Lifestyle and venous thrombosis

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Lifestyle and venous thrombosis

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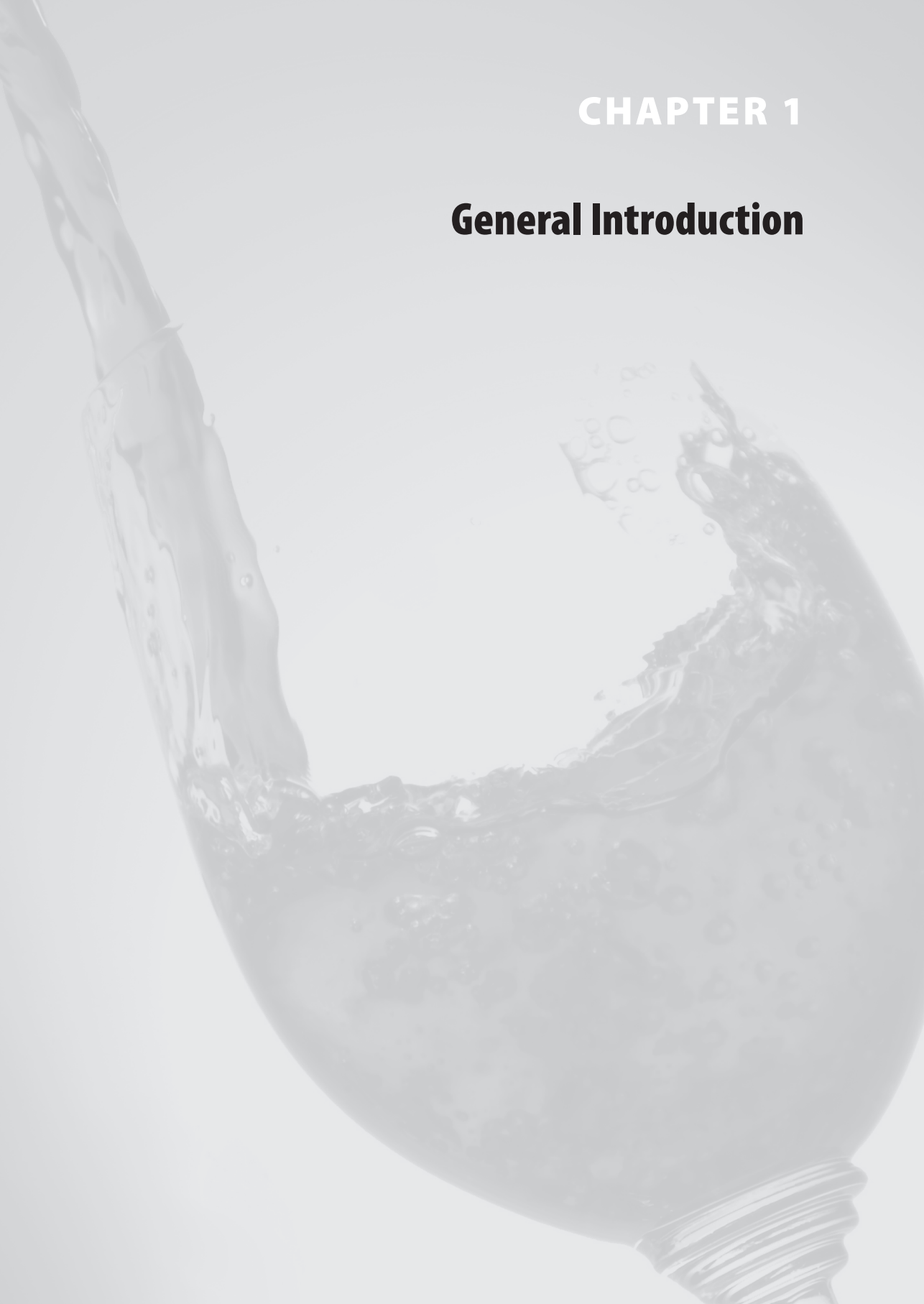
CHAPTER 1

General Introduction



VENOUS THROMBOSIS

Venous thrombosis is a condition in which an obstructive blood clot (thrombus) forms in a vein. Most commonly, venous thrombosis occurs in the deep veins of the leg. The thrombus limits blood flow through the vein, causing swelling and pain of the affected leg. A part of the thrombus may break off and travel through the bloodstream (embolize). The traveling blood clot can lodge in the lungs causing a pulmonary embolism. In the 19th century Rudolf Virchow postulated a theory, Virchow's Triad, which proposes that venous thrombosis is caused by alterations in blood flow (i.e. stasis), vascular endothelial injury or alterations in the constitution of the blood (figure 1). The triad remains clinically relevant over 150 years later.

The average annual incidence of venous thrombosis is around 2 per 1000 individuals[1;2]. The incidence rises exponentially with age, from 0.001% in childhood to nearly 1% per year in the very old[3]. Among venous thrombosis patients approximately two-third manifests deep venous thrombosis of the leg and one-third pulmonary embolism with or without deep venous thrombosis of the leg[4;5]. A common consequence of deep venous thrombosis is the post-thrombotic syndrome, which develops in 20 to 50% of patients[6]. It is characterized by pain, heaviness, swelling and cramps in the affected leg. The disease may be fatal when complicated by pulmonary embolism[5].

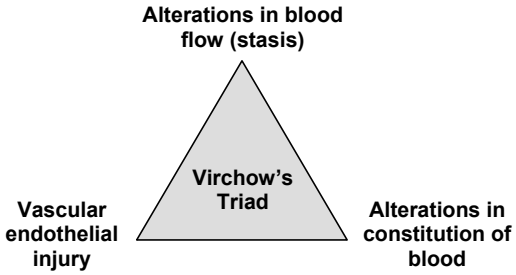


Figure 1. Proposed causes of venous thrombosis by Virchow

RISK FACTORS

Venous thrombosis is caused by both acquired and genetic risk factors[7]. Known acquired risk factors include immobilization, surgery, trauma, lupus anticoagulant, malignant disease, pregnancy, puerperium, and female hormones. Genetic risk factors are inherited abnormalities affecting blood coagulation such as deficiencies of the anticoagulants protein S, protein C and antithrombin. The factor V Leiden and the prothrombin 20210A mutation are the two most common prothrom-

botic mutations[7]. Recently the contribution of lifestyle factors to the risk of venous thrombosis has gained interest.

An increasing number of studies indicate that obesity increases the risk of venous thrombosis[8-15]. Biological support for the relationship between obesity and the risk of venous thrombosis arises from studies showing an increase of prothrombotic factors, such as factor VII, factor VIII, factor XII and fibrinogen, with increasing body mass index[16-18]. Viewed together with the association of obesity with venous stasis[12] a relation between obesity and an increased risk of venous thrombosis becomes plausible. The multicausal nature of venous thrombosis dictates that risk factors have to be present simultaneously to lead to disease. Because obesity and oral contraceptive use are common in the general population, and because factor V Leiden and the prothrombin 20210A mutation are the two most frequent prothrombotic mutations, these are good candidates to investigate gene-environment interaction.

Like obesity, smoking is a well-established risk factor for arterial disease. However, the results of studies investigating the relationship between smoking and venous thrombosis are inconsistent[9;10;12;19;20]. Results vary from an adverse to a protective effect of smoking. A possible risk increasing effect may be mediated through an increase in coagulation factors in smokers compared to non smokers. It is well-known that smokers have higher fibrinogen levels[21-25] and smoking cessation causes a rapid fall in plasma fibrinogen[22]. Supporting data for an association between fibrinogen and the risk of venous thrombosis arises from 'The Leiden Thrombophilia Study' (LETS)[26] and a study among African-Americans[27]. Given that smoking is still common worldwide[28] it is important to address the controversy between study results and elucidate if there is an effect of smoking on the risk of venous thrombosis. In addition the joint effect of smoking and oral contraceptive use on venous thrombotic risk is of interest, since for arterial disease smoking has been shown to act synergistically with oral contraceptive use[29].

Unlike obesity and smoking, moderate alcohol consumption is known for its protective effect on arterial cardiovascular disease[30]. A beneficial effect of moderate alcohol consumption on the risk of venous thrombosis is also not unlikely considering the effect of alcohol consumption on several coagulation factors. Reduced levels of fibrinogen, factor VII and von Willebrand factor are reported to be associated with moderate alcohol consumption. In contrast heavy and binge alcohol drinking is associated with increased levels of fibrinogen and factor VII[31]. The effect of alcohol on venous thrombotic risk has only been investigated in a few studies with varying outcomes[10;12;32].

In this thesis the association of obesity, smoking and alcohol consumption with the risk of venous thrombosis is investigated. The joint effect of overweight and

smoking with important other risk factors for venous thrombosis such as oral contraceptive use and the factor V Leiden mutation is assessed to identify possible high-risk groups, which could be of importance in medical practice.

There are important acquired risk factors for venous thrombosis that are limited to women e.g., oral contraceptive use, hormone replacement therapy, pregnancy and puerperium. During pregnancy, the risk of venous thrombosis is about 5-fold increased with an even higher risk in the postpartum period[33]. About 15% of maternal deaths in developed countries results from pulmonary embolism[34], which makes pulmonary embolism the most common cause of maternal mortality in these countries. In women with thrombophilia the pregnancy related risk is further increased, with varying risk estimates from studies of different designs[35]. We evaluated pregnancy and the postpartum period as risk factors for venous thrombosis and the joint effect of pregnancy with the factor V Leiden and the prothrombin 20210A mutation.

Genetic factors also contribute to the thrombotic risk as indicated above. The two most common prothrombotic mutations, factor V Leiden and the prothrombin 20210A mutation, are present in respectively five and two percent of the Caucasian population[6;7]. In addition there are various genetic variants with a lower prevalence and a smaller contribution to the risk of venous thrombosis than these mutations. A previous analysis within the LETS study found a genetic variant associated with reduced levels, but no deficiency, of the crucial anticoagulant protein C which was also associated with an increased risk of deep venous thrombosis of the leg[36]. Individuals with the homozygous CGT genotype were found to have a 50% to 100% greater risk of venous thrombosis than individuals who were homozygous for the common genotype. Two of the three polymorphisms tested in the LETS were considered as functionally different and were tested again in a French study with 394 healthy individuals aged 20 to 60 years[37]. This study confirmed the link between the protein C gene polymorphisms and circulating protein C levels, and suggested a complex effect on the risk of venous thrombosis. In this thesis we investigated these two polymorphisms within the protein C gene and different combinations of these polymorphisms as risk factors for venous thrombosis.

When designing a case-control study the choice of an appropriate control group is very important. The various sources of control subjects in the numerous case-control studies performed over the years show that several options exists. We included two separate control groups in our study on the etiology of venous thrombosis and explore the consequences of the choice for a particular control group.

MEGA STUDY

All research questions addressed in this thesis were studied in a large population-based case-control study, The Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA study). From March 1999 till September 2004, the MEGA study included consecutive patients with a first diagnosis of venous thrombosis. Patients were selected from the files of the anticoagulation clinics in Amsterdam, Amersfoort, The Hague, Leiden, Rotterdam and Utrecht. Patients with deep venous thrombosis of the leg, pulmonary embolism or a combination of these diagnoses were included in the study. Patients with deep venous thrombosis of the arm were also included, but are not part of the analyses presented in this thesis. As control subjects partners of patients were asked to participate. An additional control group was recruited from the general population using a random digit dialing method.

Patients and control subjects filled in a questionnaire about putative risk factors for venous thrombosis. Most questions referred to a period of 12 months prior to the index date, i.e. the date of diagnosis of the thrombosis for patients and their partners and the date of filling in the questionnaire for the random control subjects. At least three months after withdrawal of anticoagulation the patients and their partners were asked to visit the anticoagulation clinic where after an overnight fast a blood sample was drawn. From June 2002 onwards, blood draws were no longer performed in patients and their partners, and sampling was restricted to DNA collection by buccal swabs sent by mail. The random controls were invited for a blood draw within a few weeks after the questionnaire was sent. Within this group buccal swabs were sent when someone refused the blood draw.

OUTLINE OF THIS THESIS

Chapter 2: We describe overweight and obesity as risk factors for venous thrombosis and the joint effect of overweight and obesity together with Factor V Leiden, the prothrombin 20210A mutation and oral contraceptive use. In addition, body weight and height were also evaluated as separate risk factors for venous thrombosis.

Chapter 3: The relative risk of venous thrombosis associated with smoking is presented. We investigated smoking status, the amount of smoking, smoking duration and the number of pack-years as risk factors for venous thrombosis. By adjusting the smoking status analyses for fibrinogen levels we examined if fibrinogen levels were part of the mechanism behind the relationship between smoking and venous

thrombosis. Also the joint effect of smoking with two major risk factors for venous thrombosis, oral contraceptive use and the factor V Leiden mutation, was investigated.

Chapter 4: We report the association of a third lifestyle factor with the risk of venous thrombosis. Relative risks for different amounts of alcohol consumption were calculated.

Chapter 5: The risk of venous thrombosis in pregnant and post-partum women is presented. We studied different stages of pregnancy and the postpartum period and the risk in carriers of the factor V Leiden or the prothrombin 20210A mutation.

Chapter 6: We investigated the effect of two polymorphisms within the promoter region of the protein C gene (C/T at -2405 and A/G at -2418) on risk of venous thrombosis and on plasma protein C levels. In addition the combined effect of the two polymorphisms with factor V Leiden and oral contraceptive use was investigated.

Chapter 7: By addressing different hypotheses within the MEGA study we describe our considerations concerning control group choice and the importance of adaptation of statistical analyses to the source of controls.

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CHAPTER 2

Risk of venous thrombosis: obesity and its joint effect with oral contraceptive use and prothrombotic mutations

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SUMMARY

In the MEGA study we evaluated body weight, height and body mass index (BMI) as risk factors for venous thrombosis. Additionally we analyzed the joint effect of obesity together with oral contraceptive use and prothrombotic mutations on the risk of venous thrombosis. 3834 patients with a first venous thrombosis and 4683 control subjects were included, all non-pregnant and without active malignancies. Relative to those with a normal BMI (<25 kg/m^2), overweight (BMI≥25 and BMI<30 kg/m^2) increased the risk of venous thrombosis 1.7-fold (odds ratio (OR) $_{adj(age\ and\ sex)}$ 1.70, 95% confidence interval (95% CI) 1.55-1.87) and obesity (BMI≥30 kg/m^2) 2.4-fold (OR_{adj} 2.44, 95% CI 2.15-2.78). An increase in body weight and body height also individually increased thrombotic risk. Obese women who used oral contraceptives had a 24-fold higher thrombotic risk (OR_{adj} 23.78, 95% CI 13.35-42.34) than women with a normal BMI who did not use oral contraceptives. Relative to non-carriers of normal BMI, the joint effect of factor V Leiden and obesity led to a 7.9-fold increased risk (OR_{adj} 7.86, 95% CI 4.70-13.15); for prothrombin 20210A this was a 6.6-fold increased risk (OR_{adj} 6.58, 95% CI 2.31-18.69). Body height, weight and obesity increase the risk of venous thrombosis, especially obesity in women using oral contraceptives.

INTRODUCTION

Venous thrombosis has an average annual incidence of around 2 per 1000 individuals (Oger, 2000). The incidence rises exponentially with age, from 0.001% in childhood to nearly 1% per year in the very old (Rosendaal, 1997). Among venous thrombosis patients approximately two-thirds has deep venous thrombosis of the leg and one-third pulmonary embolism with or without deep venous thrombosis of the leg (Anderson, Jr. et al, 1991; White, 2003). The disease is potentially fatal when complicated by pulmonary embolism (White, 2003).

