contraceptive used was available in two women

‡ Age-adjusted

§ Age and BMI adjusted

Table 6.3: Overview of number of women and recurrences per combined oral contraceptive

Preparation	N*	N†	FU	IR (95%CI)	HR (95%CI)	HR§ (95%CI)
COC‡						
37.5 µg EE+LYN	5	0	7	-		
20 µg EE+LNG	3	0	6	-		
30 µg EE+LNG	67	3	141	21.3 (6.9-66.1)	1	1
50 µg EE+LNG	11	2	21	96.4 (24.1-385.4)	3.5 (0.6-20.9)	2.3 (0.3-16.7)
20 µg EE+DSG	8	0	24	-		
30 µg EE+DSG	33	5	58	86.7 (36.1-208.3)	3.7 (0.9-15.5)	3.4 (0.8-14.9)
20 µg EE+GSD	2	0	5	-		
30 µg EE+GSD	18	0	46	-		
35 µg EE+NRG	2	0	3	-		
35 µg EE+CPA	11	1	36	27.9 (3.9-197.8)	1.3 (0.1-12.7)	1.1 (0.1-11.6)
30 µg EE+DRSP	3	0	5	-		
Triphasic	17	5	25	200.0 (83.2-480.4)	8.6 (2.0-37.2)	7.5 (1.7-33.6)

COC, combined oral contraceptive; CPA, cyproterone acetate; DRSP, drospirenone; DSG, desogestrel; EE, ethinylestradiol; FU, follow-up in woman-years; GSD, gestodene; HR, hazard ratio; LNG, levonorgestrel; POP, progestagen-only pills; Triphasic, contraceptive with varying dose of ethinylestradiol over 21 days with either norethisterone (N=1) or levonorgestrel (N=16)

* Number of women

† Number of recurrences; the total number exceeds the total number of women, because a woman could have used multiple preparations during follow-up

‡ Preparation 35 µg ethinylestradiol with norethisterone was not reported because none of the women used this contraceptive during follow-up

§ Age-adjusted

tradiol and progestagen were associated with the risk of recurrent venous thrombosis. Within users of combined oral contraceptives containing levonorgestrel, users of 50 µg ethinylestradiol had a two-fold higher recurrence rate than users of 30 µg ethinylestradiol with levonorgestrel (HR 2.3, 95%CI: 0.3-16.7, adjusted for age). Within contraceptives with 30 µg ethinylestradiol, contraceptives with desogestrel were associated with a higher risk of recurrence than contraceptives with levonorgestrel (HR 3.4, 95%CI: 0.8-14.9, adjusted for age). The risk of a recurrence was about equal in users of cyproterone acetate compared with users of 30 µg ethinylestradiol with levonorgestrel (HR 1.1, 95%CI: 0.1-11.6, adjusted for age). Out of 17 users of a triphasic preparation, 5 women had a recurrence leading to an eight-fold higher rate than in users of 30 µg ethinylestradiol with levonorgestrel (HR 7.5, 95%CI: 1.7-33.6, adjusted for age). One woman was using a triphasic contraceptive with norethisterone and 16 women with levonorgestrel. After excluding the user of norethisterone, the results were unchanged (age-adjusted HR 7.5, 95%CI: 1.7-33.6).

Discussion

In a study comprising 702 premenopausal women with venous thrombosis, we assessed the association between hormonal contraceptive use and recurrent venous thrombosis. Women using hormonal contraceptives, in particular combined oral contraceptives, after a first venous thrombosis have a threefold higher risk of recurrence than non-users. The use or non-use of hormonal contraceptives at the first event did not affect the risk of recurrent venous thrombosis. Among combined oral contraceptives users during follow-up, both the dose of ethinylestradiol as well as the progestagens influenced the recurrence rates. Users of triphasic contraceptives were found to have the highest recurrence rates among combined oral contraceptives. Interestingly, among 20 levonorgestrel-releasing IUD users, none had a recurrent venous thrombosis.

To date, only one other study (LETS) evaluated the risk of recurrent venous thrombosis among women using hormonal contraceptives in a prospective follow-up study [21]. That analysis was restricted to women who used hormonal contraceptives at the first event. The authors observed a recurrence rate of 48.8 (95%CI: 24.3-87.2) among hormonal contraceptive users during follow-up and a recurrence rate of 10.5 (95%CI: 4.5-20.7) among non-users. The recurrence rate among non-users was similar as reported in the current study (10.5 versus 11.2); however, their recurrence rate in hormonal contraceptive users was slightly higher (48.8 versus 38.6). This is most likely due to differences in the distribution of types of contraceptives in the LETS and the MEGA study, between which a decade elapsed. In both the LETS and the present study, the recurrence rate among triphasic contraceptive users was increased compared with non-users (138.9 (95%CI: 16.8-501.7) versus 200.0 per 1000 women-years (95%CI: 83.2-480.4)). Why the risk of a recurrence is higher for these types of contraceptives is currently unexplained, in particular because there is no difference reported between monophasic and triphasic contraceptives regarding the risk of a first venous thrombosis.

A limitation of our study is that data on hormonal contraceptive use were obtained through a detailed questionnaire which not every patient of the MEGA follow-up study was willing to fill in, reducing the number of women included in our analyses. The reason for not returning the questionnaire may have introduced selection bias. We therefore assessed the recurrence rate in patients who filled in the detailed questionnaire (responders) and patients who did not (non-responders) and this proved to be similar ($IR_{responders}$ 17.6, 95%CI: 14.5-21.5 and $IR_{non-responders}$ 17.3, 95%CI: 12.3-24.1). Most likely, selection bias did not influence our results. A further limitation of the present study is the assessment of menopausal status. Menopausal status was based on statements from the women at baseline and not on medical data. Potentially, peri- or postmenopausal women could have

been included. However, menopausal status is to our knowledge not related to thrombotic risk and therefore, this potential misclassification cannot have affected our risk estimates. Strengths of our study are its size, and that we used a decision rule to ascertain recurrence by which we ensured that only certain recurrences were included in our analyses. Furthermore, detailed information on participants' hormonal contraceptive use obtained during follow-up made it possible to perform a time-dependent survival analysis, allowing switches from exposed to non-exposed during follow-up and vice versa.

In conclusion, hormonal contraceptive use after a first venous thrombotic event increased the risk of a recurrence. Although numbers are small, results suggest that the use of an levonorgestrel-releasing IUD may be a safe option after a venous thrombotic event. Among combined oral contraceptive users, the increase in risk depended on the ethinylestradiol dose, progestagen and whether the contraceptive was triphasic or not.

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Chapter 7

Network meta-analysis of different combined oral contraceptives and the risk of venous thrombosis

Bernardine H. Stegeman
Marcos de Bastos
Frits R. Rosendaal
Astrid van Hylckama Vlieg
Frans M. Helmerhorst
Theo Stijnen
Olaf M. Dekkers

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Abstract

Background: Oral contraceptive use is associated with venous thrombosis risk. Although the risk of venous thrombosis has been evaluated for various estrogen doses and types of progestagen, no comprehensive comparison involving commonly used oral contraceptives is available.
Methods: A network meta-analysis was performed to assess the risk of thrombosis associated with oral contraceptive use at the level of individual types of contraceptives. Electronic databases (Pubmed, Embase, Web of Science, Cochrane, CINAHL, Academic Search Premier and ScienceDirect) were searched in November 2011 to identify potential relevant articles. We selected publications that assessed the effect of combined oral contraceptives on venous thrombosis (i.e., deep venus thrombosis and pulmonary embolism) in healthy women. Two independent reviewers extracted data from included publications. The network meta-analysis was performed using an extension of frequenist random-effects models for mixed multiple treatment comparisons and relative risks (RR) with 95% confidence intervals (CI) were reported.
Results: A total of 2011 publications were retrieved through a search strategy; 24 publications reporting on 25 studies were included. Combined oral contraceptive use increased the risk of venous thrombosis compared with non-use (RR 3.6, 95 %CI: 2.9-4.6). Oral contraceptives containing a third generation progestagen (desogestrel, gestodene or norgestimate) were associated with a higher risk of venous thrombosis than oral contraceptives with levonorgestrel (second generation) (RR 1.6, 95%CI: 1.3-1.9). Sensitivity analyses stratified by funding source, study design, or confirmed venous thrombosis did not change the findings. From the network meta-analysis at the level of individual type, the highest thrombosis risk was observed among 50 µg ethinylestradiol with levonorgestrel (RR 6.7, 95%CI: 4.0-11.2, compared with non-use), 35 µg ethinylestradiol with cyproterone acetate (RR

5.5, 95%CI: 3.9-7.7) and 30 µg ethinylestradiol with drospirenone (RR 6.0, 95%CI: 4.1-8.9), while the lowest risk was observed among 20 µg with levonorgestrel (RR 2.6, 95%CI: 1.5-4.5).
Conclusion: All combined oral contraceptives are associated with an increased risk of venous thrombosis. The effect size depends on the progestagen and the dose of ethinylestradiol.

Introduction

In 1960, shortly after the introduction of the first combined oral contraceptive, a case of venous thrombosis associated with contraceptive use was reported[1]. Since then many observational studies showed that combined oral contraceptives, containing both an estrogen and a progestagen, are associated with a two-fold to six-fold increased risk of venous thrombosis[2–5]. Despite the low incidence of venous thrombosis of about 3 per 10,000 woman-years among women of reproductive age[6], the impact of combined oral contraceptives on venous thrombosis is large because worldwide many women use oral contraceptives.

Because the estrogen compound (ethinylestradiol) in combined hormonal contraceptives was thought to cause the increased thrombosis risk, the dose of ethinylestradiol has been lowered from 150-100 µg in the earliest brands to 50 µg in the 1960s to 30-35 µg and 20 µg in the 1970s[7–9]. The reduced dose of ethinylestradiol in contraceptives was indeed associated with a reduction in the risk of venous thrombosis[10–14]. Furthermore, the currently prescribed combined oral contraceptives containing 30 µg of ethinylestradiol are associated with a higher risk of venous thrombosis than contraceptives containing 20 µg[15,16]. Besides adjustments in the dose of ethinylestradiol, the progestagen compound was also changed in an effort to reduce side-effects. After the first generation progestagens (e.g., norethisterone, lynestrol), new progestagens were developed, called second (i.e., levonorgestrel (LNG)) and third generation progestagens (i.e., gestodene (GSD), desogestrel (DSG), norgestimate (NGM))[17]. However, third generation combined oral contraceptive users have a higher risk of venous thrombosis than second generation users[15,16,18,19]. Other progestagens have been developed after the introduction of the third generation progestagens, e.g. drospirenone (introduced in 2001). The use of drospirenone in a combined oral contraceptive also increased the risk of venous thrombosis compared with non-use[15,16] and compared with than

second generation contraceptives[20,21].