Venous thrombosis is a multicausal disease caused by both acquired and genetic factors. Recent studies indicate that obesity increases the risk of venous thrombosis (Abdollahi et al, 2003; Goldhaber et al, 1997; Oren et al, 2006; Samama, 2000; Stein et al, 2005; Tsai et al, 2002; Vaya et al, 2002; White et al, 2000). Biological support for the observed relationship between obesity and coagulation, and thus the risk of venous thrombosis, arises from studies showing an increase of procoagulant factors, such as factor VII, factor VIII, factor XII and fibrinogen, with increasing body mass index (BMI) (Bowles et al, 2003; Rosito et al, 2004; Chan et al, 1995; De Pergola et al, 1997). Obesity is also associated with venous stasis (Tsai et al, 2002) which may increase thrombotic risk.

Although BMI is the most widely used measure of obesity no single function of body height and weight is likely to capture fully the ways in which height and weight are related to venous thrombosis (Kronmal, 1993). For this reason we will also evaluate body weight and height as separate risk factors for venous thrombosis.

The multicausal nature of venous thrombosis dictates that risk factors are present simultaneously. We reported previously that oral contraceptives modified the effect of obesity on the risk of venous thrombosis, with a 10-fold increased risk among women with a BMI greater than 25 kg/m^2 compared to normal weight women not using oral contraceptives. A 4.6-fold increased risk was found for oral contraceptive use among women with a BMI below 25 kg/m^2 (Abdollahi et al, 2003). The Copenhagen City Heart Study led to reports on the joint effect of overweight and the factor V Leiden mutation and found a substantially increased risk in obese individuals with the mutation (Juul et al, 2004). Because obesity and oral contraceptive use are common in the general population and factor V Leiden and the prothrombin mutation are the two most frequent prothrombotic mutations, these are good candidates to investigate gene-environment interaction. Only a very large study will be able to do so.

To investigate the risk of venous thrombosis due to obesity, the separate risk contributions of body weight and body height and the combination of obesity with

other risk factors for venous thrombosis, we performed a large population-based case-control study.

METHODS

Study design

The Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA study) included consecutive patients with a first diagnosis of venous thrombosis. Between March 1999 and September 2004, patients were selected from the files of the anticoagulation clinics in Amsterdam, Amersfoort, The Hague, Leiden, Rotterdam and Utrecht. In the Netherlands, anticoagulation clinics monitor anticoagulation treatment of all patients in a geographically well-defined area. We included patients between the age of 18 and 70 years with a first deep venous thrombosis of the leg, a pulmonary embolism or a combination of these diagnoses. Idiopathic venous thrombosis was defined as venous thrombosis in patients without surgery, injury, plaster cast, immobilization in the year prior to the thrombosis or oral contraceptive use and hormone replacement therapy at the time of the event.

The diagnostic methods were verified in a random sample of the overall patient group (n=742). Within this group the diagnosis of 97% of deep venous thrombosis and 78% of pulmonary embolism was objectively confirmed. The tests included compression ultrasonography, Doppler ultrasound, impedance plethysmography and contrastvenography for diagnosis of deep venous thrombosis and perfusion and ventilation lung scanning, spiral computer tomography and pulmonary angiography for pulmonary embolism.

Patients with severe psychiatric problems or those unable to speak Dutch were considered ineligible. Of the 6331 eligible patients 276 died soon after the venous thrombosis. Of the remaining 6055 patients 5051 participated (83%). Of the non-participants 82 persons were in the end stage of disease and 922 refused to participate or could not be located. Of the participants, 4637 patients (92%) filled in and returned the questionnaire. Participants who did not return a questionnaire completed a short questionnaire by phone, which did not include questions on body weight and height, or only participated with a blood sample or buccal swab.

Partners of patients were asked to volunteer as control subjects. From January 2002 until September 2004, additional control subjects were recruited by using the random digit dialing (RDD) method (Hartge et al, 1984). Phone numbers were dialed at random within the geographical inclusion area of the patients. During

the phone call a specific person within a household (e.g. youngest woman between 20 and 50) was asked to participate. The random control subjects were frequency matched to the patients with respect to age and sex. RDD is an efficient method to collect a nearly random sample of all individuals in the population. Only control subjects with no recent history of venous thrombosis were included and the same exclusion criteria were applied as for the patients.

Of the 5051 participating patients, 3657 had an eligible partner. One partner died soon after the request for participation. Of the remaining 3656 partners 2982 participated (82%). Of the non-participants 18 were in end-stage disease, 649 refused to participate or could not be located and for 7 persons the reason for non-participation was unknown. A questionnaire was returned by 2821 participating partners (95%).

Of the 4350 eligible RDD control subject, four died before they were able to participate. Of the remaining 4346 persons 3000 participated (69%). Of the non-participants 15 were in the end stage of disease and 1331 refused to participate or could not be located. A questionnaire was returned by 2789 participants (93%). All participants gave written informed consent. The study was approved by the Medical Ethics Committee of the Leiden University Medical Center, Leiden, The Netherlands.

Data collection

Within a few weeks after diagnosis and registration at the anticoagulation clinics eligible patients received a letter with information about the MEGA study. Subsequently they were contacted by phone. If the patient was willing to participate a questionnaire was sent. The control subjects received the questionnaires immediately after inclusion by phone. The questionnaires included items on surgery, injury, plaster cast, immobilization, malignancies, pregnancy, use of oral contraceptives, hormone replacement therapy, body weight and body height. Most questions referred to a period of 12 months prior to the index date. For the patients and their partners, the index date was defined as the date of diagnosis of the thrombosis of the patient. The date of filling in the questionnaire was defined as the index date for the random control subjects.

Body mass index (BMI) was calculated by dividing body weight (kg) by squared height (m^2). BMI was categorized according to the criteria of the World Health Organization (1998), defining a BMI between 18.5 and 25 kg/m^2 in adults as normal, a BMI of 25 to 30 kg/m^2 as overweight and a BMI equal to or greater than 30 kg/m^2 as obesity (World Health Organisation, 2000).

Participants with missing data on body weight or body height were excluded from all analyses. In addition, individuals with malignancies diagnosed within 10 years prior to the index date and pregnant women or women that had been pregnant in the year before the index date were also excluded. In the analyses only partner controls with a participating patient were included leading to a total of 3834 patients, 2152 partner and 2531 random control subjects for the present analyses.

DNA collection

Within the patient group used for the analyses 3607 provided a blood sample or buccal swab (94%). In the combined control group 3830 blood samples or buccal swabs were obtained (82%). The factor V Leiden mutation was successfully determined in 3600 patients and 3809 control subjects, the prothrombin 20210A mutation in 3601 patient and 3810 control subjects. A detailed description of blood collection and DNA analysis for the factor V Leiden (G1691A) and the prothrombin mutation (G20210A) in the MEGA study has been published previously (Blom et al, 2005).

Statistical analysis

As estimates of relative risks we calculated odds ratios (ORs) and 95% confidence intervals (95% CI) according to the method of Woolf (Woolf, 1955). With a multiple logistic regression model we adjusted for age (continuous) and sex (categorical). Adjustment for age as a categorical variable resulted in the same risk estimates. In the analysis of body weight we also adjusted for body height (categorical). In the analyses with partners as control group, we performed a matched logistic analysis to adjust for similar lifestyle factors between patients and their partners (Cannegieter et al, 2006). In these matched analyses only patient-partner pairs were included (2152 pairs). In the analyses with the random control subjects an unmatched analysis including all patients and random control subjects was performed. Because the results of the matched and the unmatched analyses showed consistent elevated relative risks in all the analyses, we calculated our risk estimates with a method that combines the matched and the unmatched analyses. This analysis took into account the presence of 2152 patients in both the matched and the unmatched analysis (see appendix). When analyzing the risk in men and women separately it was not possible to perform a matched analysis with the partner controls, as control individuals were nearly always of the opposite sex to the cases. Therefore, risk estimates were calculated with an unmatched analysis with all patients and the

random control subjects. Statistical significance was considered for $P<0.05$. SAS 9.1 (SAS institute Inc, Cary, NC, USA) was used for all statistical analyses.

RESULTS

In the current analysis 3834 patients with a first venous thrombosis and 4683 control subjects were included. Mean age of 3834 patients was 48.3 (5^{th}-95^{th} percentiles, 25.9-67.5) and of 4683 control subjects 46.9 (5^{th}-95^{th} percentiles, 25.1-66.3) years. Fifty two percent (n=2008) of patients and 53% (n=2498) of control subjects were women. In the patient group 58% (n=2212) was diagnosed with deep venous thrombosis of the leg, 29% (n=1113) with a pulmonary embolism and 13% (n=509) with the combination of these diagnoses. In Table I relative risks of venous thrombosis with increasing body mass index are presented. The table presents the combined odds ratios for both control groups; the effects when each control group was used separately did not differ substantially (overweight, partner controls OR 1.45, 95% CI 1.26-1.67; overweight RDD controls OR 1.84, 95% CI 1.64-2.06; obesity partner controls OR 1.84, 95% CI 1.51-2.23; obesity RDD controls OR 2.88, 95% CI 2.47-3.37). Among patients 42% was overweight and 21% obese, which was 37% (overweight) and 13% (obese) among controls (Table I).

Overweight resulted in a 1.7-fold increased risk (OR_{adj} 1.70, 95% CI 1.55-1.87) and obesity in a 2.4-fold increased risk of venous thrombosis (OR_{adj} 2.44, 95% CI 2.15-2.78) compared to the reference category with a BMI below 25 kg/m^2 (Table I). Combining the overweight and obese categories, the odds ratio was 1.88 (95% CI 1.72-2.06).

In Figure 1 a more detailed relationship between body mass index and the risk of venous thrombosis is shown. Individuals with a body mass index between 22.5 and 25.0 kg/m^2 formed the reference category. In general the relation between BMI and thrombotic risk formed a J-shaped curve. In persons with the highest BMI (≥35 kg/m^2) the risk of venous thrombosis was 2.6 fold increased (OR_{adj} 2.62, 95% CI 2.06-3.33) compared to the reference group. With BMI as a continuous variable in the

Table I. Relative risk of venous thrombosis by categories of body mass index

BMI (kg/m^2)	Patients N=3834	Partners N=2152	RDD N=2531	OR*	95% CI
All VT					
<25	1393	948	1409	1	
≥25&<30	1629	880	848	1.70	1.55-1.87
≥30	812	324	274	2.44	2.15-2.78

VT, venous thrombosis; RDD, random digit dialing control subjects; OR, odds ratio; CI, confidence interval
*Combined OR, adjusted for age and sex

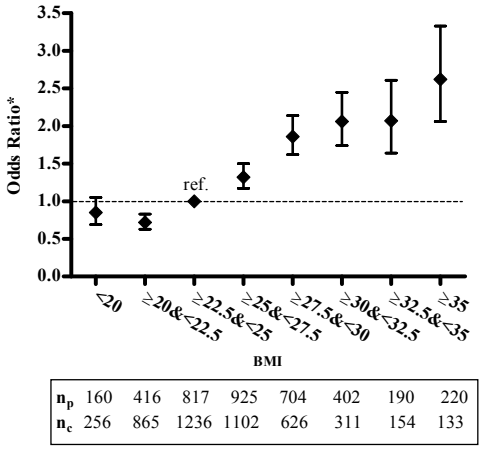

Figure 1. Relative risk of venous thrombosis by categories of body mass index (BMI) (kg/m^2).
I, 95% confidence interval; n_p, number of patients; n_c, number of control subjects; ref., reference category.
*Adjusted for age and sex

Table II. Relative risk of venous thrombosis by categories of body mass index in different subgroups

BMI (kg/m^2)	Patients	Control subjects	OR*	95% CI
DVT				
<25	772	2357	1	
≥25&<30	949	1728	1.83	1.63-2.06
≥30	491	598	2.80	2.41-3.26
PE				
<25	463	2357	1	
≥25&<30	458	1728	1.51	1.31-1.75
≥30	192	598	1.89	1.55-2.31
DVT+PE				
<25	158	2357	1	
≥25&<30	222	1728	2.05	1.64-2.57
≥30	129	598	3.77	2.89-4.93
Idiopathic VT				
<25	344	2357	1	
≥25&<30	549	1728	1.84	1.55-2.17
≥30	221	598	2.58	2.07-3.23
All VT_{women}†				
<25	823	867	1	
25-30	683	370	1.93	1.64-2.26
≥30	502	156	3.36	2.74-4.12
All VT_{men}†				
<25	569	542	1	
≥25&<30	944	478	1.72	1.46-2.03
≥30	310	118	2.32	1.82-2.97

DVT, deep venous thrombosis; PE, pulmonary embolism; OR, odds ratio; CI, confidence interval
*combined for both control groups and adjusted for age and sex; †three patients were not included in these analyses because two were transsexuals and one had Klinefelter syndrome, these analyses were performed with the random control subjects only.

logistic model a 1.1-fold increased risk (10% increase) per 1 kg/m^2 was observed (OR_{adj} 1.13, 95% CI 1.11-1.16).

Odds ratios were slightly higher for deep venous thrombosis than for pulmonary embolism and in women than in men (Table II). The odds ratio of idiopathic venous thrombosis with increasing BMI was approximately the same as the overall risk (Table II).

Table III shows the relative risk of venous thrombosis by categories of body weight (kg) and body height (m). As was to be expected, adjusted for body height, body weight again was associated with thrombotic risk, which was also evident without adjustment for body height, but less clearly. A 2.9-fold increased risk was found for body weights equal to or above 110 kg (OR_{adj} 2.93, 95% CI 2.28-3.77) relative to those between 70 to 79 kg. Body weights between 50 and 70 kg were associated with the lowest risk of venous thrombosis. Body weight was also assessed as a risk factor in men and women separately, with similar results (data not shown). Only individuals with a body height above 1.80 m had a slightly increased risk of venous thrombosis compared to those between 1.70 to 1.74 m. Short persons (<1.70 m) had a low risk of venous thrombosis. When analyzing men and women separately, the risk only appeared to be decreased for short men ($OR_{adj, \leq 1.79\ m}$ 0.77, 95% CI 0.64-0.94) and increased for very tall men ($OR_{adj, \geq 1.90\ m}$ 1.32, 95% CI 1.02-1.70) compared to men with a body height between 1.80 and 1.84 m (data not shown).

Table III. Relative risk of venous thrombosis by categories of body weight and body height

Body weight (kg)	Patients	Control subjects	OR*	95% CI
<50	27	38	0.68	0.40-1.16
50-59	206	396	0.60	0.49-0.73
60-69	595	1076	0.69	0.60-0.78
70-79	871	1209	1	
80-89	932	1038	1.43	1.26-1.62
90-99	676	586	1.88	1.63-2.17
100-109	308	212	2.45	2.01-2.99
≥110	219	128	2.93	2.28-3.77
Body height (m)				
<1.60	182	247	0.69	0.56-0.86
1.60-1.64	377	531	0.73	0.61-0.86
1.65-1.69	649	857	0.83	0.72-0.95
1.70-1.74	749	878	1	
1.75-1.79	662	809	1.01	0.87-1.16
1.80-1.84	571	671	1.14	0.98-1.33
1.85-1.89	373	438	1.17	0.98-1.41
≥1.90	271	252	1.56	1.27-1.92

OR, odds ratio; CI, confidence interval

*adjusted for age, sex and body height in body weight analyses, adjusted for age and sex in body height analyses

Joint effect of obesity with other risk factors for venous thrombosis

The combined effect of oral contraceptive use and obesity was examined in women aged 18 to 39 years (Table IV). Among women who did not use oral contraceptives the risk increased 2.5-fold for overweight women and 3.0-fold for obese women compared to normal weight women not using oral contraceptives. Relative to non-users of normal BMI, oral contraceptive users who were overweight had an 11.6-fold increased risk and those who were obese a 23.8-fold increased risk.