The aim of the present network meta-analysis was to provide an overview of the risk of venous thrombosis per combined oral contraceptive in healthy women. Furthermore, the effect of the generation of a progestagen in combined oral contraceptive was assessed. A network meta-analysis was performed because combined oral contraceptives are mostly compared with non-use or with a contraceptive containing levonorgestrel with 30 µg ethinylestradiol resulting in gaps in direct evidence. In other words, not every combined oral contraceptive has been directly compared with all other possible combined oral contraceptives. A network meta-analysis allows evidence from direct and indirect comparisons to be summarized in a weighted average for all possible comparisons.

Methods

Search strategy and selection criteria A detailed overview of the search strategy and selection criteria can be found in the supplementary data[22]. Publications of interest were observational studies (i.e., cohort or (nested) case-control studies) that included healthy women using combined oral contraceptives. The primary outcome of interest was a fatal or non-fatal first event of venous thrombosis (deep venous thrombosis or pulmonary embolism). Publications with a minimum of 10 events in total were eligible.

The following databases were searched; Pubmed (918 articles retrieved), Embase (1198), Web of Science (298), Cochrane (57), CINAHL (111), Academic Search Premier (183) and ScienceDirect (103). Our search terms consisted of MeSH (sub)headings, text words, and word variations for "combined oral contraceptive", "estrogens", "progestagens" and "venous thromboembolism". This search strategy was amended for each database. Each database was searched from inception until 22 November 2011 (date of final search). No language restriction was applied. Beside database searches, references of potential interesting publications

were searched.

A standard form was used to select publications. Two investigators (BHS, MdB) independently assessed publications for eligibility. Titles and abstracts were screened and if deemed potentially relevant, full-texts were retrieved. Any disagreements between the investigators were discussed and if necessary, a third reviewer (OMD) was asked to resolve any disagreements. In case of multiple publications from the same study, the publication with the most updated or the most inclusive data was chosen for inclusion.

Data collection Two investigators (BHS, MdB) independently extracted data using a standard form. Data were extracted on type of combined oral contraceptive (dose and type of estrogen and progestagen), crude numbers for exposure and outcome via a 2-by-2 table, crude and adjusted risk estimates, and variables adjusted for in the analysis.

Risk of bias assessment was based on design features that could potentially bias the association between exposure and outcome. We assessed adequacy of exposure (oral contraceptive) and outcome (venous thrombosis) measurement. Specific for cohort studies, loss-to-follow-up assessment was taken into account and for case-control studies, the sampling of controls. Women are more likely to remember that they used oral contraceptives than what specific preparation they used [23,24]. Therefore, assessment of type of combined oral contraceptive through an interview or questionnaire was deemed as high risk of bias, while information from a prescription database was judged as low risk. Only 25-33% of patients presenting with clinical symptoms suggestive of venous thrombosis are diagnosed with venous thrombosis [25].Therefore, objectively confirmed venous thrombosis in all patients was judged as low risk of bias. Venous thrombosis was objectively confirmed when a deep venous thrombosis was diagnosed by plethysmography, ultrasound examination, CT, or venography; or when a pulmonary embolism was diagnosed by

ventilation-perfusion (V/Q) scanning, spiral computed tomography, or pulmonary angiography[26,27]. Less than 10% loss-to-follow-up was considered to represent a low risk of bias. For case-control studies, controls selected from a hospital population was considered to confer a high risk of bias.

For sensitivity analyses, data on funding source and first-time use were abstracted.

In case of incomplete data on dose/type of estrogen or progestagen, authors were sent an email for extra information. Emails were sent on 25 July 2012 with a reminder on 20 August 2012. In total, 80% replied to our emails.

Classification of type or combined oral contraceptives For the network meta-analysis per generation of progestagen, the following progestagens were considered first generation; lynestrenol and norethisterone. Norgestrel and levonorgestrel were categorized as second generation progestagens, whereas desogestrel, gestodene and norgestimate were classified as third generation progestagens[17]. This classification was irrespective of the ethinylestradiol dose. Publications, for which our classification could not be applied but which did provided their own classification, were included irrespective of whether this classification differed from our own. To assess the influence of combining different classifications, we repeated the analysis in studies where our own classification of generations could be applied.

Many different combined oral contraceptives are available. The most commonly used combined oral contraceptives were selected for analysis, namely 20 µg ethinylestradiol with levonorgestrel (20LNG), 30 µg ethinylestradiol with levonorgestrel (30LNG), 50 µg ethinylestradiol with levonorgestrel (50LNG), 20 µgg ethinylestradiol with gestodene (20GSD), 30 µg ethinylestradiol with gestodene (30GSD), 20 µg ethinylestradiol with desogestrel (20DSG), 30 µg ethinylestradiol with desogestrel (30DSG), 35 µg ethinylestradiol with norgestimate (35NRG), 35 µg ethinylestradiol with cyproterone acetate (35CPA), and 30

µg ethinylestradiol with drospirenone (30DRSP).

Statistical analysis A network meta-analysis was conducted per generation of progestagen in a combined oral contraceptive and per selected oral contraceptive preparation.

An extension of frequentist random-effects models for mixed multiple treatment comparisons was used. Network meta-analysis was performed with the *mvmeta* command for STATA as described by White et al[28]. Crude data (i.e., data from a 2-by-2 table) were used in the analysis. Odds ratio, risk ratio or rate ratio and appropriate variances were computed and combined in the analysis leading to an overall relative risk (RR). For publications with zero events in one group, all groups in that publication were inflated by adding 0.5.

When enough studies provided data on the same stratum (i.e., data on generations of progestagen or on specific contraceptive preparations), consistency of the results was checked through interaction analysis. An interaction term was added to the model to estimate the difference in results from direct and indirect evidence. All potential interactions were tested in an overall test to determine whether there were any inconsistencies in our network meta-analysis. Inconsistencies were only checked when there was more than one study comparing the same groups.

The following sensitivity analyses were planned: per study design, per funding source (whether industry sponsored or not), within first-time users, and according to risk of bias.

All statistical analyses were performed with STATA, version 12.0 (Statacorp LP, College Station, TX, USA).

Results

Characteristics of included studies A total of 2011 publications were retrieved through electronic and references searches. 1912 were excluded after screening of title and abstract and 75 publications were excluded after detailed assessment of the full-text.

Reasons for exclusion are shown in the flow chart (Figure 7.1, Supplementary table 7.1). Overall, 25 studies published in 24 articles were included. Two publications provided important additional information to studies included in the meta-analysis (information on first-time use); data from these publications were added to respective studies already included. Details of included studies are shown in table 7.1. Seven cohort studies, three nested case-control studies and 15 case-control studies were included.

Based on data from 14 studies that included a non-user group, combined oral contraceptive use was found to be associated with a four-fold increased risk of venous thrombosis (RR 3.6, 95%CI: 2.9-4.6).

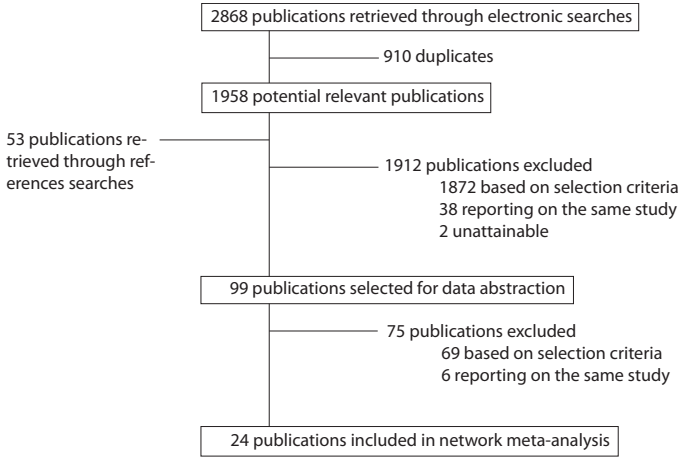

Figure 7.1: Flow chart of included and excluded publications

Table 7.1: Characteristics of included studies

Author	Year	Startdate	Enddate	Study design	No. of participants	Country
N. Gronich [29]	2011	1 Jan 2002	31 Dec 2008	Cohort	329995 women; 819749 wyrs	Israel
S.S. Jick [20]	2011	1 Jan 2002	31 Dec 2008	Case-control	186 cases; 681 controls	USA
L. Parkin [21]	2011	1 May 2002	30 Sep 2009	Case-control	61 cases; 215 controls	UK
L.A.J. Heinemann [30]	2010	Jan 2002	Feb 2006	Case-control	451 cases; 1920 controls	Austria
A. van Hylckama Vlieg [16]	2009	Mar 1999	Sep 2004	Case-control	1524 cases; 1760 controls	Netherlands
S.S. Jick [31]	2006	Jan 2000	Mar 2005	Case-control	281 cases; 1055 controls	USA
E. Samuelsson [32]	2004	1 Jan 1991	31 Dec 2000	Cohort	88 cases; 243723 wyrs	Sweden
K. Hedenmalm [33]	2004	1965	2001	Cohort	172 cases; 10016194 trtyrs	Sweden
L.A.J. Heinemann [34]	2002	Jan 1994	Jul 1999	Case-control	606 cases; 2942 controls	Germany
L. Parkin [35]	2000	Jan 1990	Aug 1998	Case-control	26 cases; 111 controls	New Zealand
R.D.T. Farmer [36]	2000	Jan 1992	Jun 1997	Cohort	287 cases; 783876 wyrs	UK
M.A. Lewis [37]	1999	Jan 1993	20 Oct 1995	Case-control	471 cases; 1702 controls	UK & Germany
R.M.C. Herings [38]	1999	1986	1995	Cohort	450000 women; 33 cases	Netherlands
J.C. Todd [39]	1999	1992	Mar 1997	Cohort	99 cases; 216356 wyrs	UK
I. Martinelli [40]	1999	Apr 1995	Apr 1998	Case-control	Unclear*	Italy
K.W.M. Bloemenkamp [41]	1999	1 Sep 1982	18 Oct 1995	Case-control	185 cases; 591 controls	Netherlands
Ø. Lidegaard [42]	1998	1994	1995	Case-control	375 cases; 1041 controls	Denmark
R.D.T. Farmer [43]	1998	Oct 1992	Sep 1995	Case-control	42 cases; 168 controls	Germany
B.S. Andersen [44]	1998	-	-	Case-control	67 cases; 134 controls	Denmark
M.A. Lewis [45]	1996	Jul 1991	Dec 1995	Case-control	505 cases; 1877 controls	UK & Germany
R. Farmer [46]	1996	-	-	Cohort	30 cases; 697000 women	UK
K.W.M. Bloemenkamp [47]	1995	1 Jan 1988	31 Dec 1995	Case-control	126 cases; 159 controls	Netherlands
WHO [48]	1995	1 Feb 1989	31 Jan 1993	Case-control	829 cases; 1979 controls	9 countries†
WHO1 [49]	1995	1 Feb 1989	3 Jan 1993	Case-control	433 cases; 1044 controls	Europe
WHO2 [49]	1995	1 Feb 1989	3 Jan 1993	Case-control	710 cases; 1954 controls	Developing

wyrs, woman-years; trtyrs, treatment-years

* Total no. of women was unclear; however, numbers were available for specific contraceptives

† Brazil, Chile, Colombia, Germany, Hong Kong, Hungary, Jamaica, Thailans, and UK

Risk of bias A total of eight studies assessed combined oral contraceptive use through an interview or questionnaire (Supplementary table 7.2). Only five studies objectively confirmed venous thrombosis in all patients, whereas eleven studies objectively confirmed venous thrombosis in a proportion of the population or subjectively confirmed venous thrombosis. Six case-control studies selected controls from a hospitalized population. Of the seven cohort studies, none reported any information about loss-to-follow-up.

Network meta-analysis comparing generations of progestagens A total of 23 studies were included for the analysis stratified per generation of progestagen in a combined oral contraceptive. Two studies reported solely on the risk of venous thrombosis in drospirenone, which is not classified as a certain generation of progestagen. Details of the number of events and total number of women or total follow-up time per group (i.e., non-use, first generation, second generation and third generation) are provided in supplementary table 7.3.

Results of the network meta-analysis according to generations of progestagen are shown in table 7.2. Users of oral contraceptives with a first generation progestagen had a 3.4-fold increased risk compared with non-users (95%CI: 2.5-4.5), for second generations the risk was 2.8-fold increased (95%CI: 2.3-3.5) and third generation users the risk was 4.5-fold increased (95%CI: 3.6-5.5). Third generation users had a higher risk of venous thrombosis than second generation users (RR 1.6, 95%CI: 1.3-1.9). Restriction to studies where our classification of generations could be applied, the results remained the same (RR_{1st} 3.6, 95%CI: 2.5-5.2; RR_{2nd} 2.9, 95%CI: 2.3-3.8; RR_{3rd} 5.2, 95%CI: 4.0-6.6 compared with non-use). A formal interaction test did not show inconsistencies in the network ($\chi^2 = 3.17$, $p = 0.67$).

Table 7.2: Overview of the results of the network meta-analysis per generation of progestagen

	Reference group			
	Non-use	**1st**	**2nd**	**3rd**
	RR (95%CI)	**RR (95%CI)**	**RR (95%CI)**	**RR (95%CI)**
Non-use	1			
1st	3.4 (2.5-4.5)	1		
2nd	2.8 (2.3-3.5)	0.8 (0.6-1.1)	1	
3rd	4.5 (3.6-5.5)	1.3 (1.0-1.7)	1.6 (1.3-1.9)	1

Network meta-analysis comparing different combined oral contraceptives Twelve studies had data available per type of oral contraceptive preparation (Supplementary table 7.4). In all these 12 studies at least one preparation was compared with non-use or two types were compared directly. Results of the network meta-analysis per combined oral contraceptive preparation are shown in table 7.3. All selected preparations were associated with a two-fold or more increased risk of venous thrombosis compared with non-use (Figure 7.2). The relative risk estimate was the highest in 50LNG users and the lowest in 20LNG and 20GSD users. There was a dose-related effect of ethinylestradiol within gestodene and levonorgestrel users, but not in desogestrel users (Table 7.3). In other words, taking either 20DSG or 30DSG as reference did not materially alter the risk estimates for other contraceptives. The risk of venous thrombosis with the use of 35CPA and 35DRSP was approximately the same as 50LNG (RR_{30CPA} 0.8, 95%CI: 0.5-1.5 and RR_{30DRSP} 0.9, 95%CI: 0.5-1.7 compared with 50LNG). A formal interaction test could not be performed because none of the studies provided data on the same groups (Supplementary table 7.3).

Sensitivity analysis Because of the low number of included studies, sensitivity analyses were limited to analyses stratified on funding source, per study design, and objectively versus subjec-

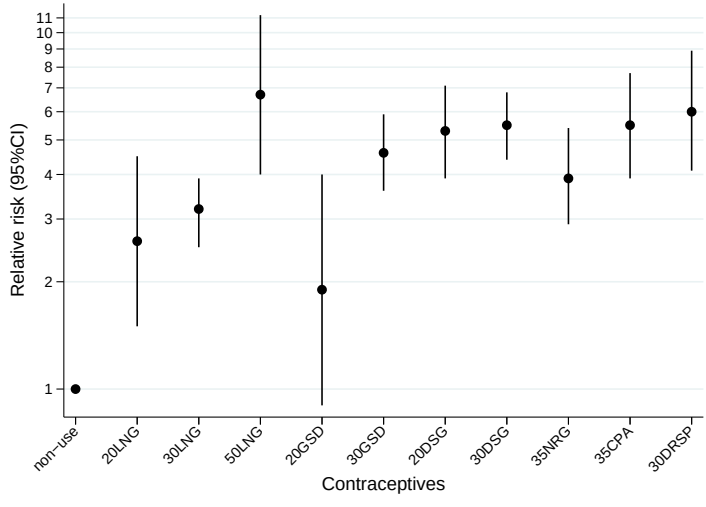

Figure 7.2: Results of the network meta-analysis per contraceptive pill on a logarithmic scale. The dots indicate the overall RR with lines representing the 95% confidence interval. Non-use was taken as the reference group

tively confirmed venous thrombosis (Table 7.4). Results from the sensitivity analysis stratified on funding source showed that the risk estimate for third generation users (compared with non-users) was lower in the industry-sponsored studies than in non-industry-sponsored studies (RR 3.3 versus RR 5.2). In cohort studies, the risk estimate for third generation users (compared with non-users) was higher than in case-control studies (RR 6.1 versus RR 4.3). All risk estimates were higher in studies with objectively confirmed venous thrombosis of which none were industry-sponsored.

Table 7.3: Overview of the results of the network meta-analysis per combined oral contraceptive pill

	Reference group Non-use RR (95%CI)	20LNG RR (95%CI)	30LNG RR (95%CI)	50LNG RR (95%CI)	20GSD RR (95%CI)	30GSD RR (95%CI)
Non-use	1					
20LNG	2.6 (1.5-4.5)	1	0.8 (0.5-1.4)	0.4 (0.2-0.8)	1.4 (0.6-3.3)	0.6 (0.3-1.0)
30LNG	3.2 (2.5-3.9)	1.2 (0.7-2.1)	1	0.5 (0.3-0.8)	1.7 (0.8-3.5)	0.7 (0.5-0.9)
50LNG	6.7 (4.0-11.2)	2.6 (1.2-5.3)	2.1 (1.2-3.6)	1	3.5 (1.5-8.4)	1.4 (0.8-2.5)
20GSD	1.9 (0.9-4.0)	0.7 (0.3-1.8)	0.6 (0.3-1.3)	0.3 (0.1-0.7)	1	0.4 (0.2-0.9)
30GSD	4.6 (3.6-5.9)	1.8 (1.0-3.1)	1.5 (1.2-1.9)	0.7 (0.4-1.2)	2.4 (1.1-5.2)	1
20DSG	5.3 (3.9-7.1)	2.0 (1.1-3.7)	1.7 (1.2-2.2)	0.8 (0.5-1.4)	2.8 (1.3-6.0)	1.1 (0.8-1.6)
30DSG	5.5 (4.4-6.8)	2.1 (1.2-3.7)	1.7 (1.4-2.1)	0.8 (0.5-1.4)	2.9 (1.4-6.1)	1.2 (0.9-1.5)
35NRG	3.9 (2.9-5.4)	1.5 (0.8-2.7)	1.2 (0.9-1.7)	0.6 (0.3-1.1)	2.1 (0.9-4.5)	0.9 (0.6-1.2)
35CPA	5.5 (3.9-7.7)	2.1 (1.2-3.9)	1.7 (1.3-2.4)	0.8 (0.5-1.5)	2.9 (1.3-6.3)	1.2 (0.8-1.7)
30DRSP	6.0 (4.1-8.9)	2.3 (1.4-3.9)	1.9 (1.4-2.7)	0.9 (0.5-1.7)	3.2 (1.4-7.1)	1.3 (0.9-2.0)

	Reference group Non-use RR (95%CI)	20DSG RR (95%CI)	30DSG RR (95%CI)	35NRG RR (95%CI)	35CPA RR (95%CI)	30DRSP RR (95%CI)
Non-use	1					
20LNG	2.6 (1.5-4.5)	0.5 (0.3-0.9)	0.5 (0.3-0.8)	0.7 (0.4-1.2)	0.5 (0.3-0.9)	0.4 (0.3-0.7)
30LNG	3.2 (2.5-3.9)	0.6 (0.5-0.8)	0.6 (0.5-0.7)	0.8 (0.6-1.1)	0.6 (0.4-0.8)	0.5 (0.4-0.7)
50LNG	6.7 (4.0-11.2)	1.3 (0.7-2.2)	1.2 (0.7-2.1)	1.7 (1.0-3.0)	1.2 (0.7-2.2)	1.1 (0.6-2.0)
20GSD	1.9 (0.9-4.0)	0.4 (0.2-0.8)	0.4 (0.2-0.7)	0.5 (0.2-1.1)	0.4 (0.2-0.8)	0.3 (0.1-0.7)
30GSD	4.6 (3.6-5.9)	0.9 (0.6-1.2)	0.8 (0.7-1.1)	1.2 (0.9-1.6)	0.8 (0.6-1.2)	0.8 (0.5-1.2)
20DSG	5.3 (3.9-7.1)	1	1.0 (0.7-1.3)	1.3 (0.9-1.9)	1.0 (0.7-1.4)	0.9 (0.6-1.4)
30DSG	5.5 (4.4-6.8)	1.0 (0.8-1.4)	1	1.4 (1.1-1.9)	1.0 (0.7-1.4)	0.9 (0.6-1.3)
35NRG	3.9 (2.9-5.4)	0.7 (0.5-1.1)	0.7 (0.5-1.0)	1	0.7 (0.5-1.1)	0.7 (0.4-1.0)
35CPA	5.5 (3.9-7.7)	1.1 (0.7-1.6)	1.0 (0.7-1.4)	1.4 (0.9-2.1)	1	0.9 (0.6-1.5)
30DRSP	6.0 (4.1-8.9)	1.1 (0.7-1.8)	1.1 (0.8-1.6)	1.5 (1.0-2.4)	1.1 (0.7-1.7)	1

Discussion

We performed a network meta-analysis based on 25 studies. Overall, combined oral contraceptive use increased the risk of venous thrombosis four-fold. We observed that all generations of progestagens were associated with an increased risk of venous thrombosis and that third generation users had an increased venous thrombosis risk compared with second generation users. All individual types of combined oral contraceptives increased thrombosis risk compared with non-use. The contraceptives 30DRSP and 35CPA increased the risk of venous thrombosis compared with any other contraceptive except for 50LNG. Users of 20LNG had the lowest risk of venous thrombosis. Whether 20GSD can be considered as safe as 20LNG remains to be determined, because only one study[16] contributed data on 32 women using 20GSD.