Among non-carriers of factor V Leiden, obesity led to a 2.5-fold increased risk (normal BMI as reference). The joint effect of factor V Leiden and obesity resulted in a 7.9-fold increased risk of venous thrombosis (Table V). For obese participants

Table IV. Combined effect of body mass index and oral contraceptive (OC) use on the risk of venous thrombosis in women aged 18 to 39

BMI (kg/m^2)	OC use	Patients	Control subjects	OR*	95% CI
<25	no	51	167	1	
≥25&<30	no	27	34	2.52	1.38-4.57
≥30	no	28	30	3.04	1.66-5.57
<25	yes	260	233	4.15	2.85-6.03
≥25&<30	yes	178	55	11.63	7.46-18.14
≥30	yes	132	19	23.78	13.35-42.34

OR, odds ratio; CI, confidence interval *analyses are performed with all patients and the random control subjects and adjusted for age

Table V. Combined effect of body mass index, the factor V Leiden (FVL) and the prothrombin (FII) 20210A mutation on the risk of venous thrombosis

BMI (kg/m^2)	FVL	Patients	Control subjects	OR*	95% CI
<25	no	1077	1631	1	
≥25&<30	no	1289	1244	1.72	1.54-1.93
≥30	no	643	423	2.48	2.13-2.88
<25	yes	217	69	4.18	3.12-5.61
≥25&<30	yes	250	58	5.77	4.20-7.93
≥30	yes	124	18	7.86	4.70-13.15
	FII 20210A	Patients	Control subjects	OR*	95% CI
<25	no	1225	1803	1	
≥25&<30	no	1455	1351	1.72	1.54-1.91
≥30	no	735	477	2.45	2.12-2.82
<25	yes	70	18	4.39	2.56-7.51
≥25&<30	yes	84	20	4.51	2.64-7.72
≥30	yes	32	4	6.58	2.31-18.69

OR, odds ratio; CI, confidence interval

*Adjusted for age and sex.

Note: The inclusion of matched case control pairs in the analyses was dependent on the category (BMI, FVL; BMI, FII 20210A) of both partners

with the prothrombin 20210A mutation the risk of venous thrombosis increased 6.6-fold (normal BMI, non-carriers as reference).

DISCUSSION

In this large population-based case-control study both overweight and obesity were associated with a two- to three-fold increased risk of venous thrombosis. Since the prevalence of obesity is increasing, this has a major impact (http://www.ic.nhs.uk/webfiles/publications/opan06/OPAN%20bulletin%20finalv2.pdf,http://www.cdc.gov/nchs/products/pubs/pubd/hestats/overweight/overwght_adult_03.htm). In this study 50% of the control subjects, who represent the general population, were overweight or obese. This suggests that almost one-third of all events of thrombosis are preventable by weight loss (population attributable risk=28%), assuming that weight loss reduces venous thrombotic risk (Ditschuneit et al, 1995; Hankey et al, 1997; Kopp et al, 2003). Prevalences of overweight and obesity reported from the UK and the USA of 60-65 percent lead to even higher preventable fractions (http://www.ic.nhs.uk/webfiles/publications/opan06/OPAN%20bulletin%20finalv2.pdf,http://www.cdc.gov/nchs/products/pubs/pubd/hestats/overweight/overwght_adult_03.htm).

We also evaluated body weight and height as separate risk factors for venous thrombosis. Body weight was positively associated with thrombotic risk in both men and women. For body height no substantial increased risks were found in women, but short men appeared to have a low risk and tall men a high risk of venous thrombosis. Particularly this latter is remarkable, since body height is not associated with the relative amount of fat, as body weight and BMI both are. The effect of obesity was more pronounced in women than in men, with high relative risks for overweight and obese women who used oral contraceptives. The joint effect of obesity with the factor V Leiden mutation or the prothrombin mutation appeared both slightly higher than the sum of the separate effects.

The association between BMI and venous thrombosis is likely to be causal because it is consistent over studies, shows a dose-response relation and is biologically plausible. Our results are consistent with previous studies demonstrating an increased risk of venous thrombosis with increasing body mass index. The Nurses Health Study found a three-fold increased risk of pulmonary embolism in women with obesity (Goldhaber et al, 1997). Another prospective follow-up study reported a hazard ratio of 2.3 for venous thrombosis among persons with a body mass index (BMI) above 30 kg/m^2 compared to persons with a BMI below 25 kg/m^2 (Tsai et al, 2002). Other studies also showed an elevated risk of venous thrombosis among

overweight persons (Abdollahi et al, 2003; Oren et al, 2006; Samama, 2000; Stein et al, 2005; Vaya et al, 2002; White et al, 2000). The magnitudes of the relative risks are largely similar. To our knowledge the only case-control study that did not found an increased risk of venous thromboses with BMI was a study with a very small sample size (n=90) performed in pregnant women and women during post partum (Danilenko-Dixon et al, 2001). It is not unlikely that BMI in these women is a poor marker for the relative amount of body fat.

There are several ideas about the mechanism behind the association between overweight and the risk of venous thrombosis. An increase in prothrombotic factors in obese persons may play a role (Bowles et al, 2003; Rosito et al, 2004; Chan et al, 1995; De Pergola et al, 1997), while obesity may also be associated with lack of exercise and venous stasis (Tsai et al, 2002). A high body mass index can be the result of excess body fat or abundant muscle development. 'The study of men born in 1913' evaluated waist circumference as a measure for abdominal obesity instead of BMI (Hansson et al, 1999). In this study, men in the highest decile of waist circumference (≥100 cm) had a relative risk for DVT of 3.9 compared to men with a waist circumference less than 100 cm. This result suggests that obesity caused by excess body fat is likely to be a risk factor for venous thrombosis.

We found a more pronounced excess risk for deep vein thrombosis than for pulmonary embolism. This is in accordance with results from the National Hospital Discharge Survey (Stein et al, 2005). An explanation may be the complexities of the diagnosis of pulmonary embolism, which may have led to misclassification, i.e. inclusion of some patients without a true pulmonary embolism (PIOPED, 1990). Alternatively, clots in obese individuals may be different from those in non-obese people and have less tendency to embolize, as has also been suggested as an explanation for the low risk of pulmonary embolism in individuals with factor V Leiden. Obesity and factor V Leiden both lead to APC resistance (Lowe et al, 1999), which lends further plausibility to a differential effect of deep venous thrombosis and pulmonary embolism. Oral contraceptive use also leads to APC-resistance, which helps to understand the syntergistic effect of obesity and factor V Leiden and obesity and oral contraceptive use. This is in line with the synergy between factor V Leiden and oral contraceptive use (Vandenbroucke et al, 1994) and a previous report on obesity and the factor V Leiden mutation (Juul et al, 2004).

A possible limitation of our study is that height and weight were self-reported. If there would be a difference between patients and control subjects in over- or underreporting body weight or height an incorrect estimate of risk would be the result. There is no reason to expect such a difference in reporting behaviour between the two groups. The number of individuals who failed to report their body weight

was similar in patients (2.2%) and control subjects (2.6%). In general, overweight individuals tend to underreport and underweight individuals tend to overreport their body weight (Gunnell et al, 2000). If this phenomenon occurred the actual relative risks would even be higher.

Control subjects were drawn from two different sources. Because partners have similar lifestyles that may result in similar body mass indices, we performed a matched analysis that takes these associations into account. The matched analysis adjusted for all similar lifestyle factors between partners, which may include some unknown, unmeasured confounders resulting in lower risk estimates for the matched analysis compared to the unmatched analysis using the random digit dialing controls. Both analyses show consistent results in terms of clearly increased risks.

In conclusion, overweight and obesity are risk factors for venous thrombosis in this large population-based case-control study. Especially obesity in women using oral contraceptives is associated with a very high risk. The 24-fold increased risk should be considered when prescribing oral contraceptives for obese women.

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APPENDIX: COMBINING THE ESTIMATES OF THE CONDITIONAL AND UNCONDITIONAL LOGISTIC REGRESSION

Combining two estimates of the odds ratio

In our approach the two estimates of the log-odds ratio are combined into one overall log-odds ratio. Since both estimates use the same subset of cases, the estimates are correlated. The correlation between the two estimates is estimated using a sandwich estimator which is the commonly used estimator in statistics (Kauermann & Carroll, 2001). Details about this calculation are given later on in this appendix. The correlation is used to combine the two estimates in the most efficient way and to calculate the correct standard errors.

We consider first the case when there is only one parameter to combine. Let $\hat{\beta}_1$ and $\hat{\beta}_2$ be the estimated log odds ratios in the two different analyses with respective standard errors s_1 and s_2 and let $\hat{\rho}$ be the estimated correlation coefficient between the two estimates. In this case the combined estimate is a weighted mean of $\hat{\beta}_1$ and $\hat{\beta}_2$: $\hat{\beta}_{com} = w\hat{\beta}_1 + (1-w)\hat{\beta}_2$ with standard error

$$s_{com} = se(\hat{\beta}_{com}) = \sqrt{w^2 s_1^2 + (1-w)^2 s_2^2 + 2w(1-w)\rho\, s_1 s_2}.$$

It is straightforward to show that the optimal weight is given by $w = (s_2^2 - \hat{\rho} s_1 s_2)/(s_1^2 + s_2^2 - 2\hat{\rho} s_1 s_2)$.

In general, there are two multidimensional parameters $\theta_1 = (\alpha_1, \beta)$ and $\theta_2 = (\alpha_2, \beta)$, respectively. The k-dimensional β-parameter is the shared part. The parameters α_1 and α_2 of dimension k_1 and k_2, respectively, are not shared, for example because of different confounding variables in the two analyses, or because the effect of a confounder is expected to act differently in the two models.

Suppose that $\hat{\beta}_1$ and $\hat{\beta}_1$ are the two correlated estimates of the shared part β with covariance matrices $\mathrm{cov}(\hat{\beta}_1) = C_1$, $\mathrm{cov}(\hat{\beta}_2) = C_2$ and $\mathrm{cov}(\hat{\beta}_1, \hat{\beta}_2) = C_{12}$.

Then the most efficient estimate of β (the weighted least square estimate) is given by $\hat{\beta}_{com} = \left(\begin{pmatrix} I_k \\ I_k \end{pmatrix}^T \begin{pmatrix} C_1 & C_{12} \\ C_{21} & C_2 \end{pmatrix}^{-1} \begin{pmatrix} I_k \\ I_k \end{pmatrix} \right)^{-1} \begin{pmatrix} I_k \\ I_k \end{pmatrix}^T \begin{pmatrix} C_1 & C_{12} \\ C_{21} & C_2 \end{pmatrix}^{-1} \begin{pmatrix} \hat{\beta}_1 \\ \hat{\beta}_1 \end{pmatrix}$ with covariance matrix

$\mathrm{cov}(\hat{\beta}_{com}) = \left(\begin{pmatrix} I_k \\ I_k \end{pmatrix}^T \begin{pmatrix} C_1 & C_{12} \\ C_{21} & C_2 \end{pmatrix}^{-1} \begin{pmatrix} I_k \\ I_k \end{pmatrix} \right)^{-1}$. Here I_k is the k-dimensional identity matrix.

Estimation of the correlation between the two estimated odds ratios

In the general situation, there are two multidimensional parameters $\theta_1 = (\alpha_1, \beta_1)$ and $\theta_2 = (\alpha_2, \beta_2)$, respectively. Assume that both parameters are estimated by multiple regression models (in our situation θ_1 is estimated by conditional logistic regression and θ_2 by unconditional logistic regression.) When fitting this models by maximum likelihood we obtain the estimated parameters $\hat{\theta}_1 = (\hat{\alpha}_1, \hat{\beta}_1)$ and $\hat{\theta}_2 = (\hat{\alpha}_2, \hat{\beta}_2)$, the Fisher-information matrices $\mathbf{I}_1$ and $\mathbf{I}_2$ and the score matrices $\mathbf{U}_1$ and $\mathbf{U}_2$, where, generally $\mathbf{I} = \frac{\partial^2 l}{\partial \theta^2}$ and $U_{ij} = \frac{\partial l_i(\hat{\theta})}{\partial \theta_j}$ is the derivative of the log-likelihood contribution of individual *i* with respect to parameter θ_j.

Due to the overlap the estimated parameters $\hat{\theta}_1 = (\hat{\alpha}_1, \hat{\beta}_1)$ and $\hat{\theta}_2 = (\hat{\alpha}_2, \hat{\beta}_2)$ are dependent. Their covariance matrix can be estimated by a sandwich estimator: $\text{cov}(\hat{\theta}_1, \hat{\theta}_2) = \mathbf{I}_1^{-1}\, \mathbf{U}_{1,overlap}{}^T\, \mathbf{U}_{2,overlap} \mathbf{I}_2^{-1}$ using only the rows of $\mathbf{U}_1$ and $\mathbf{U}_2$ that correspond to the overlapping observations. From the estimated covariance matrix $\text{cov}(\hat{\theta}_1, \hat{\theta}_2)$ we can obtain the covariance matrix of the common part $\text{cov}(\hat{\beta}_1, \hat{\beta}_2)$.

CHAPTER 3

Smoking increases the risk of venous thrombosis and acts synergistically with oral contraceptive use

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ABSTRACT

The results of studies investigating the relationship of smoking with venous thrombosis are inconsistent. Therefore, in the MEGA study, a large population-based case-control study, we evaluated smoking as a risk factor for venous thrombosis and the joint effect with oral contraceptive use and the factor V Leiden mutation.

Consecutive patients with a first venous thrombosis were included from six anticoagulation clinics. Partners of patients were asked to participate and additional controls were recruited using a random digit dialing method. Participants completed a standardized questionnaire. Individuals with known malignancies were excluded from the analyses, leaving a total of 3989 patients and 4900 controls.

Current and former smoking resulted in a moderately increased risk of venous thrombosis (odds ratio $(OR)_{current}$ 1.43, 95% confidence interval (CI95) 1.28-1.60, OR_{former} 1.23, CI95 1.09-1.38) compared to non-smoking. Adjustment for fibrinogen levels did not substantially change these risk estimates. A high number of pack-years resulted in the highest risk among young current smokers ($OR_{\geq 20\ pack\text{-}years}$ 4.30, CI95 2.59-7.14) compared to young non-smokers. Women who were current smokers and used oral contraceptives had an 8.8-fold higher risk (OR 8.79, CI95 5.73-13.49) than non-smoking women who did not use oral contraceptives. Relative to non-smoking non-carriers, the joint effect of factor V Leiden and current smoking led to a 5.0-fold increased risk; for the prothrombin 20210A mutation this was a 6.0-fold increased risk.

In conclusion, smoking appears to be a risk factor for venous thrombosis with the greatest relative effect among young women using oral contraceptives.

INTRODUCTION

Venous thrombosis is a common and serious disorder with acquired and genetic risk factors [1]. Several of these risk factors are common for arterial and venous thrombosis, e.g. oral contraceptive use [2]. Factors that promote atherosclerosis are thought not to have an effect on venous thrombosis. Smoking is directly related to vessel-wall damage [3], but may also increase the risk of cardiovascular disease through other mechanisms, such as inflammation and increased fibrinogen levels [4-9]. These may lead to arterial as well as venous thrombotic disease. Results of studies investigating the relationship between smoking and venous thrombosis are inconsistent and vary from an adverse to a protective effect of smoking. In the 'The Nurses Health Study' a two-fold increased risk of pulmonary embolism was reported in women who smoked more than 35 cigarettes per day compared to never smokers [10]. 'The Study of Men born in 1913' reported a three-fold increased risk of venous thrombotic events in men smoking more than 15 cigarettes per day [11]. In contrast, The Framingham study showed that cigarette use had no association with pulmonary embolism found at autopsy [12]. A follow-up study of middle-aged and elderly individuals also found no effect of smoking on venous thrombosis [13]. In a case-control study from France regular smoking was protective for deep venous thrombosis of the leg [14]. This finding may be explained by the nature of the control group that consisted of individuals with influenzal or rhinopharyngeal syndrome, i.e. which may have had an excess of smokers. The reason for the discrepancy between the other study results is unclear.

A risk-increasing effect of smoking may be mediated through an increase in coagulation factors [8]. It is well known that smokers have higher fibrinogen levels [5-9] and that smoking cessation causes a rapid fall in plasma fibrinogen [6]. Elevated levels of fibrinogen were related to the risk of venous thrombosis in the 'The Leiden Trombophilia Study' (LETS), where we reported a 2.8-fold increased risk for individuals with fibrinogen levels above the 95th percentile (4.49 g/L) [15]. A case-control study among African-Americans found a 1.5-fold increased risk of venous thrombosis for fibrinogen levels above 5 g/L [16].