In this meta-analysis, bias was potentially introduced by the lack of objectively confirming venous thrombosis in all studies. Only 25-33% of patients with clinical symptoms of thrombosis are diagnosed with venous thrombosis[25]. Including cases in a study without objectively confirmed venous thrombosis would lead to overestimating the association when oral contraceptive users were more likely to be diagnosed than non-users. However, two studies showed that this bias is unlikely to depend on combined oral contraceptive use[18,19]. In studies with clinically diagnosed venous thrombosis without objective confirmation, women are misclassified irrespective of their contraceptive use leading to non-differential misclassification. Therefore, results of such studies may be an underestimation of the true association. This was confirmed by the results from our sensitivity analysis where the risk estimates were higher in studies with objectively confirmed venous thrombosis.

Strengths and limitations The internal validity of the network meta-analysis was assessed through interaction analysis modelling potential inconsistencies in the network. Our results in-

Table 7.4: Results of sensitivity analyses

	Industry N=8 RR (95%CI)	**Non-industry N=9 RR (95%CI)**	**Cohort N=7 RR (95%CI)**	**Case-control N=16 RR (95%CI)**	**Confirmed VT N=5 RR (95%CI)**	**Not confirmed VT N=10 RR (95%CI)**
Non-use	1	1	1	1	1	1
1st	3.5 (2.1-5.6)	3.3 (2.4-4.6)	3.9 (1.0-14.4)	3.5 (2.6-4.7)	4.6 (3.2-6.5)	2.8 (1.8-4.3)
2nd	2.4 (1.7-3.5)	3.1 (2.5-3.8)	3.7 (1.1-12.2)	2.8 (2.3-3.4)	3.3 (2.8-4.0)	2.6 (1.9-3.6)
3rd	3.3 (2.3-4.8)	5.2 (4.2-6.5)	6.1 (2.0-18.8)	4.3 (3.5-5.2)	6.2 (5.2-7.4)	4.3 (3.1-6.0)

dicated that potential inconsistencies are likely the results of chance. A limitation of our network meta-analysis is that the publications had to provide the number of users with number of events per type of combined oral contraceptive. A total of 16 studies provided information on combined oral contraceptive use and risk of venous thrombosis without specifying which contraceptive types were used. Therefore, the number of publications included for the meta-analysis per contraceptive is relatively low. A further limitation may be that the classification of generations of progestagen was not the same in every publication. However, no clear consensus exists on the classification into generations of progestagen. For instance, norgestimate can be categorised as a second or a third generation progestagen. However, restriction on studies where our classification could be applied did not change the results. Network meta-analysis summarizes data from direct and indirect comparisons in a weighted average. A strength of our analysis is that through a network meta-analysis the data on cimbined oral contraceptives were most effectively used. This resulted in a comprehensive overview of the risk of venous thrombosis in commonly used oral contraceptives.

Two other meta-analyses[18,50] have evaluated the risk of venous thrombosis in third generation contraceptive users versus second generation users. Both found an increased risk in third generation users (RR 1.5 95%CI: 1.2-1.8[18] and RR 1.57 95%CI: 1.24-1.98[50]) in line with our result. Although they differed in their included studies, the majority of included studies from both meta-analyses were included in our analysis. To date, no other meta-analysis summarized the data on venous thrombosis risk per combined oral contraceptive preparation.

Meaning of the study Although we observed that the risk of venous thrombosis increased with the dose of ethinylestradiol, this was dependent on the progestagen provided. There was no difference in the venous thrombosis risk between 20DSG and 30DSG, whereas oral contraceptives with other progestagens with differ-

ent doses of ethinylestradiol did show a difference in risk. It is unclear why the dose effect of ethinylestradiol might depend on the progestagen. A possibility is that there is a difference in inhibitory effects of the progestagen on the procoagulant effect of ethinylestradiol. Oral contraceptive use increases the levels of factors II, VII, VIII, protein C and decreases the levels of antithrombin and protein S. Clinical studies have showed that this effect on coagulation factors was more pronounced in desogestrel users than in levonorgestrel users, and limited to combined oral contraceptives[51,52].

The results per combined oral contraceptive preparation showed that combining different preparations into generations may not be an appropriate way to present the risk of thrombosis. The risk of venous thrombosis depended both on the dose of ethinylestradiol as well as on the progestagen provided. We suggest abstaining from any classification of contraceptives, but to compare the risk of venous thrombosis per oral contraceptive preparation.

What do these results mean for clinical practice? Prescribing combined oral contraceptives may not be a good choice with regard to the risk of venous thrombosis. However, if a woman persists in using combined oral contraceptives, only the contraceptive with the lowest risk of venous thrombosis and good compliance should be prescribed, namely levonorgestrel with 30 µg ethinylestradiol. Current practice is to increase the dose of ethinylestradiol in case of disruptions in bleeding patterns[53]. In all likelihood, our results indicate that prescribing 50LNG in case of spotting during the use of 30LNG might carry a serious adverse effect.

Conclusions Combined oral contraceptives were associated with an increased risk of venous thrombosis. The effect size may depend both on the progestagen and the dose of ethinylestradiol.

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Supplementary data

Search strategy of the review

PubMed (“Contraceptives, Oral"[mesh] OR "Contraceptives, Oral"[Pharmacological Action] OR "oral contraceptives" OR "oral contraceptive" OR "Contraceptives, Oral, Combined"[Mesh] OR "combined oral contraceptives" OR "combined oral contraceptive" OR ((norethisterone OR norethisteron* OR norethindrone OR norethindron* OR "ethynodiol diacetate" OR lynestrenol OR lynestrenol* OR norethynodrel OR norethynodrel* OR dienogest OR dienogest* OR levonorgestrel OR levonorgestrel* OR norgestrel OR norgestrel* OR dl-norgestrel OR dl-norgestrel* OR desogestrel OR desogestrel* OR norgestimate OR norgestimat* OR gestodene OR gestoden* OR "medroxyprogesterone acetate" OR "chlormadinone acetate" OR nomegestrol OR nomegestrol* OR nestorone OR nestoron* OR "Cyproterone acetate" OR Drospirenone OR Drospirenon* OR oestrogen*[ti] OR estrogen[ti]) AND ("Ethinyl Estradiol"[mesh] OR "Ethinyl Estradiol" OR ethinylestradiol OR ethinylestradiol* OR Mestranol OR Mestranol* OR "estradiol valerate"[Supplementary Concept] OR "estradiol valerate" OR progestogen*[ti]))) AND ("deep vein thrombosis"[ti] OR "deep venous thrombosis"[ti] OR "Venous Thrombosis"[ti] OR "Vein Thrombosis"[ti] OR "Venous Thrombosis"[mesh:noexp] OR "Thrombophlebitis"[mesh] OR "Upper Extremity Deep Vein Thrombosis"[mesh] OR Thrombophlebitis[ti] OR "pulmonary embolism"[ti] OR "pulmonary embolism"[mesh] OR "venous thromboembolism"[ti] OR "Venous Thromboembolism"[mesh] OR "venous thromboembolic disorders"[ti] OR (venous[ti] AND thromboembolic[ti] AND disorder[ti]) OR "venous thromboembolic diseases"[ti] OR "venous thromboembolic disease"[ti] OR "venous thrombotic"[ti] OR ("Thromboembolism"[mesh:noexp] AND (venous[tiab] OR vein[tiab] OR veins[tiab]))) AND (risk OR risks OR risk factor OR risk factors) AND (women OR woman OR woman* OR women* OR girl OR girls OR female) NOT (animals NOT (human AND animals))

EMBASE (exp oral contraceptive agent/ OR "oral contraceptives".mp OR "oral contraceptive".mp OR "combined oral contraceptives".mp OR "combined oral contraceptive".mp OR (((norethisterone OR norethisteron* OR norethindrone OR norethindron* OR "ethynodiol diacetate" OR lynestrenol OR lynestrenol* OR norethynodrel OR norethynodrel* OR dienogest OR dienogest* OR levonorgestrel OR levonorgestrel* OR norgestrel OR norgestrel* OR dl-norgestrel OR dl-norgestrel* OR desogestrel OR desogestrel* OR norgestimate OR norgestimat* OR gestodene OR gestoden* OR "medroxyprogesterone acetate" OR "chlormadinone acetate" OR nomegestrol OR nomegestrol* OR nestorone OR nestoron* OR "Cyproterone ac-

etate" OR Drospirenone OR Drospirenon*).mp OR oestrogen*.ti OR estrogen.ti) AND (("Ethinyl Estradiol" OR ethinylestradiol OR ethinylestradiol* OR Mestranol OR Mestranol* OR "estradiol valerate" OR "estradiol valerate").mp OR progestogen*.ti))) AND (("deep vein thrombosis" OR "deep venous thrombosis" OR "Venous Thrombosis" OR "Vein Thrombosis").ti OR exp deep vein thrombosis/ OR Vein Thrombosis/ OR Thrombophlebitis/ OR Thrombophlebitis.ti OR "pulmonary embolism".ti OR exp lung embolism/ OR "venous thromboembolism".ti OR exp Venous Thromboembolism/ OR "venous thromboembolic disorder*".ti OR "venous thromboembolic disease*".ti OR "venous thrombotic".ti) AND (exp risk/ OR risk*.mp OR exp risk factor/) AND ((women OR woman OR woman* OR women* OR girl OR girls OR female).mp OR exp female/) AND (exp human/ OR human.ti OR patient.ti OR patients.ti)

Web of Science TS=("oral contraceptives" OR "oral contraceptive" OR combined oral contraceptive* OR ((norethisterone OR norethisteron* OR norethindrone OR norethindron* OR "ethynodiol diacetate" OR lynestrenol OR lynestrenol* OR norethynodrel OR norethynodrel* OR dienogest OR dienogest* OR levonorgestrel OR levonorgestrel* OR norgestrel OR norgestrel* OR dl-norgestrel OR dl-norgestrel* OR desogestrel OR desogestrel* OR norgestimate OR norgestimat* OR gestodene OR gestoden* OR "medroxyprogesterone acetate" OR "chlormadinone acetate" OR nomegestrol OR nomegestrol* OR nestorone OR nestoron* OR "Cyproterone acetate" OR Drospirenone OR Drospirenon*) AND ("Ethinyl Estradiol" OR ethinylestradiol OR ethinylestradiol* OR Mestranol OR Mestranol* OR "estradiol valerate" OR "estradiol valerate"))) AND TI=("deep vein thrombosis" OR "deep venous thrombosis" OR "Venous Thrombosis" OR "Vein Thrombosis" OR "Vein Thrombosis" OR Thrombophlebitis OR "pulmonary embolism" OR "venous thromboembolism" OR "venous thromboembolic disorder*" OR "venous thromboembolic disease*" OR "venous thrombotic") AND TS=risk* AND TS=(women OR woman OR woman* OR women* OR girl OR girls OR female) Cochrane (http://www3.interscience.wiley.com/cgi-bin/mrwhome/106568753/HOME) ("oral contraceptives" OR "oral contraceptive" OR combined oral contraceptive* OR ((norethisterone OR norethisteron* OR norethindrone OR norethindron* OR "ethynodiol diacetate" OR lynestrenol OR lynestrenol* OR norethynodrel OR norethynodrel* OR dienogest OR dienogest* OR levonorgestrel OR levonorgestrel* OR norgestrel OR norgestrel* OR dl-norgestrel OR dl-norgestrel* OR desogestrel OR desogestrel* OR norgestimate OR norgestimat* OR gestodene OR gestoden* OR "medroxyprogesterone acetate" OR "chlormadinone acetate" OR nomegestrol OR nomegestrol* OR nestorone OR nestoron* OR "Cyproterone acetate" OR Drospirenone OR Drospirenon*) AND ("Ethinyl Estradiol" OR ethinylestradiol OR ethinylestradiol* OR Mestranol OR Mes-

tranol* OR "estradiol valerate" OR "estradiol valerate"))) AND ("deep vein thrombosis" OR "deep venous thrombosis" OR "Venous Thrombosis" OR "Vein Thrombosis" OR "Vein Thrombosis" OR Thrombophlebitis OR "pulmonary embolism" OR "venous thromboembolism" OR "venous thromboembolic disorder*" OR "venous thromboembolic disease*" OR "venous thrombotic") AND risk* AND (women OR woman OR woman* OR women* OR girl OR girls OR female)

CINAHL TITLE/ABSTRACT/KEYWORD (oral contraceptives OR oral contraceptive OR combined oral contraceptive* OR ((norethisterone OR norethisteron* OR norethindrone OR norethindron* OR ethynodiol diacetate OR lynestrenol OR lynestrenol* OR norethynodrel OR norethynodrel* OR dienogest OR dienogest* OR levonorgestrel OR levonorgestrel* OR norgestrel OR norgestrel* OR dl-norgestrel OR dl-norgestrel* OR desogestrel OR desogestrel* OR norgestimate OR norgestimat* OR gestodene OR gestoden* OR medroxyprogesterone acetate OR chlormadinone acetate OR nomegestrol OR nomegestrol* OR nestorone OR nestoron* OR Cyproterone acetate OR Drospirenone OR Drospirenon*) AND (Ethinyl Estradiol OR ethinylestradiol OR ethinylestradiol* OR Mestranol OR Mestranol* OR estradiol valerate OR estradiol valerate))) AND (deep vein thrombosis OR deep venous thrombosis OR Venous Thrombosis OR Vein Thrombosis OR Vein Thrombosis OR Thrombophlebitis OR pulmonary embolism OR venous thromboembolism OR venous thromboembolic disorder* OR venous thromboembolic disease* OR venous thrombotic) AND risk* AND (women OR woman OR woman* OR women* OR girl OR girls OR female)

Academic Search Premier (oral contraceptives OR oral contraceptive OR combined oral contraceptive* OR ((norethisterone OR norethisteron* OR norethindrone OR norethindron* OR ethynodiol diacetate OR lynestrenol OR lynestrenol* OR norethynodrel OR norethynodrel* OR dienogest OR dienogest* OR levonorgestrel OR levonorgestrel* OR norgestrel OR norgestrel* OR dl-norgestrel OR dl-norgestrel* OR desogestrel OR desogestrel* OR norgestimate OR norgestimat* OR gestodene OR gestoden* OR medroxyprogesterone acetate OR chlormadinone acetate OR nomegestrol OR nomegestrol* OR nestorone OR nestoron* OR Cyproterone acetate OR Drospirenone OR Drospirenon*) AND (Ethinyl Estradiol OR ethinylestradiol OR ethinylestradiol* OR Mestranol OR Mestranol* OR estradiol valerate OR estradiol valerate))) AND (deep vein thrombosis OR deep venous thrombosis OR Venous Thrombosis OR Vein Thrombosis OR Vein Thrombosis OR Thrombophlebitis OR pulmonary embolism OR venous thromboembolism OR venous thromboembolic disorder* OR venous thromboembolic disease* OR venous thrombotic) AND risk* AND (women OR woman OR woman* OR women* OR girl OR girls OR female)

ScienceDirect TITLE((oral contraceptives OR oral contraceptive OR combined oral contraceptive* OR ((norethisterone OR norethisteron* OR norethindrone OR norethindron* OR ethynodiol diacetate OR lynestrenol OR lynestrenol* OR norethynodrel OR norethynodrel* OR dienogest OR dienogest* OR levonorgestrel OR levonorgestrel* OR norgestrel OR norgestrel* OR dl-norgestrel OR dl-norgestrel* OR desogestrel OR desogestrel* OR norgestimate OR norgestimat* OR gestodene OR gestoden* OR medroxyprogesterone acetate OR chlormadinone acetate OR nomegestrol OR nomegestrol* OR nestorone OR nestoron* OR Cyproterone acetate OR Drospirenone OR Drospirenon*) AND (Ethinyl Estradiol OR ethinylestradiol OR ethinylestradiol* OR Mestranol OR Mestranol* OR estradiol valerate OR estradiol valerate))) AND (deep vein thrombosis OR deep venous thrombosis OR Venous Thrombosis OR Vein Thrombosis OR Vein Thrombosis OR Thrombophlebitis OR pulmonary embolism OR venous thromboembolism OR venous thromboembolic disorder* OR venous thromboembolic disease* OR venous thrombotic) AND risk* AND (women OR woman OR woman* OR women* OR girl OR girls OR female))

Criteria for considering studies for this review

Types of studies Observational studies in this review will include case-control, cohort and nested case-control designs. If available, RCT will also be evaluated and included.

Types of participants Participants will be healthy women taking combined oral contraceptives. We will exclude studies of women on hormone replacement therapy, studies of women taking non-oral or progestagen-only contraceptives and studies of women with recurrent venous thrombosis.

Types of interventions Combined oral contraceptive use will be compared to non-use or to a reference combined oral contraceptive (for example, levonorgestrel with 30 µg of ethinylestradiol). We define a woman as a non-user when either she has never been exposed to a combined oral contraceptive or she was a former/previous combined oral contraceptive user. We will categorize the combined oral contraceptive type according to the estrogen type and dose and to the progestagen type.

Types of outcome measures The outcome will be fatal or non-fatal first venous thrombosis event (deep vein thrombosis or pulmonary embolism).

Supplementary table 7.1 Characteristics of excluded studies

Author	Year	Reason for exclusion
Ø. Lidegaard	2011	Included cerebral vein thrombosis
M.K. Barsoum	2010	No data on progestagen type or ethinylestradiol dose
J.C. Dinger	2010	Included recurrent venous thrombosis
V. Tsankova	2010	Compared ever users versus never users
V. Tsankova	2010	No data on progestagen type or ethinylestradiol dose
H. Austin	2009	Other hormonal contraceptives, such as transdermal patch, vaginal ring, were included
P.G. Lindqvist	2009	No data on progestagen type or ethinylestradiol dose
P.M. Eng	2008	Compared drospirenone versus other oral contraceptive users
J.C. Dinger	2007	Included recurrent venous thrombosis
C. Huerta	2007	No data on progestagen type or ethinylestradiol dose
J.D. Seeger	2007	Included recurrent venous thrombosis
C.C. Yang	2007	Exposed consisted of hormone replacement therapy users and oral contraceptive users
K. Hedenmalm	2005	Included recurrent venous thrombosis and cerebral vein thrombosis
H.M. Pearce	2005	No comparison was included
M. Primignani	2005	Included not only venous thrombosis
C. Worralurt	2005	Included recurrent venous thrombosis and no data on progestagen type or ethinylestradiol dose
A. Girolami	2004	Included not only venous thrombosis
P. Heuser	2004	No extractable number of exposed and non-exposed women
H.E. Seaman	2004	Included recurrent venous thrombosis
S. Sidney	2004	Incomplete data on contraceptive use
H. Kieler	2003	Included recurrent venous thrombosis
I. Martinelli	2003	No data on progestagen type or ethinylestradiol dose
A. Tosetto	2003	No data on progestagen type or ethinylestradiol dose
C. Legnani	2002	Included recurrent venous thrombosis
Ø. Lidegaard	2001	Review
L. N. Meurer	2001	Review

Continued on next page

Supplementary table 7.1 – continued from previous page

Author	Year	Reason for exclusion
J.P. Vallée	2001	Review
T. Amundsen	2000	No data on progestagen type or ethinylestradiol dose
R.D. Farmer	2000	No data on progestagen type or ethinylestradiol dose
L.A. Heinemann	2000	Report on Transnational study, already included
R. Lawrenson	2000	Review
A.L. Nightingale	2000	Commentary
M.S. Burnhill	1999	Included progestagen-only contraceptives and retinal vein thrombosis
R.M. Herings	1999	Data already included
M.A. Lewis	1999	Report on Transnational study, already included
Ø. Lidegaard	1998	Review
C. Bonifacj	1997	Included recurrent venous thrombosis
R.D. Farmer	1997	Ecologic study
M.A. Lewis	1997	Report on Transnational study, already included
F.J. van der Meer	1997	Review
J.P. Realini	1997	Less than 10 venous thrombosis events
S. Suissa	1997	Duration of contraceptive use
H. Ulmer	1997	No data on progestagen type or ethinylestradiol dose
F. Grodstein	1996	No data on progestagen type or ethinylestradiol dose
M. Pini	1996	Included not only venous thrombosis and included recurrent venous thrombosis
N.R. Poulter	1996	Data already included
Y. Lis	1993	Publication of study protocol
W.O. Spitzer	1993	Publication of study protocol
D.A. Quinn	1992	No data on progestagen type or ethinylestradiol dose
M. Thorogood	1992	Included recurrent venous thrombosis and no data on progestagen type or ethinylestradiol dose
B.B. Gerstman	1991	Incomplete data on contraceptive use
E. Hirvonen	1990	No data on progestagen type or ethinylestradiol dose
WHO	1989	No data on progestagen type or ethinylestradiol dose
H. Meinel	1988	Included not only venous thrombosis and no data on progestagen type or ethinylestradiol dose
S.P. Helmrich	1987	Incomplete data on contraceptive use

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Supplementary table 7.1 – continued from previous page

Author	Year	Reason for exclusion
D. Bernstein	1986	No data on progestagen type or ethinylestradiol dose
R. Lambrekht	1986	No data on venous thrombosis
K. Overgaard	1986	No data on progestagen type or ethinylestradiol dose
M.P. Vessey	1986	Incomplete data on contraceptive use
J.B. Porter	1985	Less than 10 venous thrombosis events
J.B. Porter	1982	Less than 10 venous thrombosis events
L.E. Bottiger	1980	No data on ethinylestradiol dose
T.W. Meade	1980	Included not only venous thrombosis
D.B. Pettiti	1979	No data on progestagen type or ethinylestradiol dose
A.W. Diddle	1978	Less than 10 venous thrombosis cases
RCGP	1978	Included not only venous thrombosis
IPPF	1976	Communication to the editor
P.D. Stolley	1975	Included not only venous thrombosis
M. Grounds	1974	Included not only venous thrombosis
BCDS	1973	No data on progestagen type or ethinylestradiol dose
W.H. Inman	1970	No data on progestagen type or ethinylestradiol dose
H. Ludwig	1970	Unclear what is defined as high progestagen
H.A. Siegel	1969	No data on progestagen type or ethinylestradiol dose
M.P. Vessey	1969	Included recurrent venous thrombosis
W.H. Inman	1968	No data on progestagen type or ethinylestradiol dose