Since smoking is still common worldwide [17] it is important to address the contradictory study results and assess whether smoking affects the risk of venous thrombosis. In addition, the multicausal nature of venous thrombosis makes it important to investigate the effect of smoking in the presence of other risk factors. For arterial disease, smoking has been shown to act synergistically with oral contraceptive use [18]. Therefore we assessed the joint effect of smoking and oral contraceptive use on the risk of venous thrombosis. Factor V Leiden and the prothrombin mutation are the two most frequent prothrombotic mutations and are therefore

good candidates to investigate gene-environment interaction. To investigate the risk of venous thrombosis due to smoking, the possible role of fibrinogen in this relationship and the combination of smoking with oral contraceptive use, factor V Leiden and the prothrombin 20210A mutation, we performed a large population-based case-control study.

METHODS

Study Design

Between March 1999 and September 2004, we included consecutive patients with a first diagnosis of venous thrombosis. Patients were selected from the files of the Anticoagulation Clinics in Amsterdam, Amersfoort, The Hague, Leiden, Rotterdam and Utrecht. In the Netherlands, Anticoagulation Clinics monitor anticoagulation treatment in all patients in a geographically well-defined area. Patients between the age of 18 and 70 with deep venous thrombosis of the leg, pulmonary embolism or a combination of these diagnoses were included. The diagnostic methods were verified in a random sample of the overall patient group (n=742). Within this group the diagnosis of 97% of deep venous thrombosis and 78% of pulmonary embolism had been objectively confirmed. The tests included compression ultrasonography, Doppler ultrasound, impedance plethysmography and contrastvenography for the diagnosis of deep venous thrombosis and perfusion and ventilation lung scanning, spiral computer tomography and pulmonary angiography for pulmonary embolism.

Patients with severe psychiatric problems or those unable to speak Dutch were considered as ineligible. Of the 6331 eligible patients, 276 died soon after the venous thrombosis. Of the remaining 6055 patients 5051 participated (83%). Of the non-participants 82 persons were in the end stage of disease and 922 refused to participate or could not be located. Of the participants, 4637 (77%) patients returned the questionnaire. Participants who did not return a questionnaire completed a short questionnaire by phone, which did not include questions on smoking habits.

Partners of patients were asked to volunteer as control subjects. Of the 5051 participating patients, 3657 had an eligible partner. One partner died soon after the request for participation. Of the remaining 3656 partners, 2982 participated (82%). Of the non-participants 18 were in end-stage disease, 649 refused to participate or could not be located and for seven persons the reason for non-participation was unknown. A questionnaire was returned by 2821 participating partners (77%).

From January 2002 until September 2004, additional control subjects were recruited by random digit dialing (RDD) [21]. Phone numbers were dialed at random within the geographical inclusion area of the patients. The random controls were frequency matched to the patients with respect to age and sex. Only control subjects between the age of 18 and 70 years with no history of deep venous thrombosis were included and the same exclusion criteria were applied as for the patients.

Of the 4350 eligible random control subject, four died before they were able to participate. Of the remaining 4346 persons 3000 participated (69%). Of the non-participants 15 were in the end stage of disease and 1331 refused to participate or could not be located. A questionnaire was returned by 2789 participating random control subjects (64%).

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

Data collection

Within a few weeks after diagnosis and registration at the anticoagulation clinics patients received a letter with information about the study and were subsequently contacted by phone. Both patient and control subjects received the questionnaire shortly after inclusion. The questionnaires included items on smoking habits, body weight and body height, malignancies, pregnancies and use of oral contraceptives. Most questions referred to a period of 12 months prior to the index date, i.e. the date of diagnosis of the thrombosis of the patient for patients and partners and the date of filling in the questionnaire for the random control subjects.

When someone reported to smoke one cigarette per month or more the person was considered a smoker. Smokers were asked to report the age at which they started smoking, the age they quitted smoking, if there was a period in-between they did not smoke and the (cumulative) duration of such periods. Smokers were divided in current, former and never smokers. When the difference between the age at index date and the age of smoking cessation was 1 year or less, the person was considered a current smoker. The average number of cigarettes, self-rolled cigarettes, cigars or pipes smoked per day was also asked for. Because only a minor difference was found between different types of smoking and their risk of venous thrombosis, cigar and pipe smoking were included in the analysis by arbitrarily counting 1 cigar as 3 cigarettes and 1 pipeful as 2 ½ cigarettes. Several individuals wrote down the number of packages instead of the number of cigarettes smoked. In this case, the number of cigarettes was calculated with one package counted as 20 cigarettes. For smokers of self-rolled cigarettes one package was counted as 50

cigarettes. Pack-years were defined as the average number of cigarettes per day divided by 20 and multiplied by the number of smoking years.

Individuals with malignancies diagnosed within 10 years before the index date (active malignancies) were excluded from all analyses. In addition, participants with missing data regarding items of the smoking questions, body weight and height or pregnancy were excluded from the analyses. In the analyses only partner controls with a participating patient were included, leading to a total of 3989 patients, 2288 partner and 2612 random control subjects in the present analyses.

Blood collection

At least three months after withdrawal of anticoagulation the patients and their partners were asked to visit the anticoagulation clinic after an overnight fast and a blood sample was drawn. Only in case of continuous use for more than one year a blood sample was taken during anticoagulation therapy. From December 1999 onwards, we obtained self-administered buccal swabs by mail when participants were unable or unwilling to come for a blood draw. From June 2002 onwards, blood draws were no longer performed in patients and their partners, and the study was restricted to DNA collection by buccal swabs sent by mail. The random controls were invited for a blood draw within a few weeks after the questionnaire was sent. Within this group buccal swabs were sent when someone refused the blood draw. During the blood draws information on smoking habits after the index date was obtained. In case of DNA collection by mailed buccal swabs a short interview was performed by phone.

Within the patient group 3745 provided a blood sample or buccal swab (94%). In the control subjects 4004 blood samples or buccal swabs were obtained (82%). Genotyping was successful in 3739 patients and 3983 control subjects for factor V Leiden and in 3739 patients and 3984 control subjects for the prothrombin 20210A mutation [22]. Fibrinogen levels were successfully determined in all blood samples, consisting of 2118 patient en 2485 control samples. Fibrinogen activity was measured according to the method of Clauss [23]. Calibration was performed using STA preciclot plus I en II. The intra-assay coefficient of variation (CV) was 1.81, the inter-assay CV was 3.78.

Statistical analysis

As estimates of relative risks we calculated odds ratios (ORs) and 95% confidence intervals (CI95) according to the method of Woolf [24]. With a multiple logistic regression model ORs were adjusted for age (continuous), sex (categorical), body

mass index (BMI=kg/m^2) (continuous) and pregnancy (categorical). Adjustment for age (10 categories) and body mass index (8 categories) as categorical variables resulted in approximately the same risk estimates. In the analyses with partners as the control group, we performed a matched analysis to adjust for similar lifestyle factors between patients and their partners (2288 pairs) [25]. In the analyses with the random control subjects an unmatched analysis including all patients and random control subjects was performed. Because the results of the matched and unmatched analyses showed consistently elevated relative risks in all the analyses, we calculated pooled risk estimates with a method that combines the matched and unmatched analyses. This analysis takes into account the presence of 2288 patients in both the matched and unmatched analyses (see appendix chapter 2). When analyzing the risk in men and women separately it was not possible to perform a matched analysis with the partner controls, as control individuals were nearly always of the opposite sex. Therefore, risk estimates were calculated with an unmatched analysis with all patients and the random control subjects.

In the analyses adjusted for fibrinogen levels (categorical), individuals who quitted smoking after the index date but before the blood draw were included in the former smoking category. Individuals who started smoking in the period between the index date and the blood draw were included as current smokers. To further remove any effect of starters and quitters, we restricted an analysis adjusted for fibrinogen levels to individuals who consistently either smoked or did not smoke at the index date and the time of the blood draw. SAS 9.1 (SAS institute Inc, Cary, NC, USA) was used for all statistical analyses.

RESULTS

In these analyses data on 3989 patients and 4900 control subjects were included. Mean age of patients was 47.5 (5^{th}-95^{th} percentiles, 25.3-67.4) and of control subjects 46.0 (5^{th}-95^{th} percentiles, 25.1-66.2) years old. Fifty five percent (n=2185) of patients and 53% (n=2606) of control subjects were women. In the patient group 58% (n=2305) was diagnosed with deep venous thrombosis of the leg, 29% (n=1168) with pulmonary embolism and 13% (n=516) with the combination.

In table 1 relative risks of venous thrombosis with smoking status are presented. Among patients 37% was current and 28% was former smoker, in the control subjects 32% was current and 28% was former smoker. Current and former smoking were both associated with a moderately increased risk of venous thrombosis compared with never smoking ($OR_{current}$ 1.43, CI95 1.28-1.60, OR_{former} 1.23, CI95 1.09-1.38). The table presents the pooled odds ratios with both control groups. The effects

Table I. Relative risk of venous thrombosis by smoking status

Smoking status	Patients	Partners	RDD	$OR_{Combined}$[a] (CI95)	$OR_{Combined}$[b] (CI95)	$OR_{Combined}$[c] (CI95)
Never	1391	867	1109	1	1	1
Former	1136	665	692	1.23 (1.09-1.38)	1.20 (1.03-1.41)	1.22 (1.04-1.43)
Current	1462	756	811	1.43 (1.28-1.60)	1.40 (1.19-1.63)	1.34 (1.15-1.57)

RDD, random digit dialing controls; OR, odds ratio; CI, confidence interval.
[a]Adjusted for age, sex, BMI and pregnancy.
[b]Adjusted for age, sex, BMI and pregnancy in participants with measured fibrinogen levels (53% of patients, 50% of control subjects).
[c]Adjusted for age, sex, BMI, pregnancy *and fibrinogen levels* in participants with measured fibrinogen levels.

contrasting the patients to each control group separately did not materially differ from the pooled results (current smoking, partner controls OR 1.20, CI95 1.01-1.44; current smoking, RDD controls OR 1.52, CI95 1.34-1.71; former smoking, partner controls OR 1.33, CI95 1.13-1.56; former smoking, RDD controls OR 1.17, CI95 1.03-1.33). To investigate causal mechanisms we adjusted the associations

Table II. Relative risk of venous thrombosis by smoking status in different subgroups

	Patients	Control subjects	OR[a] (CI95)
DVT			
Never	802	1976	1
Former	642	1357	1.18 (1.02-1.36)
Current	861	1567	1.50 (1.31-1.71)
PE			
Never	391	1976	1
Former	349	1357	1.38 (1.15-1.64)
Current	428	1567	1.53 (1.29-1.80)
DVT+PE			
Never	198	1976	1
Former	145	1357	0.99 (0.78-1.27)
Current	173	1567	1.21 (0.96-1.53)
All VT_{women}[b]			
Never	877	706	1
Former	516	334	1.22 (1.02-1.46)
Current	792	443	1.55 (1.33-1.82)
All VT_{men}[b]			
Never	514	403	1
Former	618	358	1.03 (0.85-1.26)
Current	669	368	1.42 (1.18-1.71)

DVT, deep venous thrombosis; PE, pulmonary embolism; OR, odds ratio; CI, confidence interval; [a]adjusted for age, sex, BMI and pregnancy; [b]three patients were not included in these analyses because two were transsexuals and one had Klinefelter syndrome, this analysis is performed using the random control subjects only

for fibrinogen levels. We found slightly attenuated risk estimates after adjustment (table 1). Adjustment for fibrinogen in the analyses comparing consistent current smokers (at the index date and time of blood draw) to consistent non-smokers, resulted in only slightly lower risk estimates than before adjustment ($OR_{current}$ 1.46, CI95 1.25-1.71; $OR_{current, adj}$ 1.41, CI95 1.20-1.65).

For pulmonary embolism and deep venous thrombosis, current smoking resulted in the same relative risk (OR_{PE} 1.53, CI95 1.29-1.80; OR_{DVT} 1.50, CI95 1.31-1.71). Former smoking was associated with a higher relative risk of pulmonary embolism (OR 1.38, CI95 1.15-1.64) than of deep venous thrombosis (OR 1.18, CI95 1.02-1.36) (table 2). Also, smoking increased the risk of thrombosis more in women than men.

In table 3 relative risks are presented for the number of cigarettes smoked per day and the number of smoking-years. In current smokers, daily amount smoked was associated with the risk of venous thrombosis in a dose-dependent manner. Smoking 20 or more cigarettes per day resulted in a 1.6-fold increased risk among current smokers compared to never smokers (OR 1.64, CI95 1.41-1.90). No dose

Table III. Relative risk of venous thrombosis by number of cigarettes smoked per day and smoking period

Smoking amount (cigarettes/day)	Patients	Control subjects	OR[a] (CI95)
Current			
never	1391	1696	1
1-9	242	277	1.23 (1.00-1.50)
10-19	524	528	1.41 (1.21-1.64)
≥ 20	676	589	1.64 (1.41-1.90)
Former			
never	1391	1781	1
1-9	286	321	1.20 (1.00-1.45)
10-19	372	400	1.22 (1.03-1.45)
≥ 20	444	474	1.08 (0.92-1.28)
Smoking period (years)			
Current			
never	1391	1689	1
1-9	162	166	1.54 (1.19-2.01)
10-19	279	324	1.37 (1.13-1.67)
≥ 20	935	834	1.46 (1.27-1.67)
Former			
never	1391	1748	1
1-9	226	243	1.33 (1.08-1.64)
10-19	325	382	1.09 (0.91-1.31)
≥ 20	425	420	1.11 (0.93-1.32)

OR, odds ratio; CI, confidence interval; [a]adjusted for age, sex, BMI and pregnancy

Table IV. Relative risk of venous thrombosis by number of pack-years in three age categories (tertiles)

Pack-years	OR[a] (CI95)	OR[a] (CI95)	OR[a] (CI95)
Current smokers	< 37.8 yrs	37.8-51.1 yrs	≥ 51.1 yrs
never	1	1	1
1-9	1.38 (1.07-1.77)	0.94 (0.66-1.36)	1.25 (0.76-2.07)
10-19	2.76 (1.99-3.83)	1.32 (0.97-1.79)	1.06 (0.71-1.59)
≥ 20	4.30 (2.59-7.14)	1.34 (1.05-1.72)	1.14 (0.91-1.42)

OR, odds ratio; CI, confidence interval; [a]adjusted for age, sex, BMI and pregnancy
Note: The pack-year analyses were performed in three different age categories because the number of pack-years was dependent on the age of the participants. We established the categories by dividing the age distribution of the current smokers into tertiles.

Table V. Combined effect of smoking status with oral contraceptive (OC) use on the risk of venous thrombosis in women aged 18 to 39

Smoking status	OC use	Patients	Control subjects	OR[a] (CI95)
Never	no	105	168	1
Former	no	54	52	1.63 (1.00-2.67)
Current	no	87	93	2.03 (1.33-3.11)
Never	yes	257	189	3.90 (2.63-5.79)
Former	yes	82	40	4.83 (2.89-8.08)
Current	yes	271	94	8.79 (5.73-13.49)

OR, odds ratio; CI, confidence interval; [a]adjusted for age, BMI and pregnancy;
Note: The OC analyses were performed with random control subjects only

response relation was found for the number of smoking-years in either current or former smokers.

Table 4 shows the effects of the number of pack-years for three age categories in current smokers. In the youngest age category the risk of thrombosis increased with pack-years smoked, with a 4.3-fold increased risk for smokers with 20 or more pack-years (OR 4.30, CI95 2.59-7.14). In those aged over 38, we saw no association between pack-years and the risk of venous thrombosis.

We also investigated the joint effect of smoking with oral contraceptive use in women aged 18 to 39 years (table 5). Among non-users, smoking was associated with a 2.0-fold increased risk. Women who used oral contraceptives and did not smoke had a 3.9-fold increased risk, while those who also smoked had an 8.8-fold increased risk (compared to never smokers not using oral contraceptives).