Supplementary table 7.2 Overview of risk of bias per study

Study	Source population*	Exposure assessment	Outcome assessment	Follow-up
N. Gronich 2011	NA	Low risk	Unclear	Unclear
S.S. Jick 2011	Low risk	Low risk	Unclear	NA
L. Parkin 2011	Low risk	Low risk	High risk	NA
L.A.J. Heinemann 2010	Low risk	High risk	High risk	NA
A. van Hylckama Vlieg 2009	Low risk	High risk	Low risk	NA
S.S. Jick 2006	Low risk	Low risk	Unclear	NA
E. Samuelsson 2004	NA	Low risk	Low risk	Unclear
K. Hedenmalm 2004	NA	Low risk	High risk	Unclear
L.A.J. Heinemann 2002	High risk	High risk	High risk	NA
L. Parkin 2000	Low risk	High risk	High risk	NA
R.D.T. Farmer 2000	NA	Low risk	High risk	Unclear
M.A. Lewis 1999	High risk	Low risk	Unclear	NA
R.M.C. Herings 1999	NA	Low risk	Unclear	Unclear
J.C. Todd 1999	NA	Low risk	High risk	Unclear
I. Martinelli 1999	Low risk	Unclear	Unclear	NA
K.W.M. Bloemenkamp 1999	Low risk	High risk	Low risk	NA
Ø. Lidegaard 1998	Low risk	High risk	High risk	NA
R.D.T. Farmer 1998	Low risk	Low risk	Unclear	NA
B.S. Andersen 1998	Low risk	Low risk	Low risk	NA
M.A. Lewis 1996	High risk	High risk	Unclear	NA
R. Farmer 1996	NA	Low risk	Unclear	Unclear
K.W.M. Bloemenkamp 1995	Low risk	Low risk	Low risk	NA
WHO 1995	High risk	Low risk	High risk	NA
WHO1 1995	High risk	Low risk	High risk	NA
WHO2 1995	High risk	Low risk	High risk	NA

NA, not applicable due to cohort design in case of source population or due to case-control design in case of follow-up

* Case-control studies: population of controls, hospitalized or community-based.

Supplementary table 7.3 Included publications with data on generation of progestagens

Design	Study	Non-use*	1st*	2nd*	3rd*
1	Hylckama 2009	421/1523	55/81	382/672	412/582
	Heinemann 2002	246/2115	45/190	131/865	28/195
	Lewis 1999	171/1268	38/97	142/562	137/401
	Bloemenkamp 1999	83/511	18/46	8/22	33/67
	Bloemenkamp 1995	46/150	8/13	20/38	37/52
	WHO1 1995	168/855	29/74	156/392	53/104
	WHO2 1995	505/2220	26/65	153/337	18/25
2	Heinemann 2010	70/1215	-	61/245	62/238
	Parkin 2000	9/95	-	3/11	12/27
	Lidegaard 1998	203/1037	-	31/85	120/244
	WHO 1995	397/1916	-	137/340	71/127
3	Samuelsson 2004	32/171206	-	-	17/14819
	Martinelli 1999	41/179	-	-	43/79
	Andersen 1998	27/133	-	-	16/23
4	Hedenmalm 2004	-	36/1898899	74/6343562	83/1739393
	Farmer 2000	-	12/39421	98/307070	161/374129
5	Gronich 2011	-	-	23/33187	384/651455
	Jick 2006	-	-	70/386	211/950
	Herrings 1999	-	-	29/121411	49/88295
	Todd 1999	-	-	32/76993	53/92052
	Farmer 1998	-	-	27/116	15/79
	Lewis 1996	-	-	96/419	156/451
	Farmer 1996	-	-	14/76600	15/65100

1st, first generation; 2nd, second generation; 3rd, third generation

* Number of cases/ total number of women in the group or total follow-up time

Supplementary table 7.4 Included publications with specific combined oral contraceptive pills

Design	Study	Non-use*	20LNG*	30LNG*	50LNG*	20GSD*	30GSD*
1	Hylckama 2009	421/1523	8/14	485/858	60/80	14/32	119/186
2	Parkin 2000	9/95	-	2/6	0/2	-	5/10
3	Lidegaard1998	203/1037	-	-	4/8	-	69/146
4	Bloemenkamp 1999	83/511	-	18/46	-	-	5/9
5	Bloemenkamp 1995	46/150	-	20/38	-	-	-
6	Farmer 2000	-	-	64/190191	-	-	63/143581
7	Todd 1999	-	-	22/49484	-	-	21/41947
8	Farmer 1996	-	-	5/35800	-	-	5/30500
9	Jick 2006	-	-	70/386	-	-	-
10	Jick 2011	-	20/151	45/282	-	-	-
11	Parkin 2011	-	-	44/233	-	-	-
12	Lewis 1996	-	-	-	-	-	-

Design	Study	20DSG*	30DSG*	35NRG*	35CPA*	30DRSP*
1	Hylckama 2009	58/85	289/397	9/13	125/187	19/33
2	Parkin 2000	4/9	3/8	-	2/3	-
3	Lidegaard1998	24/46	24/43	3/9	-	-
4	Bloemenkamp 1999	6/7	22/51	-	-	-
5	Bloemenkamp 1995	-	37/52	-	-	-
6	Farmer 2000	18/37584	65/152524	15/40440	16/25709	-
7	Todd 1999	9/10426	23/39679	-	-	-
8	Farmer 1996	-	10/34600	-	-	-
9	Jick 2006	-	87/315	124/635	-	-
10	Jick 2011	-	-	-	-	121/434
11	Parkin 2011	-	-	-	-	17/43
12	Lewis 1996	15/51	64/174	19/50	-	-

* Number of cases/ total number of women in the group or total follow-up time

General discussion

Chapter 8

Discussion and recommendations

This thesis focussed on the role of hormonal contraceptives in the pathogenesis of venous thrombosis and its association with a first and recurrent venous thrombosis. We set out to research the mechanisms behind combined oral contraceptive-associated venous thrombosis, to assess the association between hormonal contraceptive use and recurrent venous thrombosis and to provide an overview of the risk of a first venous thrombosis per type of combined oral contraceptive. In this section, the research questions of the thesis and its implications will be discussed.

Mechanism behind combined oral contraceptive-associated venous thrombosis

Sex hormone binding globulin (SHBG) levels are said to be a marker of venous thrombosis in combined oral contraceptive users reflecting the difference in risk between different contraceptives[1,2]. For instance, SHBG levels are higher in users of contraceptives with a third generation progestagenn (desogestrel, gestodene and norgestimate) than in users of a second generation (levonorgestrel), reflecting the difference in venous thrombosis risk. Although the association between SHBG levels and contraceptive use has been established, the question remained whether SHBG level is an intermediate, i.e., meaning both a marker and a cause, or only a marker for venous thrombosis risk. In chapter 2, we used a Mendelian randomization approach to show that SHBG level was only a marker and not a cause of venous thrombosis. A disadvantage of this analysis is the requirement that the changes in protein levels caused by genetic variation, which may be minor, are also causative of the disease of interest. However, we observed changes in SHBG levels caused by genetic variation in the SHBG gene in the same magnitude as changes caused by oral contraceptive use, rendering this approach feasible. We were able to include only 23 women with venous thrombosis, and over 200 women without venous thrombosis. Despite this

low number of patients, our three-pronged approach consistently showed that increased SHBG levels were not causally related with venous thrombosis.

The association between SHBG levels and different progestagens in combined oral contraceptives has been well established [1,2], but the association with the ethinylestradiol dose remains to be determined. The ethinylestradiol dose in combined oral contraceptives is positively associated with the risk of venous thrombosis [3,4]. For SHBG levels to be considered as a useful marker of venous thrombosis in combined oral contraceptive users, the ethinylestradiol dose should also be reflected in SHBG levels. In chapter 3, we were able to show that with increasing ethinylestradiol dose, SHBG levels increased as well. Although we were not able to restrict our analysis to one specific progestagen, we did adjust for the progestagen. Furthermore, the observed SHBG levels were similar as previously reported.

The relevance of the first-pass metabolism of ethinylestradiol for the risk of venous thrombosis was assessed through haplotypes in genes coding for enzymes involved in this metabolism and their effect on SHBG levels. We found that carrying two copies of haplotype D in the gene *UGT2B7* was associated with an increased risk of venous thrombosis, described in chapter 4. We only assessed genetic variation in this metabolism through common variation (SNPs with a minor allele frequency of more than 5%). However, these common SNPs could code for rare haplotypes (frequency below 5%). The first-pass metabolism may not be as pivotal in the pathogenesis of venous thrombosis as previously thought. Other contraceptives were developed to bypass the first-pass metabolism of ethinylestradiol, namely the transdermal patch and the vaginal ring. Users of these contraceptives, however, still have an increased risk of venous thrombosis [5]. Therefore, although we show that genetic variation in the first pass metabolism may explain part of the pathogenesis of combined oral contraceptive-associated venous thrombosis, currently we cannot fully explain how combined oral contraceptives cause

venous thrombosis.

Hormonal contraceptive use after a first event

Guidelines[6–8] recommend that women should refrain from using combined preparations after a first event of venous thrombosis. Progestagen-only preparations could be used instead. Are these guidelines followed in daily practice? We found that about 25% of women continued using hormonal contraception after a venous thrombotic event, either by continuing their oral contraceptive or by switching. This includes women changing to a progestagen-only contraceptive which is according to the guidelines. However, we found that 21% continued or changed to another combined oral contraceptive. Women without a positive family history of venous thrombosis, or women with thrombosis following surgery or a plaster cast were more likely to continue or switch contraceptives. Because venous thrombosis is a multicausal disease, it is difficult to indicate one factor that solely causes venous thrombosis. From our data it is not possible to know exactly why decisions to continue or stop were made. However, 12% of women who received advice from a physician to stop their contraceptive, still continued or changed to another combined oral contraceptive.

Current guidelines are from 2009 and do not include a study from 2010 showing that continuing or restarting hormonal contraceptives after a first event increases the risk of recurrence[9]. In our study, we found 21 recurrences while women were using hormonal contraceptives. In line with this previous publication, we observed a three-fold increased risk of a recurrent venous thrombosis while using hormonal contraceptives. Whether or not the first event occurred during use of hormonal contraceptives did not affect the risk of recurrence. Regarding different preparations, we found that combined oral contraceptive use increased the risk of a recurrence about three-fold. The risk of a recurrence was highest (about eight times increased) in users

of triphasic contraceptives. Furthermore, results indicated that a levonorgestrel-releasing IUD could be safely used after a first event.