Among non-carriers of factor V Leiden current smoking resulted in a 1.4-fold increased risk. The joint effect of factor V Leiden and current smoking resulted in a 5.0-fold increased risk compared to never smokers without the mutation (table 6). For current smokers with the prothrombin 20210A mutation the risk of venous thrombosis increased 6.0-fold compared to never smokers without the mutation.

Table VI. Combined effect of smoking status with factor V Leiden (FVL) and the prothrombin (FII) 20210A mutation on the risk of venous thrombosis

Smoking status	FVL	Patients	Control subjects	OR[a] (CI95)
Never	no	1085	1375	1
Former	no	930	1017	1.21 (1.06-1.39)
Current	no	1106	1048	1.43 (1.26-1.63)
Never	yes	234	70	3.41 (2.53-4.58)
Former	yes	161	41	3.76 (2.58-5.49)
Current	yes	223	42	5.05 (3.46-7.38)
	FII 20210A	Patients	Control subjects	OR[a] (CI95)
Never	no	1238	1517	1
Former	no	1039	1111	1.21 (1.06-1.37)
Current	no	1269	1168	1.41 (1.25-1.60)
Never	yes	81	16	3.17 (1.94-5.18)
Former	yes	52	11	3.01 (1.60-5.68)
Current	yes	60	5	6.06 (2.67-13.76)

OR, odds ratio; CI, confidence interval; [a]adjusted for age, sex, BMI and pregnancy; Note: the inclusion of matched case control pairs in the analyses was dependent on the category (BMI, FVL; BMI, FII) of both individuals.

DISCUSSION

In this large population-based case-control study smoking was associated with a moderately increased risk of venous thrombosis, in current and former smokers. In current smokers, who had the highest risk, the risk increased with the amount of smoking. This is in accordance with the results of two follow-up studies, 'The Nurses Health Study' and 'The study of Men born in 1913' [10,11]. These studies, as well as those that did not found an association [12,13], all included less than 700 patients with venous thrombosis, while ours included almost 4000 patients.

We assessed the number of years someone had smoked, and found no association with thrombotic risk. It seems that the effect of smoking on venous thrombosis is largely an acute effect. This is illustrated by the presence of a dose response relationship between the amount of smoking and thrombotic risk in current smokers. Furthermore this is supported by the absence of a dose response relationship for smoking duration, the higher risk estimates in current compared to former smokers and the finding of a dose response relationship with pack-years in young individuals only.

In our study, the risk estimates in the current smoking category may be somewhat underestimated, because we included persons who quit smoking up to one year before the index date in the current smoking group. In case people underreported the amount of smoking some non-differential misclassification may also have occurred.

Former smoking resulted in a more pronounced risk of pulmonary embolism than of deep venous thrombosis of the leg. This finding may reflect local inflammatory effects in the lungs. The effect of smoking was also more pronounced in women than men. An explanation is our finding of a synergistic effect of smoking with oral contraceptive use, which is in accordance with the results of studies on myocardial infarction [18]. An evaluation of the effects of oral contraceptives on coagulation in smokers compared to non-smokers showed that changes in coagulation in women taking oral contraceptives were mainly evident in smoking women [19].

To investigate a mechanism for the association between smoking and venous thrombosis we adjusted our analyses for fibrinogen levels, hypothesizing that the risk was mediated via elevated fibrinogen levels. This adjustment, however, resulted only in slightly decreased risk estimates for current smoking, and therefore fibrinogen levels are not a crucial part of the mechanism. The question remains which other factors affected by smoking lead to the increased risk of venous thrombosis. A study that investigated the effect of smoking on the coagulation system found increased levels of factor VII, prothrombin, factor XI peptide and factor X peptide in smokers [8]. Besides coagulation factors, inflammatory factors may be involved. Interleukine-6 has been shown to be elevated in smokers [4] and is also associated with the risk of recurrent venous thrombosis [20].

Control subjects were drawn from two different sources. There were only minor differences when estimates were obtained with each control group separately. These differences are likely to be chance variations, although minor true differences cannot be ruled out, possibly related to differences in non-response.

In conclusion, in this large population-based case-control study we found smoking to be a moderate risk factor for venous thrombosis, that acts synergistically with oral contraceptive use. The joint effect of smoking with the factor V Leiden mutation or the prothrombin 20210A mutation was also slightly higher than the sum of the separate effects. Our findings suggest that fibrinogen levels are not an important mediator of the effect of smoking on the risk of venous thrombosis.

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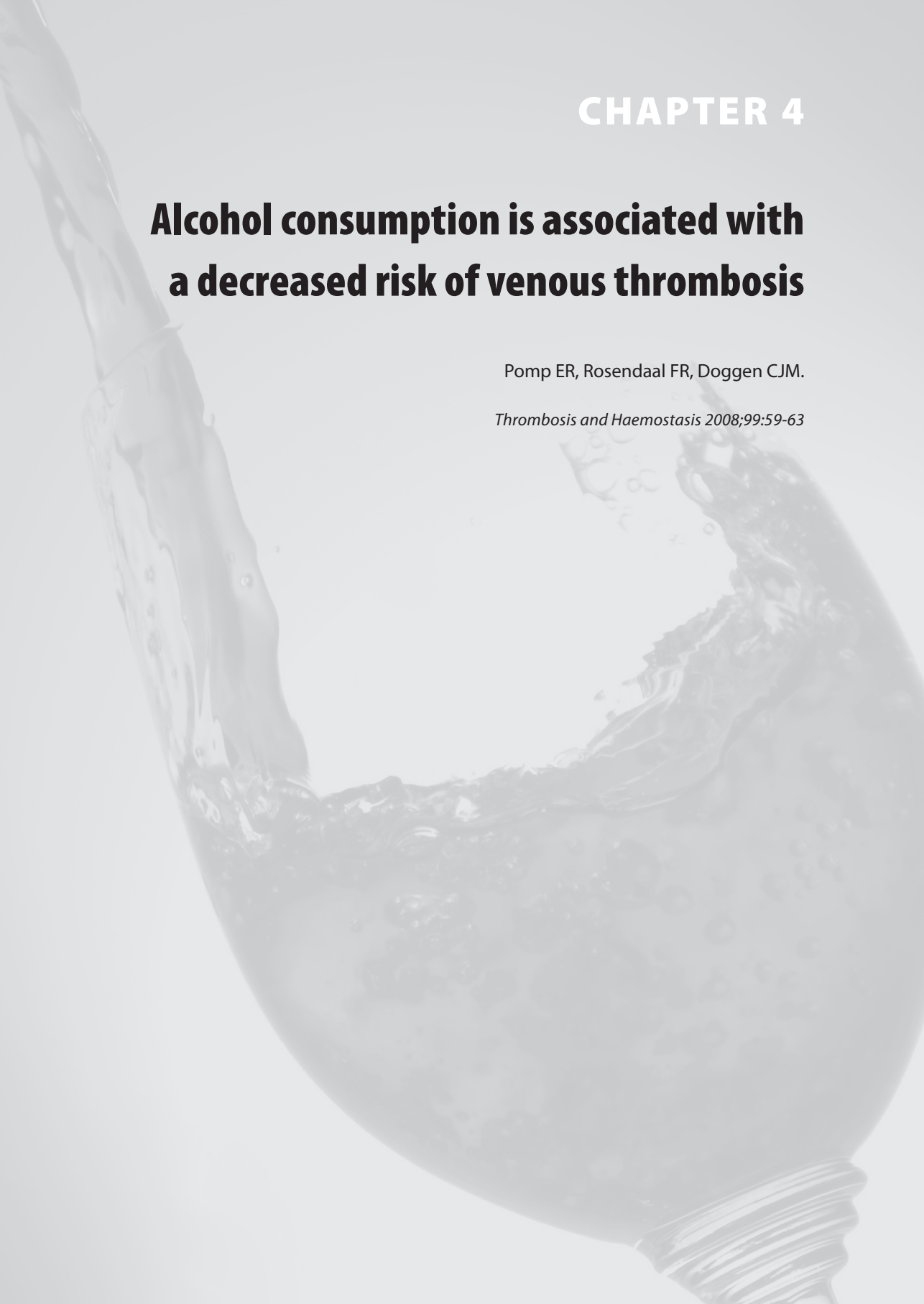
CHAPTER 4

Alcohol consumption is associated with a decreased risk of venous thrombosis

Pomp ER, Rosendaal FR, Doggen CJM.

Thrombosis and Haemostasis 2008;99:59-63



SUMMARY

Moderate alcohol consumption is associated with lower levels of several coagulation factors. It is an established protective factor for cardiovascular disease; however the effect on venous thrombosis is unknown. In a large population-based case-control study, we evaluated the association between alcohol consumption and the risk of venous thrombosis.

The MEGA study included consecutive patients with a first venous thrombosis between March 1999 and September 2004 from six anticoagulation clinics in the Netherlands. Partners of patients were asked to participate and additional controls were recruited using a random digit dialing method. All participants completed a standardized questionnaire and blood samples were collected. A total of 4423 patients and 5235 controls were included in the analyses.

Alcohol consumption was associated with a reduced risk of venous thrombosis, with two to four glasses per day resulting in the largest beneficial effect (Odds Ratio (OR) 0.67, 95% confidence interval (CI95) 0.58-0.77) compared to abstainers. The effect was more pronounced in women (OR 0.66, CI95 0.53-0.84) than men (OR 0.82, CI95 0.63-1.07) and also more striking for pulmonary embolism (OR 0.56, CI95 0.46-0.70) than for deep venous thrombosis of the leg (OR 0.74, CI95 0.63-0.88).

Compared to abstainers, fibrinogen levels were decreased in individuals who consumed alcohol (maximum decrease: 0.30 g/l). Factor VII and von Willebrand levels were mildly decreased in these individuals but not consistently over the categories of alcohol consumption.

In conclusion, alcohol consumption is associated with a reduced risk of venous thrombosis, which may be in part mediated by decreased fibrinogen levels.

INTRODUCTION

The protective effect of moderate and the harmful effect of heavy alcohol consumption on the risk of arterial disease has been shown in many epidemiological studies (1). Similar effects of alcohol consumption on the risk of venous thrombosis are also not unlikely considering the effect of alcohol consumption on coagulation factors. A systematic review reported an association between moderate alcohol intake and reduced levels of fibrinogen, factor VII and von Willebrand factor, whereas heavy and binge alcohol drinking was associated with increased levels of fibrinogen and factor VII (2).

Few studies have reported on the relationship between alcohol consumption and venous thrombosis. In an Italian cohort study of elderly individuals low to moderate alcohol consumption appeared beneficial with relative risks of 0.7 for less than one drink a month, 0.6 for less than one ounce per day and 0.5 for one or more than one ounce per day (3). In contrast, two US cohort studies found no effect of alcohol consumption on the risk of venous thrombosis (4, 5). In these cohort studies alcohol intake was only assessed at baseline and variations of alcohol intake during follow-up may have resulted in misclassification of alcohol levels and spurious estimates. In a French case-control study no effect of alcohol consumption was found (6). In this study, the control group consisted of patients with influenzal or rhino pharyngeal symptoms in whom alcohol consumption may differ from the base population of cases. Alcohol is consumed regularly by 2 billion people worldwide (7), which makes it important to elucidate the relationship between alcohol consumption and the risk of venous thrombosis. In the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA study), a large population-based case-control study, we investigated alcohol use as a risk factor for venous thrombosis. In addition we analyzed the association of alcohol consumption with fibrinogen, factor VII and von Willebrand factor levels to verify if a protective effect could be explained by changes in these coagulation parameters.

METHODS

Study Design

Details of the MEGA study have been published (8). Between March 1999 and September 2004, consecutive patients with a first deep venous thrombosis of the leg or a pulmonary embolism were included from six anticoagulation clinics.

All patients were between the age of 18 and 70. Patients with severe psychiatric problems or those unable to speak Dutch were considered as ineligible. Partners of patients were asked to participate as control subjects. From January 2002 until September 2004, an additional control group was recruited using a random digit dialing method. Phone numbers were dialed at random within the geographical inclusion area of the patients. During the phone call a specific person within a household (e.g. youngest woman between 20 and 50) was asked to participate. The random control subjects were frequency matched to the patients with respect to age and sex. Only control subjects with no recent history of venous thrombosis were included and the same exclusion criteria applied as for the patients.

Among the 6055 eligible patients 5051 participated (83%). Of the 5051 participating patients, 3656 had an eligible partner of whom 2982 participated (82%). Of the 4346 eligible random control subjects 3000 participated (69%).

All participants gave written informed consent and the study was approved by the Medical Ethics Committee of the Leiden University Medical Center, Leiden, The Netherlands.

Data collection

Within a few weeks after diagnosis and registration at the anticoagulation clinics patients received a letter with information about the study and were subsequently contacted by phone. If the patient was willing to participate a questionnaire was sent. The control subjects received the questionnaires immediately after inclusion by phone. The questionnaire was returned by 4637 patients (77%), 2821 partners (77%) and 2789 random control subjects (64%). The participants who did not return a questionnaire were asked questions by phone. This short interview did not include questions regarding alcohol consumption.

The question referring to alcohol consumption included ten categories of alcohol consumption: none, 1 glass or less per week, 2 to 6 glasses per week, 1 glass per day, 2 to 4 glasses per day, 5 to 9 glasses per day, 10 to 19 glasses per day, 20 to 29 glasses per day, 30 to 39 glasses per day and 40 or more glasses per day. Because only 85 individuals filled in an amount in the highest four categories of alcohol consumption these categories were taken together in the analysis. The questionnaire did not ask about kind of alcohol used. Participants who returned the questionnaire with missing data on alcohol consumption, body weight or height, smoking or pregnancy were excluded from all analyses. The total excluded proportion was the same in patients (4.6%) and control subjects (4.7%). In the analyses only partner controls with a participating patient were included leading to a total of 4423 patients, 2576 partner and 2659 random control subjects for the present analyses.

Blood collection

At least three months after withdrawal of anticoagulation the patients and their partners were asked to visit the anticoagulation clinic after an overnight fast where a blood sample was drawn. Only in case of continuous use for more than one year a blood sample was taken during anticoagulation therapy. From December 1999 onwards, we obtained self-administered buccal swabs by mail when participants were unable or unwilling to come for a blood draw. From June 2002 onwards, blood draws were no longer performed in patients and their partners, and the study was restricted to DNA collection by buccal swabs sent by mail. The random controls were invited for a blood draw within a few weeks after the questionnaire was sent. Within this group buccal swabs were sent when someone refused the blood draw.

In the control subjects 2614 blood samples were obtained (50%). Fibrinogen, von Willebrand factor and factor VII were successfully determined in 2612 samples. Fibrinogen activity was measured on the STA-R analyzer according to methods of Clauss (9). The intra-assay coefficient of variation (CV) was 1.8, the inter-assay CV was 3.8. Factor VII activity (FVII) was measured with a mechanical clot detection method on the STA-R analyzer following the instructions of the manufacturer (Diagnostica Stago, Asnieres, France). The intra-assay CV was 3.4, the inter-assay CV was 4.0. Von Willebrand factor antigen (vWF) was measured with the immuno-turbidimetric method, using the STA liatest kit (rabbit anti-human vWF antibodies), following the instructions of the manufacturer. For vWF the intra- and inter-assay CV were 3.6 and 2.6.

Statistical analysis

Odds ratios (OR) were calculated as estimates of the relative risk with 95% confidence intervals (CI95) according to the method of Woolf. Using a multiple logistic regression model ORs were adjusted for age (continuous), sex (categorical), body mass index (BMI=kg/m^2) (continuous), pregnancy (categorical) and smoking (categorical). Adjustment for age (10 categories) and body mass index (8 categories) as categorical variables resulted in approximately the same risk estimates. Additional adjustment for disease history, including malignancies, did not change the risk estimates. In the analyses with the random control subjects an unmatched analysis including 4423 patients and 2659 random control subjects was performed. In the analyses with partners as the control group (2576 pairs), we performed a matched analysis which adjusts for similar lifestyle factors between patients and their partners (10). Because the results of the matched and the unmatched analyses showed consistently protective relative risks in all analyses, we calculated pooled

risk estimates with a method that combines the matched and unmatched analyses (11). When analyzing the risk in men and women separately it was not possible to perform a matched analysis with the partner controls, therefore risk estimates were calculated with an unmatched analysis with all patients and the random control subjects.