Overview per type of contraceptive

The literature was searched for studies that assessed the risk of a first venous thrombosis per type of combined oral contraceptive. Through a network meta-analysis, we were able to combine direct and indirect evidence in a weighted average. All hormonal contraceptives increased the risk of venous thrombosis. The dose effect of ethinylestradiol depended on the progestagen. We observed no difference in risk between 20 µg ethinylestradiol with desogestrel or 30 µg ethinylestradiol with desogestrel users. Furthermore, users of 20 µg ethinylestradiol with gestodene seemed to have the same risk of venous thrombosis as 20 µg ethinylestradiol with levonorgestrel. Although only one study had data on 20 µg ethinylestradiol with gestodene users, it is worth investigating this observation further. Previous reports have already suggested the risk of venous thrombosis for gestodene users is not equal to desogestrel but somewhere between levonorgestrel and desogestrel use, for an equal dose of ethinylestradiol[3]. The effects of these compounds on SHBG levels point in the same direction[1,2].

Conclusion

The research outlined in this thesis has contributed to our understanding of the pathogenesis of venous thrombosis in combined oral contraceptive users. SHBG levels are only marker for venous thrombosis and not a cause and are affected not only by the type of progestagen but also by the ethinylestradiol dose in combined oral contraceptive. Genetic variation in the first-pass metabolism of ethinylestradiol was associated with venous thrombosis in combined oral contraceptive users. A substantial

proportion of women disregard the advice of a physician to stop their contraceptive use after a venous thrombosis, while continuing hormonal contraceptive use, in particular combined oral contraceptives, after a first venous thrombotic event increased the risk of a recurrence. A levonorgestrel-releasing intra-uterine device may be a safe option after a venous thrombotic event.

Recommendations

Clinical recommendations

- Regardless of exposure to risk factors for venous thrombosis, women should stop using combined hormonal contraceptives after a venous thrombosis.
- After a venous thrombosis, the use of a levonorgestrel-releasing IUD is likely to be a safe option.
- In women without a history of venous thrombosis, switching to a contraceptive with a higher ethinylestradiol dose, for control of breaktrhough bleeding, is not rational and likely to be detrimental.

Scientific recommendations

- When evaluating the risk of venous thrombosis, refrain from any classification of contraceptives.
- Regarding the mechanism behind combined oral contraceptive-associated venous thrombosis, variation in protein levels, caused by factors other than genetic, or variation in ethinylestradiol levels may be pivotal in the pathogenesis of combined oral contraceptive-associated venous thrombosis.
- A proportion of women disregards the advice of a physician to stop their contraceptive use after a venous thrombosis. It would useful to know why they disregard this advice.
- Triphasic contraceptives increase the risk of recurrence in hormonal contraceptives more than monophasic preparations containing the same progestagen. The mechanism behind this is unknown.

- Literature on progestagen-only contraceptives is sparse, because only a small group of women are using these contraceptives. Therefore, large studies are needed to evaluate the effect of these contraceptives, both for a first event and a second event of venous thrombosis

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Summary

Combined oral contraceptive use is associated with an increased risk of venous thrombosis which has consistently been shown in several observational studies. Over time, many different combined oral contraceptives, i.e., different doses of estrogen (ethinylestradiol) and different types of progestagen, were developed to reduce the risk of venous thrombosis and other side effects. Use of combined oral contraceptives affects the levels of several coagulation factors and thereby shifts the balance in coagulation towards a prothrombotic state. However, the mechanism behind these changes remains unclear. In **chapter 1**, the available literature about steroid hormones use for different applications was evaluated. The use of orally administered synthetic sex steroid hormones (combined therapy either for contraceptive use or for hormone replacement therapy) was associated with an increased risk of venous thrombosis. It remains unclear whether estrogens, progestagens, or both were involved in the pathogenesis of venous thrombosis. Because combined oral contraceptives are orally administered, it has been reasoned that the first-pass metabolism in the liver may play a role. The association between ethinylestradiol dose and the risk of venous thrombosis in contraceptives with the same progestagen indicated that ethinylestradiol may be pivotal in the pathogenesis.

The aim of the research presented in this thesis was to evaluate the mechanism behind combined oral contraceptive-associated venous thrombosis, to identify the clinical implications of hormonal contraceptive use after a venous thrombotic event and to provide an overview of the risk of venous thrombosis per combined oral contraceptive.

Biochemical aspects

The European Medicines Agency recommends to measure sex hormone binding globulin (SHBG) levels to evaluate the risk of venous thrombosis in newly developed contraceptives. SHBG levels are positively associated with venous thrombosis risk and

are said to reflect the estrogenicity of a contraceptive. The effect on SHBG levels of combined oral contraceptives is seen as a marker for venous thrombosis risk, but whether these levels are also a cause of venous thrombosis remained to be determined. We determined whether SHBG levels above normal were a risk factor for venous thrombosis or that it was merely a marker of venous thrombosis risk in women using hormonal contraceptives. In **chapter 2**, we used a mendelian randomization analysis to show that SHBG levels above normal in women not using hormonal contraceptives are not a cause of venous thrombosis. We showed that SHBG levels above normal were associated with an increased risk of venous thrombosis of about 2-fold, adjusted for age and BMI. However, residual confounding could have influenced these results. Therefore, we determined six SNPs in the SHBG gene of which two were found to affect SHBG levels. These SNPs were not associated with the risk of venous thrombosis. We concluded that SHBG levels were not causally related with the risk of venous thrombosis in women not using hormonal contraceptives. Although an effect of progestagens on SHBG levels has been shown in many studies, the effect of the dose of ethinylestradiol remained to be determined. In **chapter 3**, we showed that the dose of ethinylestradiol in combined oral contraceptives was positively associated with the SHBG levels. This was not affected by the type of progestagen in the contraceptives. We studied the association between genetic variation in the first-pass metabolism of ethinylestradiol, SHBG levels and the risk of venous thrombosis in premenopausal women in **chapter 4**. Genetic variation was evaluated through haplotypes in selected genes. Several criteria were devised to ensure that the observed effect was through a change in the first-pass metabolism of ethinylestradiol. First, the same association between haplotype and venous thrombosis had to be observed in two case-control studies, namely the LETS and MEGA study. Second, no association between this haplotype and venous thrombosis in non-users should be observed in either study. Third, the direction of the associa-

tion with venous thrombosis should be reflected in SHBG levels in combined oral contraceptive users. Carriers of two copies of haplotype D in the *UGT2B7* gene had a threefold increased risk of venous thrombosis, increased SHBG levels in combined oral contraceptive users and no association was observed in non-users, i.e. all three criteria were fulfilled. Genetic variation in the *UGT2B7* gene may at least in part explain the risk of venous thrombosis in combined oral contraceptive users.

Clinical aspects

National and international guidelines state that women should refrain from using combined hormonal contraceptives (i.e., oral contraceptives, transdermal patches or vaginal rings) after a venous thrombosis. Progestagen-only contraceptives (i.e., implants, intra-uterine devices, injectables or progestagen-only pills) may be used instead. In **chapter 5**, the proportion of women who stop, change or continue their hormonal contraceptive after they experienced a hormonal contraceptive-associated venous thrombosis was evaluated. Although the majority of women stop using a hormonal contraceptive after a thrombotic event, about 20% continued to use combined oral contraceptives or switched to a different combined oral contraceptive. Women were more likely to continue their contraceptive use when they were without a positive family history of venous thrombosis, or if they were exposed to a surgery or a plaster cast within three months prior to the initial venous thrombotic event. However, exposure to risk factors for venous thrombosis has not been taken into account in the guidelines. The majority of women (82%) were advised to stop their hormonal contraceptive. 12% of women who received advice either continued or switched to another combined hormonal contraceptive. It posed a real concern that some women seemed to disregard the advice from a physician, emphasized by the potential increased risk of a recurrence when they continue using hormonal contraceptives. The next step was to evaluate

the risk of a recurrent event when hormonal contraceptives are used after a venous thrombosis, described in **chapter 6**. The recurrence rate was similar in women who used hormonal contraceptive at the first event versus those who did not. The use of hormonal contraceptives, mainly combined oral contraceptives, during follow-up, however, increased the recurrence rate about three-fold. Combined oral contraceptive use increased the risk for a recurrence about three-fold compared with non-use. We also found that among 20 intra-uterine device users with a total follow-up of 70 woman-years, none experienced a recurrent event. These results suggest that the use of a levonorgestrel-releasing intra-uterine device may be a safe method for contraception after a venous thrombosis. Among combined oral contraceptive users, the increase in risk depended on the ethinylestradiol dose, type of progestagen and whether the contraceptive was triphasic or not.

To distinguish the risk of venous thrombosis between different contraceptives, combined oral contraceptives are mostly divided into different categories according to either the dose of ethinylestradiol (i.e., 20, 30 or ≥35 µg) or into different generations of progestagens (i.e., first (norethisterone, lynestrenol), second (levonorgestrel, norgestrel) and third generation (gestodene, desogestrel, norgestimate)), as well as 'other' (cyproterone acetate and drospirenone, of which the latter sometimes is erroneously referred to as 'fourth generation'). To evaluate the risk of venous thrombosis in a newly developed combined oral contraceptive, it is mostly compared with non-use or with the most commonly prescribed contraceptive, namely levonorgestrel with 30 µg ethinylestradiol. To assess the risk of venous thrombosis per combined oral contraceptive, a network meta-analysis was performed, described in **chapter 7**. All ten combined oral contraceptives commonly used and included in the analysis increased the risk of venous thrombosis. The highest risk of venous thrombosis was found among users of drospirenone with 30 µg ethinylestradiol, cyproterone acetate with 35 µg ethinyles-

tradiol and levonorgestrel with 50 µg ethinylestradiol. Users of levonorgestrel with 20 µg ethinylestradiol had the lowest risk of venous thrombosis among combined oral contraceptive users.

Samenvatting

In de laatste vijf decennia is de associatie tussen combinatiepreparaten (progestagenen en oestrogenen) van orale anticonceptiva en veneuze trombose langzaam maar zeker vastgesteld. In de loop van deze tijd zijn er aanpassingen aan die combinatie geweest om zowel het risico op veneuze trombose als op andere bijwerkingen te verlagen. Hiertoe werd de oestrogeendosis (ethinyloestradiol) verlaagd en werden er nieuwe typen progestagenen ontwikkeld. Hormonaal anticonceptivumgebruik beënvloedt de spiegels van verschillende stollingsfactoren waardoor de stollingsbalans naar een protrombotische staat verschuift. Hoe deze veranderingen tot stand komen, is grotendeels onbekend. In **hoofdstuk 1** is de literatuur over het gebruik van exogene geslachtshormonen door vrouwen voor verschillende toepassingen en het risico op veneuze trombose geëvalueerd. Daaruit bleek dat zowel het gebruik van orale anticonceptiva als oraal hormoongebruik ter verlichting van menopausale symptomen het risico op veneuze trombose verhoogde. Tot op heden is het onduidelijk of de oestrogene component, de progestagene component of de combinatie van beide essentieel is bij het ontstaan van een trombose. Echter, de eerder beschreven associatie tussen de dosis van ethinyloestradiol in combinatiepreparaten en het risico op veneuze trombose bevestigt dat ethinyloestradiol een belangrijke rol speelt bij het ontstaan van een trombose. In het geval van oraal gebruik is het mogelijk dat, naast andere biologische processen, het afbraakmechanisme in de lever een rol speelt. Het doel van het onderzoek gepresenteerd in dit proefschrift was

1. het mechanisme van veneuze trombose in vrouwen die gecombineerde orale anticonceptiva gebruiken te evalueren,

2. de klinische implicatie van anticonceptivumgebruik na een eerste trombose te beoordelen en

3. een overzicht van het risico op veneuze trombose per gecombineerd orale anticonceptivum te geven.