A χ^2-test was used to compare alcohol consumption between patients with deep venous thrombosis of the leg with those in patients with a pulmonary embolism.

SAS 9.1 (SAS institute Inc, Cary, NC, USA) was used for all statistical analyses.

RESULTS

In the current analysis 4423 patients with a first venous thrombosis and 5235 control subjects were included. Mean age of the patients was 48.5 years (5^{th}-95^{th} percentiles, 25.8-67.7) and the control subjects were on average 46.8 years (5^{th}-95^{th} percentiles, 25.4-66.4). In the patient and in the control group 54% were women ($n_{patient}$=2400, $n_{control}$=2816). In the patient group 57% (n=2528) was diagnosed with deep venous thrombosis of the leg, nearly a third (n=1340) with pulmonary embolism and 13% (n=555) with the combined diagnosis of deep venous thrombosis and pulmonary embolism.

Figure 1 shows the relationship between alcohol consumption and the risk of venous thrombosis. Moderate alcohol consumption was associated with a decreased

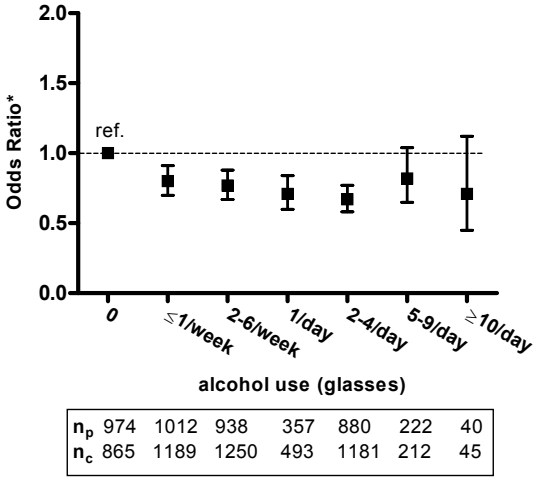


Figure 1. Relative risk of venous thrombosis by level of alcohol consumption
*adjusted for age, sex, body mass index, smoking and pregnancy;
I, CI95; n_p, number of patients; n_c, number of control subjects;
ref., reference category

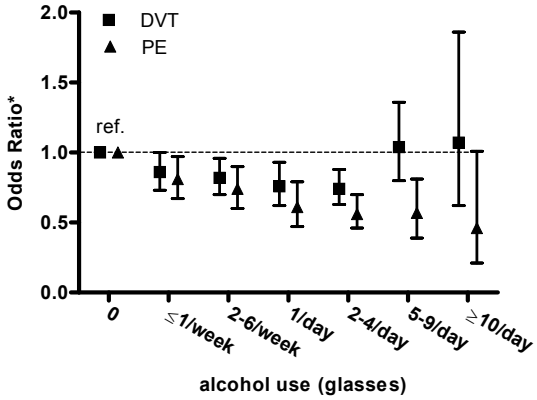


Figure 2. Relative risk of deep venous thrombosis (DVT) and pulmonary embolism (PE) by level of alcohol consumption
*adjusted for age, sex, body mass index, smoking and pregnancy;
I, CI95; ref., reference category

risk of venous thrombosis, with two to four glasses per day resulting in the strongest effect on the risk of venous thrombosis ($OR_{2\text{-}4/day}$ 0.67, CI95 0.58-0.77) compared to abstainers. Even drinking more than four glasses per day appeared to be still somewhat protective ($OR_{5\text{-}9/day}$ 0.82, CI95 0.65-1.04, $OR_{\geq 10/day}$ 0.71, CI95 0.45-1.12). Moderate alcohol consumption was associated with a somewhat more decreased risk for women ($OR_{2\text{-}4/day}$ 0.66, CI95 0.53-0.84) than men ($OR_{2\text{-}4/day}$ 0.82, CI95 0.63-1.07).

The associations of alcohol consumption with the risk of deep venous thrombosis and pulmonary embolism separately are presented in figure 2. The protective effect of alcohol consumption appeared to be more pronounced for the diagnosis of pulmonary embolism ($OR_{2\text{-}4/day}$ 0.56, CI95 0.46-0.70) than for deep venous thrombosis ($OR_{2\text{-}4/day}$ 0.74, CI95 0.63-0.88). Drinking two glasses per week or more clearly protected more against pulmonary embolism than against deep venous thrombosis (P=0.02).

In figure 3 mean fibrinogen, factor VII and von Willebrand factor levels are presented for different categories of alcohol consumption in control subjects. Alcohol consumption categories which were associated with the most pronounced reduction in venous thrombotic risk were also associated with reduced levels of fibrinogen. Factor VII levels and von Willebrand factor levels were also lower in drinkers than non-drinkers, but there were no consistent patterns over the categories of alcohol consumption.

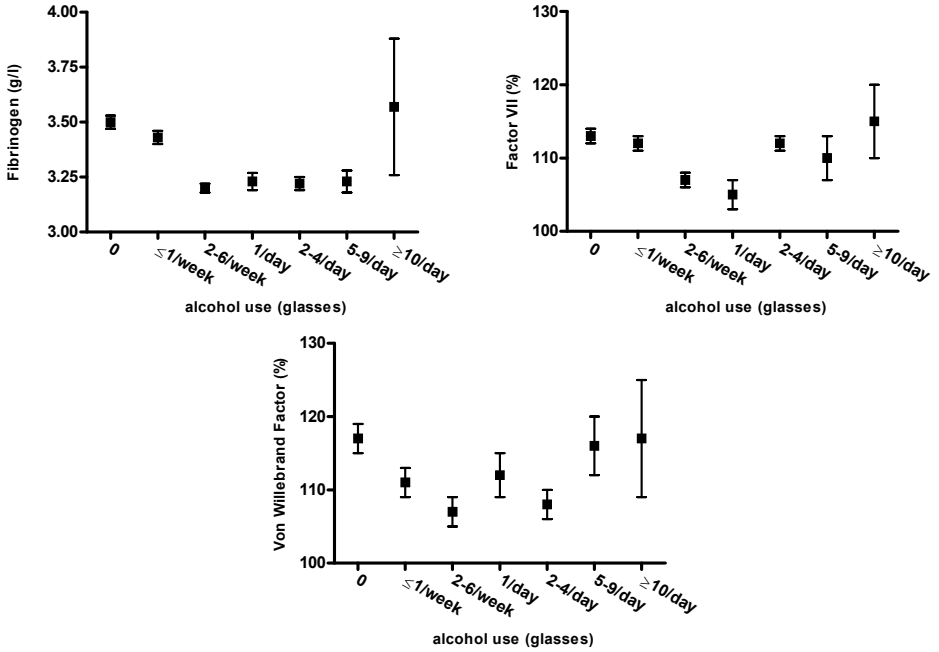


Figure 3. Mean levels of haemostatic variables according to alcohol consumption in control subjects
I, standard error of the mean
N=2612

DISCUSSION

In this large case-control study moderate alcohol consumption was associated with a decreased risk of venous thrombosis. This potential beneficial effect of moderate alcohol consumption appeared to be more pronounced in women than men and for pulmonary embolism than for deep venous thrombosis of the leg.

Very little is known about the mechanisms by which alcohol may exert anti-thrombotic effects. Some studies have shown that moderate alcohol consumption is associated with a more favorable coagulation profile, indicated by lower levels of fibrinogen, factor VII and von Willebrand factor (12, 13). In accordance with these studies we found reduced levels of fibrinogen, factor VII and von Willebrand factor in moderate alcohol drinkers. Reduced levels were most striking for fibrinogen, where levels were even decreased up to high levels of alcohol consumption (5-9 glasses per day).

The difference between men and women in the alcohol-related risk of venous thrombosis may be explained by the differential effects of wine and beer (14), the latter of which is consumed more by men than women. Unfortunately, in our study we had no information about the kind of alcoholic drinks the participants con-

sumed. A question about type of alcoholic drinks could have provided important additional information. A recent study however, showed that wine, beer and spirits were to the same extend protective for myocardial infarction, suggesting that type of alcohol drink did not influence the effect (15).

It was striking that the protective effect of drinking two or more glasses of alcohol per week was higher for pulmonary embolism than for deep venous thrombosis of the leg. We do not have an explanation for these findings.

A limitation of our study is that alcohol consumption was self-reported. Although it is possible that individuals underreport alcohol consumption, this is mainly a problem when there is a difference between patients and control subjects in reporting behavior. If patients and controls both had underreported, the protective effect we observed was underestimated. If patients underreported more than controls, the true effect would have been less pronounced. The proportion of individuals who failed to report their alcohol consumption was the same in patients (0.71%) and controls (0.66%), which suggests that the two groups behaved similarly in answering this question.

In conclusion, in this large population-based case-control study alcohol consumption is associated with a decreased risk of venous thrombosis, with two to four glasses per day resulting in the largest effect. This effect may be mediated by a decrease in coagulation factors, especially fibrinogen.

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J.W. Blom, MD, A. van Hylckama Vlieg, PhD, L.W. Tick, MD, and K.J. van Stralen, MSc took part in every step of the data collection. R. van Eck, J. van der Meijden, P.J. Noordijk, and T. Visser performed the laboratory measurements. H.L. Vos, PhD supervised the technical aspects of DNA analysis. We thank S. le Cessie, PhD for her support with the statistical analyses. We express our gratitude to all individuals who participated in the MEGA study. This research was supported by

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CHAPTER 5

Pregnancy, the postpartum period and prothrombotic defects: risk of venous thrombosis in the MEGA study

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SUMMARY

Background: Venous thrombosis is one of the leading causes of maternal morbidity and mortality.

Objective: In the MEGA study, we evaluated pregnancy and the postpartum period as risk factors for venous thrombosis in 285 patients and 857 control subjects. Patients/Methods: Between March 1999 and September 2004, consecutive patients with a first episode of venous thrombosis were included from six anticoagulation clinics. Partners of patients and a random digit dialing group were included as control subjects. Participants completed a questionnaire and DNA was collected.

Results: The risk of venous thrombosis was five-fold (OR 4.6, 95%CI 2.7-7.8) increased during pregnancy and sixty-fold (OR 60.1, 95%CI 26.5-135.9) increased during the first three months after delivery compared to non-pregnant women. A 14-fold increased risk of deep venous thrombosis of the leg was found compared to a six-fold increased risk of pulmonary embolism. The risk was highest in the third trimester of pregnancy (OR 8.8, 95%CI 4.5-17.3) and during the first six weeks after delivery (OR 84.0, 95%CI 31.7-222.6). The risk of pregnancy-associated venous thrombosis was 52-fold increased in factor V Leiden carriers (OR 52.2, 95%CI 12.4-219.5) and 31-fold increased in carriers of the prothrombin 20210A mutation (OR 30.7, 95%CI 4.6-203.6) compared to non-pregnant women without the mutation.

Conclusion: We found an increased risk of venous thrombosis during pregnancy and the postpartum period, with an especially high risk during the first six weeks postpartum. The risk of pregnancy-associated venous thrombosis was highly increased in carriers of factor V Leiden or the prothrombin 20210A mutation.

INTRODUCTION

Venous thrombosis is one of the leading causes of maternal morbidity and mortality[1, 2]. In developed countries, about 15% of maternal deaths results from pulmonary embolism[3]. In women of reproductive age, over half of all venous thrombotic events are related to pregnancy[4].

A large study of pregnancy associated venous thrombosis is the Glasgow study, a retrospective study of over 72000 deliveries[5]. This study reported an incidence of pregnancy-associated venous thrombosis of 3.24 per 1000 women years, with an incidence of 2.45 per 1000 women years for deep venous thrombosis of the leg and an incidence of 0.79 per 1000 women years for pulmonary embolism. For deep venous thrombosis of the leg the majority of cases (84%) occurred in the left leg, which is in accordance with the findings of other studies[6, 7]. The mechanism behind this propensity for the left leg is still under debate[8]. During pregnancy, the risk was highest during the third trimester[5]. Findings of other studies addressing risk differences in the three trimesters of pregnancy are inconsistent. An equal risk distribution during all three trimesters of pregnancy has been reported but there are also studies showing the highest risk during the first or second trimester of pregnancy[6-10]. The incidence of thrombosis was highest during the first six weeks after delivery both for deep venous thrombosis of the leg and for pulmonary embolism[5]. A higher risk during the postpartum period compared to pregnancy is reported by many other studies[10, 11].

As women with thrombophilia are at increased risk of venous thrombosis, a number of studies have been carried out to study the effect of pregnancy and the postpartum period in these women[12-17]. The most common inherited thrombophilias are the factor V Leiden and the prothrombin 20210A mutation. A meta-analysis of thrombophilias in pregnant women has shown the risk to be over eight-fold higher for heterozygous factor V Leiden carriers and almost seven-fold higher for heterozygous prothrombin 20210A mutation carriers than in pregnant women without thrombophilia[18].

In the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA study), a large population-based case-control study, we evaluated pregnancy and the postpartum period as risk factors for venous thrombosis. We were able to identify a sufficient number of patients in their pregnancy or postpartum period to allow for subgroup analyses; we evaluated the pregnancy-associated risk of deep venous thrombosis of the leg and pulmonary embolism separately and also analysed the risk of specific time frames within the pregnant and postpartum period. In addition the joint effect of pregnancy with factor V Leiden and the prothrombin 20210A mutation was addressed.

METHODS

Participants

The MEGA study included consecutive patients with a first diagnosis of venous thrombosis. Between March 1999 and September 2004, patients were recruited from six regional anticoagulation clinics (Amersfoort, Amsterdam, Den Haag, Leiden, Rotterdam and Utrecht) which monitor the anticoagulant therapy of all patients within a well-defined geographical area in the Netherlands. In order to participate, patients were required to be between the age of 18 and 70. Patients with severe psychiatric problems or those unable to speak Dutch were for practical reasons considered ineligible. Within the total patient group the diagnosis of 97% of deep venous thrombosis and 79% of pulmonary embolism was objectively confirmed. Ninety percent of patients used in the final analysis had an objectively confirmed diagnosis. Seven out of 285 patients (2.5%) had no objectively confirmed diagnosis and twenty-one out of 285 patients did not provide permission to obtain their medical records (7.4%). The tests included compression ultrasonography, Doppler ultrasound, impedance plethysmography and contrast venography for diagnosis of deep venous thrombosis and perfusion and ventilation lung scanning, spiral computer tomography and pulmonary angiography for pulmonary embolism.

Partners of patients were asked to participate as control subjects and an additional control group was obtained using the random digit dialing (RDD) method[19]. Only control subjects with no recent history of venous thrombosis were included and the same exclusion criteria as for patients were applied. Details of the MEGA study have been published previously[20].

Of 6055 eligible patients, 5051 participated (83%). Within this group 2737 were women and 2714 provided information on whether they had been pregnant or not before the thrombotic event. Of the 5051 participating patients, 3656 had an eligible partner of whom 2982 participated (82%). An additional 314 partners were included of whom the patient was either excluded for the final analysis, or had deep venous thrombosis of the arm. Thus a total of 3298 partners were willing to participate. Within this group 1665 were women of whom 1645 provided pregnancy related information. Out of 4350 eligible RDD control subjects, 3000 were willing to participate (69%). Information on pregnancy was obtained from 1710 out of 1719 women in this group. Individuals who were over 50 years of age, had no partner, used oral contraceptives or hormone replacement therapy or had malignancy or a partner with malignancy (for patients and partner controls) were excluded from the analyses leading to 285 patients and 857 control subjects.

The study was approved by the Medical Ethics Committee of the Leiden University Medical Center, the Netherlands. Written informed consent was obtained from all participants.