Biochemische aspecten

Ter evaluatie van nieuw ontwikkelde hormonale anticonceptiva adviseert het Europees Geneesmiddelen Agentschap (EMA) sinds 2005 om 'sex hormoon binding globuline' (SHBG) te meten. SHBG is positief geassocieerd met veneuze trombose. Daarnaast zijn SHBG spiegels mogelijk een weergave van de totale oestrogeniciteit van een hormonaal anticonceptivum. Het effect van gecombineerde orale anticonceptiva op SHBG wordt gezien als een marker van het risico op veneuze trombose. Of dit effect op SHBG ook zelf veneuze trombose kan veroorzaken, is nog onbekend. De vraag is of SHBG een risicofactor is voor veneuze trombose of alleen een marker van een verhoogde kans op veneuze trombose in vrouwen die orale anticonceptiva gebruiken. In **hoofdstuk 2** is de associatie tussen SHBG en het risico op veneuze trombose nader bestudeerd in vrouwen die geen hormonaal anticonceptivum gebruikten. Verhoogd SHBG (>70 nmol/L) bleek met een tweemaal verhoogd risico op veneuze trombose geassocieerd te zijn. Echter, andere risicofactoren, naast leeftijd en BMI, hebben mogelijk dit resultaat beïnvloed. Daartoe hebben wij een Mendeliaanse randomisatie-analyse gebruikt. Hierbij is de associatie tussen genetische varianten ('single nucleotide polymorphism', SNPs), die geassocieerd zijn met SHBG, en het risico op veneuze trombose bestudeerd. Er zijn zes SNPs in het *SHBG* gen bepaald, waarvan twee SHBG spiegels beïnvloedden. Deze twee SNPs bleken niet geassocieerd te zijn met het risico op veneuze trombose. Verhoogd SHBG is derhalve niet causaal gerelateerd aan het risico op veneuze trombose in vrouwen die geen orale anticonceptiva gebruiken. Het effect van progestagenen op SHBG spiegels is in verscheidene studies aangetoond. Daarentegen is het effect van de dosis van ethinyloestradiol op SHBG spiegels nog onbekend. In **hoofdstuk 3** wordt duidelijk dat de dosis van ethinyloestradiol in gecombineerde orale anticonceptiva positief geassocieerd is met SHBG spiegels. Dit resultaat was onafhankelijk van het type progestageen in

gecombineerde orale anticonceptiva. In **hoofdstuk 4** is de associatie tussen genetische variatie in het afbraakmechanisme van ethinyloestradiol, SHBG en veneuze trombose in premenopausale vrouwen bestudeerd. Ter beoordeling van genetische variatie in geselecteerde genen zijn haplotypes gebruikt. Voorafgaande aan de analyses, werden drie criteria vastgesteld om te bepalen of een haplotype geassocieerd was met het risico op veneuze trombose door middel van veranderingen in het afbraakmechanisme van ethinyloestradiol. Als eerste moest in twee patiënt-controle studies, de LETS en MEGA studie, dezelfde associatie tussen haplotype en veneuze trombose worden waargenomen. Als tweede mocht er geen associatie tussen haplotype en veneuze trombose in niet-gebruiksters van anticonceptiva worden waargenomen. Als derde moest in gebruiksters van gecombineerde orale anticonceptiva de associatie tussen haplotype en veneuze trombose worden gereflecteerd in de associatie tussen haplotype en SHBG. In totaal zijn er 74 haplotype bepalende SNPs gemeten in de volgende 11 kandidaatgenen *COMT*, *CYP1A2*, *CYP2C9*, *CYP3A4*, *CYP3A5*, *SULT1A1*, *SULT1E1*, *UGT1A1*, *UGT1A3*, *UGT1A9* en *UGT2B7*. Enkel haplotype D in het *UGT2B7* gen voldeed aan alle drie vooraf gestelde criteria. Draagsters van twee kopieën van haplotype D in het *UGT2B7* gen hadden een driemaal verhoogd risico op veneuze trombose én een verhoogd SHBG spiegel. Er werd geen associatie tussen haplotype D en veneuze trombose in niet-gebruiksters van anticonceptiva gevonden. Genetische variatie in het *UGT2B7* gen kan in ieder geval een deel van het risico op veneuze trombose in gecombineerde orale anticonceptiva gebruiksters verklaren.

Klinische aspecten

In nationale en internationale richtlijnen wordt geadviseerd dat vrouwen na een veneuze trombose geen gecombineerde hormonale anticonceptiva (anticonceptiepil, pleister of vaginale ring) gebruiken. Daarentegen kunnen progestageen-alleen anticonceptiva

(implantaat, spiraal, injectie of pil) gebruikt worden. Tot dusver is het onduidelijk in hoeverre deze richtlijnen ook daadwerkelijk gevolgd worden. In **hoofdstuk 5** is geëvalueerd hoeveel vrouwen na een veneuze trombose tijdens hormonaal anticonceptivumgebruik stoppen, veranderen of doorgaan met hun hormonale anticonceptiva. Alhoewel de meerderheid van vrouwen stopt met het gebruik van hormonale anticonceptie na een veneuze trombose, gaat ongeveer 20% door met het gebruik of verandert naar een ander type oraal combinatiepreparaat. Vrouwen waren meer geneigd om door te gaan met hun hormonale anticonceptiva als zij geen familieleden hadden met veneuze trombose of als zij waren blootgesteld aan een operatie of gipsbeen in de drie maanden voorafgaande aan de trombose. In de huidige richtlijnen wordt echter geen advies over anticonceptivumgebruik gegeven bij een blootstelling aan een ander risicofactor naast hormoongebruik. Naast het hormoongebruik van vrouwen na een trombose, is er ook gekeken of advies over hormoongebruik was gegeven door de behandelend arts. De meerderheid van vrouwen (82%) had het advies van de arts gekregen om te stoppen met hormonale anticonceptivum gebruik. Van hen ging 12% ondanks dit advies toch door met het gebruik of veranderde naar een ander oraal combinatiepreparaat. Met in ogenschouw nemend het mogelijk verhoogde risico op een tweede trombose bij anticonceptivumgebruik is het verontrustend dat een substantieel aantal vrouwen het advies van de arts niet volgen. Echter, tot op heden is er weinig bekend over het risico op een tweede trombose bij hormoongebruik na een eerste trombose. In **hoofdstuk 6** is het risico op een tweede trombose bij gebruik van hormonale anticonceptiva na een eerste trombose bekeken. De incidentie van een tweede trombose in vrouwen die hormonale anticonceptiva gebruikten ten tijde van de eerste trombose was gelijk aan de incidentie in vrouwen die geen hormonale anticonceptiva gebruikten. Het risico op een tweede trombose was driemaal verhoogd in gebruiksters van hormonale anticonceptiva na een eerste trombose, in het bijzonder in gebruiksters van gecombineerde orale

anticonceptiva. Opvallend was dat onder 20 gebruiksters van een levonorgestrel-houdend spiraaltje met een totale follow-up tijd van 70 jaar geen tweede trombose is waargenomen. Dit resultaat suggereert, ondanks de kleine aantallen vrouwen op wie de waarneming is gebaseerd, dat het gebruik van een levonorgestrel-houdend spiraaltje een veilige methode is na een eerste trombose. Het risico op een tweede trombose onder gecombineerde orale anticonceptiva gebruiksters bleek af te hangen van de ethinyloestradiol dosis, progestageen type en of de pil monofasisch of trifasisch was.

Gecombineerde oral anticonceptiva worden meestal onderverdeeld naar de dosis van ethinyloestradiol (oftewel 20, 30 of $\geq$35 µg) of in generaties van progestageen (eerste generatie gestagenen: norethisteron, lynestrenol; tweede: levonorgestrel, norgestrel; en derde generatie: gestodeen, desogestrel, norgestimaat), als ook 'andere' typen (cyproteron acetaat en drospirenon, de laatste worden soms geclassificeerd als een vierde generatie). Ter evaluatie van het risico op veneuze trombose voor een nieuw anticonceptivum wordt het risico vergeleken met niet-gebruiksters of met gebruiksters van levonogestrel met 30 µg ethinyloestradiol. In **hoofdstuk 7** is een netwerk meta-analyse uitgevoerd om het risico van veneuze trombose per anticonceptiepil te beoordelen. Alle tien geselecteerde anticonceptiepillen verhoogden het risico op veneuze trombose. De hoogste risico's werden in gebruiksters van drospirenon met 30 µg ethinyloestradiol, cyproterone acetaat met 35 µg ethinyloestradiol of levonorgestrel met 50 µg ethinyloestradiol gevonden. Het laagste risico op veneuze trombose werd in gebruiksters van levonorgestrel met 20 µg ethinyloestradiol geobserveerd. Het gebruik van een combinatiepreparaat met levonorgestrel en de laagst mogelijke dosis van ethinyloestradiol, met in ogenschouw nemend de compliantie, is de veiligste optie als het gaat om het risico van veneuze trombose.

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Curriculum Vitae

Bernardine Stegeman was born on 25 december 1983 in Dordrecht, the Netherlands. In 2003, she graduated from the Insula College in Dordrecht. She studied Clinical Technology at University Twente in Enschede, the Netherlands. After receiving her Bachelor degree in 2006, she started with a Master degree in Biomedical Sciences at the Radboud University Nijmegen, the Netherlands. During her Masters degree, she completed two internships. She researched genetic variation in genes involved in vitamin B12 metabolism and vitamin B12 deficiency at the gastrointestinal department at the University Medical Center Nijmegen. Her final internship was at the department of Non-Communicable Diseases Epidemiology of the London School of Hygiene and Tropical Medicine, London, United Kingdom. She assessed the influence of socioeconomic status on the mammographic density in screened women. She received her Master of Science in December 2008.

In January 2009, she started her PhD project researching combined oral contraceptive-associated venous thrombosis at the Department of Thrombosis and Haemostasis under supervision of Prof. Frits Rosendaal, Prof. Frans Helmerhorst and Dr. Hans Vos and later of Dr. Astrid van Hylckama Vlieg. She also worked closely together with the Department of Clinical Epidemiology. The results of her thesis has been presented on several congresses. She was awarded a travel award at the NVTH, Koudekerke, the Netherlands and the Young Investigator Award at the ISTH in Kyoto, Japan.

As of January 2013, she is working at the department of Epidemiology and Public Health at University College London, United Kingdom.

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