Data collection

Participants completed a detailed questionnaire on risk factors for venous thrombosis. Items covered in the questionnaire included oral contraceptive use, hormone replacement therapy, pregnancies, malignancies and civil status. The questionnaire covered a one year period prior to the index date, i.e. the date of diagnosis of the thrombosis for patients and the date of filling in the questionnaire for partners and the random control subjects. When participants were not willing to or unable to fill in the questionnaire, a standardized mini-questionnaire was taken by telephone, which also included pregnancy related questions. Participants were asked if they had been pregnant in the year before the index date or if they were still pregnant, and what the (expected) date of delivery was. We defined postpartum as the period up to three months after delivery. Information on the location of the affected leg in patients with a deep venous thrombosis of the leg was retrieved from the questionnaire and discharge letters. Out of 285 patients, 176 had a deep venous thrombosis of the leg (with or without pulmonary embolism) of whom 173 had information regarding the affected leg.

DNA collection

Three months after discontinuation of anticoagulant therapy patients and partner controls were invited for an interview and blood draw. In patients who continued anticoagulant therapy for over a year after the event, blood was drawn during anticoagulant therapy. When the participant was unable to come to the clinic a buccal swab was sent. From June 2002 onwards, blood draws were no longer performed in patients and their partners and blood draws were replaced by buccal swabs. Upon completion of the questionnaire, RDD controls were invited for an interview and blood draw. A detailed description of blood collection and DNA analysis for the factor V Leiden (G1691A) and the prothrombin mutation (G20210A) in the MEGA study has been published previously[20].

Within the patient group used for the present analyses 256 provided a blood sample or buccal swab (90%). In the control group 681 blood samples or buccal swabs were obtained (79%). Factor V Leiden and the prothrombin 20210A mutation were successfully determined in all patients and 679 control subjects.

Statistical analysis

As estimates of relative risks odds ratios (ORs) and 95% confidence intervals (95%CI) were calculated according to the method of Woolf. With a multiple logistic regression model we adjusted for age (categorical, seven classes). Because none of the control subjects in the analysis were matched to patients (they were either random population controls or partners of other (male) patients) all analyses were unmatched, with unconditional logistic regression. SPSS for Windows version 12.0.1 (SPSS Inc, Chicago, Ill) was used for all statistical analyses.

RESULTS

A group of 285 women aged 18 to 50 with venous thrombosis and 857 control subjects in the same age group were included in the analysis, with a mean age of respectively 38.3 (5^{th} -95^{th} percentile, 25.7-49.6) and 39.9 years (5^{th} -95^{th} percentile, 27.0-49.8). In the patient group 55% (n=158) was diagnosed with a deep venous thrombosis of the leg, 38% (n=109) with a pulmonary embolism and 6% (n=18) with the combined diagnosis.

Within the patient group, 116 out of 285 women (41%) were pregnant at the time of thrombosis or had been pregnant the three months before the thrombosis, compared to 82 out of 857 (9.6%) control subjects at the index date. The risk of venous thrombosis was five-fold (OR 4.6, 95%CI 2.7-7.8) increased during pregnancy and sixty-fold (OR 60.1, 95%CI 26.5-135.9) increased during the first three months after delivery compared to non-pregnant women (Table 1).

Odds ratios were higher in young women than in older women: in women aged 18 to 29 the risk of venous thrombosis during pregnancy was almost thirteen-fold increased (OR 12.5, 95%CI 4.0-39.5) whereas in women aged 30 to 50 the risk was three-fold increased (OR 3.3, 95%CI 1.8-6.1). Postpartum the risk was also more pronounced in women age 18 to 29 (OR 299.3, 95%CI 49.4-1813.1) than in women aged 30 to 50 (OR 29.4, 95%CI 12.1-71.5) (Table 1).

The risk of venous thrombosis during the first two trimesters of pregnancy appeared to be only slightly increased, with an odds ratio of 1.6. However, the risk was increased nine-fold (OR 8.8, 95%CI 4.5-17.3) during the third trimester compared to non-pregnant women. During the first six weeks after delivery the risk was highest (OR 84.0, 95%CI 31.7 – 222.6). Most cases of venous thrombosis during this period occurred within the first four weeks (95%), with the highest number of cases in the second week (42%) compared to 18%, 20% and 15% in the first, third and fourth week. The risk remained increased up to three months postpartum (Table 2).

Table 1. Relative risk of venous thrombosis during pregnancy and postpartum; overall and by age category

Age group (yrs)	Status	Patients (n)	Control subjects (n)	OR*	95%CI
18 to 50	Neither	169	775	1	Ref.
	Pregnant†	36	58	4.6	2.7-7.8
	Postpartum‡	69	10	60.1	26.5-135.9
	Overall§	116	82	9.7	6.4-14.9
18 to 29	Neither	7	86	1	Ref.
	Pregnant	14	18	12.5	4.0-39.5
	Postpartum	34	3	299.3	49.4-1813.1
30 to 50	Neither	162	689	1	Ref.
	Pregnant	22	40	3.3	1.8-6.1
	Postpartum	35	7	29.4	12.1-71.5

Ref., Reference category; OR, odds ratio; 95%CI, 95% confidence interval
*adjusted for age
†four women who currently were and had previously been pregnant are included in the pregnant group
‡period up to 3 months after delivery §included the pregnant and postpartum category and an additional 11 cases and 14 control subjects of whom delivery dates were unavailable

Table 2. Relative risk of venous thrombosis by different stages of pregnancy and postpartum

Status	Patients		Control subjects		OR*	95%CI
	n	(%)	n	(%)		
Neither	167	60.9	735	87.2	1	Ref.
1st and 2nd trimester	8	2.9	36	4.3	1.6	0.7-3.7
3rd trimester	28	10.2	22	2.6	8.8	4.5-17.3
1 to 6 weeks postpartum	66	24.1	6	0.7	84.0	31.7-222.6
7 weeks to 3rd month postpartum	3	1.1	4	0.5	8.9	1.7-48.1
4th month to 1 year postpartum	2	0.8	40	4.7	0.3	0.1-1.4

Ref., Reference category; OR, odds ratio; 95%CI, 95% confidence interval
*adjusted for age
Note: Information on delivery dates was unavailable for 11 cases and 14 controls

Overall pregnancy associated risk was most pronounced for deep vein thrombosis of the leg (OR 14.3; 95%CI 8.3-24.5) and six-fold increased for pulmonary embolism (OR 5.8; 95%CI 3.3-10.3. During pregnancy, the risk of deep venous thrombosis of the leg was clearly increased (OR 7.8; 95%CI 4.1-15.0), whereas that of pulmonary embolism was at most weakly increased (OR 2.3; 95%CI 1.0-5.2). In the postpartum period the risk for both was increased, with a relative risk of 72.6 for deep venous thrombosis of the leg and a relative risk of 34.4 for pulmonary embolism (Table 3).

The majority of pregnancy-associated deep venous thrombosis cases occurred in the left leg. During pregnancy 85% of women (23 out of 27) had a left-sided deep venous thrombosis, compared to 68% (32 out of 47) of women in the postpartum

Table 3. Risk of deep venous thrombosis, pulmonary embolism and the combined diagnosis by pregnancy status

	Patients (n)	Control subjects (n)	OR*	95%CI
DVT				
Neither	83	775	1	Ref.
Pregnant	27	58	7.8	4.1-15.0
Postpartum	42	10	72.6	30.1-175.4
Overall†	75	82	14.3	8.3-24.5
PE				
Neither	73	775	1	Ref.
Pregnant	9	58	2.3	1.0-5.2
Postpartum	22	10	34.4	13.3-88.5
Overall‡	36	82	5.8	3.3-10.3
DVT+PE				
Neither	13	775	1	Ref.
Pregnant	0	58	--	--
Postpartum	5	10	46.4	10.0-214.7
Overall§	5	82	5.5	1.4-21.1

DVT, deep venous thrombosis of the leg; PE, pulmonary embolism; Ref., Reference category; OR, odds ratio ; 95%CI, 95% confidence interval; *adjusted for age

†included the pregnant and postpartum category and an additional 6 cases and 14 control subjects of whom delivery dates were unavailable

‡included the pregnant and postpartum category and an additional 5 cases and 14 control subjects of whom delivery dates were unavailable

§included the pregnant and postpartum category and an additional 14 control subjects of whom delivery dates were unavailable

Table 4. The joint effect of pregnancy status and the factor V Leiden mutation (FVL) or the prothrombin 20210A (FII) mutation

Pregnant or postpartum	FVL	Patients (n)	Control subjects (n)	OR*	95%CI
-	-	144	580	1	Ref.
+	-	81	56	8.6	5.2-14.3
-	+	12	40	1.3	0.6-2.5
+	+	19	3	52.2	12.4-219.5
	FII				
-	-	141	605	1	Ref.
+	-	94	57	10.1	6.2-16.4
-	+	15	15	4.4	2.1-9.4
+	+	6	2	30.7	4.6-203.6

Ref., Reference category; OR, odds ratio; 95%CI, 95% confidence interval
*adjusted for age

period. In women who were not pregnant the right-left distribution was almost even, with 53% (52 out of 99) diagnosed with a left-sided deep venous thrombosis.

Among non-carriers of factor V Leiden, pregnancy and the postpartum period resulted in a nine-fold increased risk of venous thrombosis (OR 8.6, 95%CI 5.2-14.3). The joint effect of factor V Leiden and pregnancy resulted in a 52-fold

increased risk (OR 52.2, 95%CI 12.4-219.5), compared to non-carriers who had not been pregnant (Table 4). The risk of pregnancy-associated venous thrombosis was 31-fold increased (OR 30.7, 95%CI 4.6-203.6) in carriers of the prothrombin 20210A mutation, compared to non-pregnant, non-carriers (Table 4).

DISCUSSION

In this population-based case-control study we found a five-fold increased risk of venous thrombosis during pregnancy and a sixty-fold increased risk of venous thrombosis in the postpartum period. The risk was especially high during the first six weeks after delivery. The risk of both deep venous thrombosis of the leg and pulmonary embolism was increased during pregnancy and the postpartum period. During pregnancy venous thrombosis occurred far more often in the left than in the right leg. In carriers of the factor V Leiden mutation the risk of pregnancy-associated venous thrombosis increased markedly to about 52-fold compared to non-carriers who had not been pregnant. A somewhat lower increase in risk was found in prothrombin 20210A carriers, in whom the risk was 31-fold increased, compared to non-carrying, non-pregnant women.

Our finding of a five-fold increased risk in women who were pregnant is in accordance with the results of other studies[10, 11]. The higher relative risks of pregnancy in younger women compared to older women were in contrast with previous follow-up studies. However, one should bear in mind the difference between relative and absolute risks. Since thrombosis is age-dependent, these two will never both be constant over age, and a similar absolute increase will lead to much higher relative risks in young than in older women. Hence, one cannot conclude from our data that the influence is lower in older than in younger women and the reverse is probably true, also based on these data.

While previous reports were conflicting about the risk per trimester of pregnancy[6-10], we found the highest risk during the third trimester. These findings should be interpreted with some caution, because the higher risk during the third trimester might reflect a relatively high number of misdiagnoses in this trimester due to compression issues by the gravid uterus that leads to symptoms similar to venous thrombosis[8]. However, this is not very likely in our study, since 97% of patients with deep venous thrombosis were objectively diagnosed. A more important consideration is the inclusion of patients through anticoagulation clinics. Some women with venous thrombosis during pregnancy are initially treated without involvement of the anticoagulation clinic and receive low molecular weight heparin (LMWH). Women who had their venous thrombosis during the first or second trimester are

more likely to be treated with LMWH only than women with a venous thrombosis during the third trimester, who are referred to the anticoagulation clinic for additional treatment after child delivery. This might have led to an underestimate of the risk of thrombosis during early stages of pregnancy, thus no firm conclusion can be drawn about lower risks in the first two trimesters compared with the third trimester.

The risk of venous thrombosis during the first six weeks after delivery was very high compared to the overall pregnancy-associated risk. Our finding of a 84-fold higher risk during this period is within the range of findings from the majority of other studies, that reported a two- to fifteen-fold increased risk during the first six weeks after delivery compared to pregnancy[7, 14, 21]. The Glasgow study found 2.51 cases of venous thrombosis per 1000 person years in the first six weeks after delivery[5]. When we contrast this figure to the baseline risk of venous thrombosis of 0.08 per 1000 in these young women[22], these data point to a relative risk of 31 during this period. A case-control study in which control subjects were subject to the same referral and diagnostic procedures as patients found, however, less difference in the thrombotic risks during the first month after delivery and pregnancy[23]. A high risk of venous thrombosis during the first weeks after delivery may be explained by coagulation changes due to operative delivery, postnatal infections or immobility[24].

For a correct calculation of relative risks during different stages of pregnancy and the postpartum period it is important that the proportion of control subjects in each time frame is a good reflection of the source population. To verify this, we calculated the expected number of controls in each period, using data from the general population[25]. The percentage of pregnant or postpartum women was higher in the random digit dialing control group (12.3%) than in the partner control group (3.8%). In the overall control group the prevalence of pregnant or postpartum women (8.1%) was similar to the general population (8.8%). During pregnancy the proportion of controls was similar to what we would expect to find (6.9% compared to an expected 6.6%). In the first three months postpartum we observed a lower proportion of controls (1.2% compared to an expected 2.2%), possibly due to a reduced motivation to participate in our study after child delivery. In the period from four months up to one year postpartum the proportion of controls was still somewhat reduced (4.7% compared to an expected 6.6%). These lower proportions have probably resulted in a slight overestimation of relative risks in the postpartum period.

Furthermore, the time needed for control subjects to return the questionnaire could have influenced the percentage of pregnant controls assigned to each period. As controls returned the questionnaire more quickly than patients and 61% of the

controls had replied within a week (86% within a month) this is unlikely to have affected results.

Not much is known about the relative risks of the separate diagnoses of deep venous thrombosis of the leg and pulmonary embolism, during and after pregnancy. An American cohort study reported an increased risk of deep venous thrombosis and pulmonary embolism during pregnancy. Postpartum the risks were further increased with a four-fold higher risk of deep venous thrombosis and a 15-fold increased risk of pulmonary embolism compared to the pregnant period[10]. Also a Danish cohort study reported an increased risk of both deep venous thrombosis and pulmonary embolism during pregnancy and the first six weeks after delivery, with again a higher risk of pulmonary embolism during the postpartum period compared to the pregnant period[26]. We found increased risks of deep venous thrombosis of the leg and pulmonary embolism postpartum, while during pregnancy the risk of pulmonary embolism was only slightly increased.

When analysing the combined effect of pregnancy and the postpartum period with the factor V Leiden mutation or the prothrombin 20210A mutation, we found substantial increased risks for the combination of these risk factors. A meta-analysis of thrombophilias in pregnancy has found an eight-fold higher risk for heterozygous factor V Leiden carriers and an almost seven-fold higher risk for heterozygous prothrombin 20210A mutation carriers than pregnant women without thrombophilia[18]. Performing our analysis within pregnant women only, we found a five-fold increased risk for factor V Leiden carriers and a two-fold increased risk for prothrombin 20210A carriers.

In these young women, we found a low relative risk of 1.3 in carriers of factor V Leiden who had not been pregnant, which is lower than the overall risk of venous thrombosis due to factor V Leiden (three- or more fold increased)[27]. To investigate if the low risk was due to a too large proportion of non-pregnant factor V Leiden carriers among control subjects, we calculated the relative risk of venous thrombosis that one would expect in these control subjects using data from the general Dutch population as control situation. Using general data on live birth, stillbirth and use of oral contraceptives we calculated that 8.8% of these young women were expected to be pregnant or in the postpartum period[25]. Together with a prevalence of 5% for factor V Leiden[28], the calculated relative risk would be 1.5 in carriers of factor V Leiden compared to non-carriers who had not been pregnant; a similar relative risk as our finding (with 144 patients of the reference category and the 12 non-pregnant patients with factor V Leiden the following calculation was performed: 144*(5*(1-0.088)/95*(1-0.088))=7.76, 12/7.76=1.5).

We performed several subgroup analyses with relatively small numbers of patients and control subjects. Several confidence intervals were wide, but results were

in accordance with previous studies and the lower boundaries of many confidence intervals were above 2.1, with odds ratios of 4.4 or higher, indicating that the true effects were likely to be substantial.

A limitation of our study was the absence of data on the mode of delivery. It would have been interesting to investigate if we could replicate or refute previous findings that reported an increased risk of venous thrombosis from vaginal delivery to elective caesarean section to emergency caesarean section[2].

In conclusion, we found an increased risk of venous thrombosis during both pregnancy and the postpartum period, with an especially high risk during the first six weeks after delivery. Women with either factor V Leiden or prothrombin 20210A thrombophilia had a substantially increased risk of pregnancy-associated venous thrombosis compared to women without these mutations.

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CHAPTER 6

Polymorphisms in the protein C gene as risk factor for venous thrombosis

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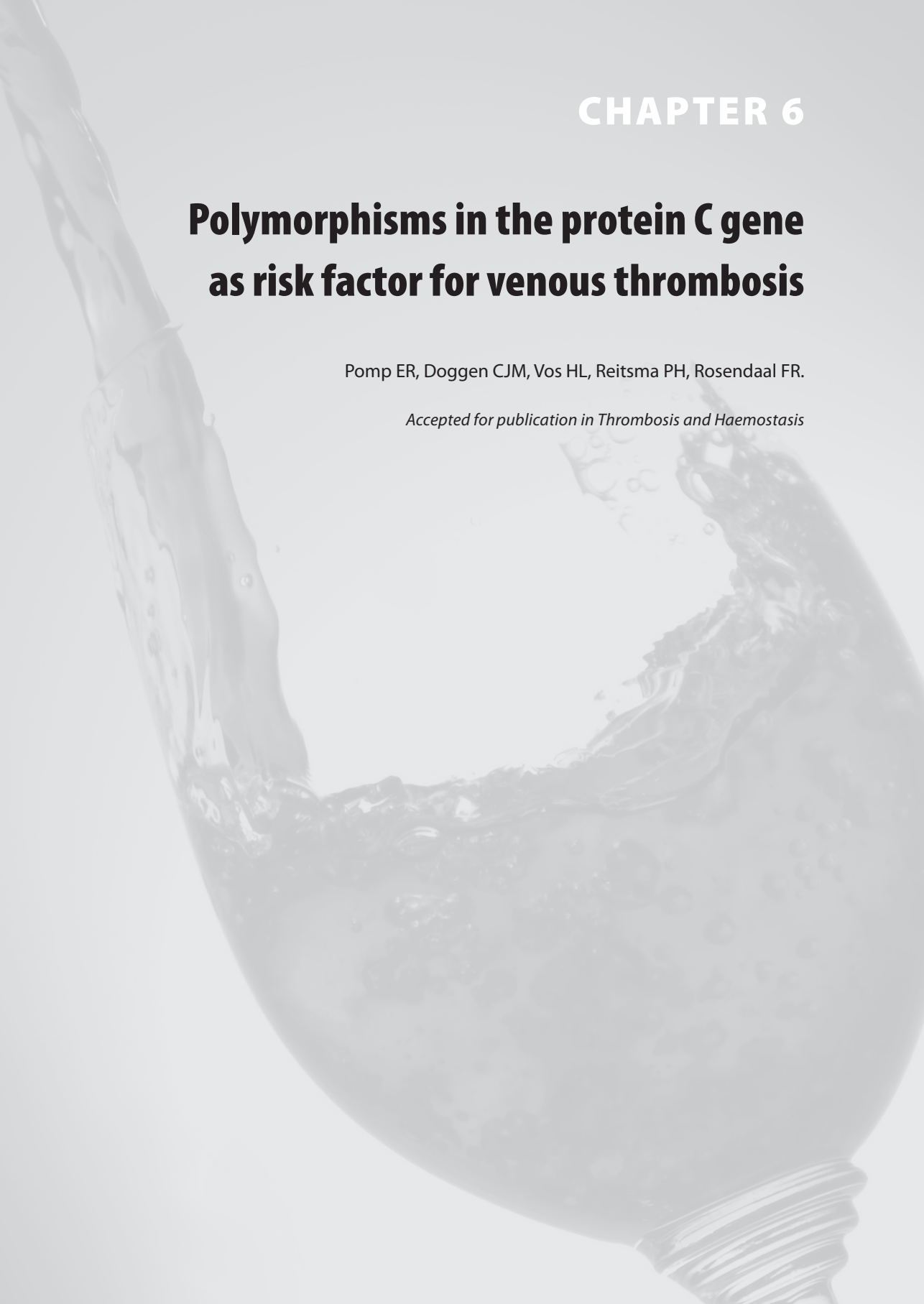

SUMMARY

Background/Objectives: Protein C is an important inhibitor of blood coagulation. We investigated the effect of two polymorphisms within the promoter region of the protein C gene (C/T at -2405 and A/G at -2418) on risk of venous thrombosis and on plasma protein C levels. In addition the combined effect of the two polymorphisms with factor V Leiden and oral contraceptive use was investigated. Previous studies on these polymorphisms were small and were not able to investigate synergistic effects.

Patients/Methods: In the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA study), protein C levels were determined in 2043 patients with venous thrombosis and 2857 controls, and the two polymorphisms in 4285 patients and 4863 controls.

Results: The CC/GG genotype was associated with the lowest protein C levels. Compared to carriers of the TT/AA genotype - a genotype associated with higher protein C levels - the risk of venous thrombosis in CC/GG carriers was 1.3-fold increased (CI95 1.09-1.48). The combination of factor V Leiden with the CC/GG genotype led to a 4.7-fold increased risk, compared to non-carriers with the TT/AA genotype. Oral contraceptive use together with the CC/GG genotype resulted in a 5.2-fold increased risk.

Conclusions: The CC/GG genotype is associated with lower levels of protein C and an elevated risk of venous thrombosis compared to the TT/AA genotype. There is no clear synergistic effect of the CC/GG genotype with factor V Leiden or oral contraceptive use on thrombotic risk.

INTRODUCTION

Activated protein C is a vitamin-K dependent natural anticoagulant. The anticoagulant effect of protein C is a result of the selective inactivation of coagulation factors V and VIII, with protein S serving as a cofactor[1].

The crucial role of protein C as an anticoagulant has been shown in many studies. Patients born with a homozygous protein C deficiency often have a severe form of thrombosis, called purpura fulminans[2]. In the early 1980s, several studies showed that heterozygous deficiencies, with fifty percent reduced protein C levels resulted in an increased risk of venous thrombosis[3-5]. Many families have been reported in the literature with recurrent thrombotic events due to this type of protein C deficiency[7]. More recently, the thrombotic risk associated with low protein C levels was confirmed in the Leiden Thrombophilia Study (LETS). In this case-control study, including 474 patients and control subjects, the risk of venous thrombosis appeared to be four times higher in persons with protein C levels below 65% compared to individuals with a protein C level equal to or above 85%[6].

Age, sex and lifestyle or biochemical factors such as body mass index, LDL-cholesterol, HDL cholesterol, triglycerides, oral contraceptive use and cigarette smoking may influence protein C levels[8,9]. Protein C levels can also be influenced by genetic factors. An analysis within the LETS showed a genetic variant in the promoter region of the protein C gene which was associated with low protein C levels and an increased risk of deep venous thrombosis of the leg[10]. Individuals with the homozygous CGT genotype of the polymorphisms at -2405, -2418 and -2583 (in LETS defined as polymorphisms at -1654, -1641 and -1476) were found to have a 1.5- to two-fold greater risk of venous thrombosis than individuals with the homozygous TAA genotype. Two out of three polymorphisms investigated in the LETS (2405C/T and 2418A/G) were considered as functionally different and were evaluated again in a study including 242 patients with deep venous thrombosis and 394 healthy individuals[11]. This study confirmed the link between these protein C gene polymorphisms and circulating protein C levels. Both studies did not evaluate the relationship between protein C levels and the risk of venous thrombosis.

Due to the relatively small number of participants in these previous studies, the risk estimates from these studies were imprecise and the joint effect of the protein C polymorphisms with other risk factors for venous thrombosis could not be investigated. The factor V Leiden mutation, which is the most common known cause of inherited thrombophilia, causes activated protein C resistance[12]. Oral contraceptive use, another important risk factor for venous thrombosis, also contributes to activated protein C resistance[13]. Since a previous study showed that deficiency of protein C and the factor V Leiden mutation had synergistic effects[14], as does the

combination of factor V Leiden and oral contraceptive use[15], it is of interest to investigate the risk of venous thrombosis in individuals with factor V Leiden or oral contraceptive use and the genotype associated with low protein C levels.

In the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA study), a large population-based case-control study, we investigated the two polymorphisms within the protein C gene (2405C/T and 2418A/G) as risk factors for venous thrombosis. We also investigated the influence of genotypic variation on plasma protein C levels and the effect of protein C levels on the risk of venous thrombosis. In addition, the joint effect of the low protein C genotype with the factor V Leiden mutation and oral contraceptive use was investigated.

METHODS

Study Design

The MEGA study included consecutive patients with a first diagnosis of venous thrombosis. Patients were selected from the files of the anticoagulation clinics in Amsterdam, Amersfoort, The Hague, Leiden, Rotterdam and Utrecht between March 1999 and September 2004. In the Netherlands, anticoagulation clinics monitor anticoagulation treatment in all patients in a geographically well-defined area. Patients between the age of 18 and 70 with a deep venous thrombosis of the leg, a pulmonary embolism or a combination of these diagnoses were included in our study. For practical reasons, patients with severe psychiatric problems or those unable to speak Dutch were considered as ineligible.

Partners of patients were asked to participate as control subjects. From January 2002 until September 2004, additional control subjects were recruited by using the random digit dialing (RDD) method[16]. The random control subjects were frequency matched on age and sex to the patients that provided a blood sample. Only control subjects without a history of venous thrombosis were included and the same exclusion criteria were applied as for the patients.

All participants were asked to fill in a questionnaire about possible risk factors for venous thrombosis. Most questions referred to a period of 12 months prior to the index date, i.e. the date of diagnosis of the thrombosis of the patient for patients and partners and the date of filling in the questionnaire for the random control subjects. Of the variables known to influence protein C levels we collected information on age, sex, body mass index (weight/height^2, kg/m^2), smoking and oral contraceptive use.

Blood collection

At least three months after withdrawal of anticoagulation the patients and their partners were asked to visit the anticoagulation clinic after an overnight fast, and a blood sample was drawn. Only in case of continuous use of anticoagulants for more than one year a blood sample was taken during anticoagulation therapy. From December 1999 onwards, we obtained self-administered buccal swabs by mail when participants were unable or unwilling to come for a blood draw. From June 2002 onwards, blood draws were no longer performed in patients and their partners, and the study was restricted to DNA collection by buccal swabs sent by mail. The random controls were invited for a blood draw within a few weeks after the questionnaire was sent. Within this group buccal swabs were sent when someone refused the blood draw.

Of the 6237 eligible patients, 276 died soon after the venous thrombosis. Of the remaining 5961 patients 4957 participated (83%). A blood sample was provided by 2349 patients, a buccal swab was obtained from an additional 1940 patients. Genotyping was successful in 4285 patient samples for the 2405C/T polymorphism (rs1799808) and in all 4289 samples for the 2418A/G polymorphism (rs1799809). Numbering of the polymorphisms was according to GB:AF378903. Protein C levels were successfully measured in 2347 out of 2349 blood samples.

Of the 4957 participating patients, 3581 had an eligible partner. One partner died soon after the request for participation. Of the remaining 3580 partners, 2917 participated (81%). An additional 173 control subjects were included of whom the partner was excluded for the final patient analysis, 121 partners were included of whom the patient had a deep venous thrombosis of the arm and 20 partners of non-participating patients were included, resulting in a total of 3231 partners. Within the partner group, 1465 blood samples and 1377 buccal swabs were obtained. Genotyping was successful in 2840 partners for the 2405C/T polymorphism and in all 2842 samples for the 2418A/G polymorphism. Protein C levels were successfully measured in 1463 out of 1465 blood samples.

Of the 4350 eligible RDD control subject, four died before they were able to participate. Of the remaining 4346 persons, 3000 participated (69%). A blood sample was provided by 1437 RDD control subjects, a buccal swab by 586 RDD controls. The 2405C/T and 2418A/G polymorphisms were successfully determined in all 2023 DNA samples and protein C levels in 1436 out of 1437 blood samples.

The 2405C/T and 2418A/G polymorphisms were determined by 5'nuclease (Taqman; Applied Biosystems, Foster City, CA) assays using a standard PCR reaction mix (Eurogentec, Seraing, Belgium) and allele-specific fluorescent probes equipped with a minor groove binding moiety (Applied Biosystem). A detailed description

of blood collection and DNA analysis for the factor V Leiden (G1691A) mutation in the MEGA study has been published previously[17]. For practical reasons, we only included 4285 patients and 4863 control subjects in the analyses with complete data for both polymorphisms. Measurement of protein C level was done with a chromogenic assay on a STA-R coagulation analyzer following the instructions of the manufacturer (Diagnostica Stago, Asnières, France). The mean intra- and inter-assay coefficients of variation were 1.4 % and 3.5 %. In the analyses with protein C levels, individuals using oral anticoagulation or with protein C deficiency (protein C levels below 66%, according to the clinical cut-off value) were excluded, resulting in 2043 patients and 2857 control subjects. In this excluded group, frequencies of the genotypes were comparable to those in the general population.

All participants gave written informed consent. The study was approved by the Medical Ethics Committee, Leiden University Medical Center, The Netherlands.

Statistical analysis

We pooled the control groups and calculated unmatched odds ratios (ORs). ORs and 95% confidence intervals (CI95) were calculated according to the method of Woolf[18]. With a multiple logistic regression model ORs were adjusted for age (continuous) and sex (categorical). SPSS for Windows version 14.0.1 (SPSS Inc, Chicago, Ill) was used for all statistical analyses.

RESULTS

In the present analyses 4285 patients and 4863 control subjects were included. Mean age of patients was 48.5 years (5^{th}-95^{th} percentiles, 26.1-67.7) and control subjects were on average 47.9 years old (5^{th}-95^{th} percentiles, 26.8-66.7). In the patients 58% (n=2491) were diagnosed with a deep venous thrombosis of the leg, 33% (n=1398) with a pulmonary embolism and 9% (n=396) with both. Fifty-four percent of patients (n=2324) and 53% of control subjects (n=2590) were women. Within the patient group with measured protein C levels, 281 out of 2347 patients (12%) were using oral anticoagulation compared to 28 out of 2899 (1%) in the control group at the time of blood draw. An additional 23 patients and 14 control subjects were potentially protein C deficient (protein C levels below 66%). When participants who used oral anticoagulation therapy or who were potentially protein C deficient were excluded, protein C levels were 118.2% (5^{th}-95^{th} percentiles, 87.0-160.0%) in patients and 117.9% (5^{th}-95^{th} percentiles, 88.0-155.0%) in control subjects.

Table 1. Frequency of protein C polymorphisms 2405C/T (rs1799808) and 2418A/G (rs1799809) in patients and control subjects and their relative risk of venous thrombosis

	Patients		Control subjects		OR (CI95)*
	n	%	n	%	
2405C/T					
CC	1887	44.0	2037	41.9	1 (ref.)
CT	1913	44.7	2208	54.4	0.93 (0.86-1.02)
TT	485	11.3	618	12.7	0.85 (0.74-0.97)
T allele freq.		33.6		35.4	
2418A/G					
AA	1230	28.7	1575	32.4	1 (ref.)
AG	2122	49.5	2358	48.5	1.15 (1.05-1.27)
GG	933	21.8	930	19.1	1.29 (1.14-1.45)
G allele freq.		46.5		43.4	

OR, odds ratio; CI95, 95% confidence interval; ref., reference category
* adjusted for age and sex

Table 2. Protein C (PC) levels in different genotypes/haplotype combinations

Genotype	Haplotype Combinations	Patients	Mean PC level		Control subjects	Mean PC level	
SNP 2405/2418		n	%	SD	n	%	SD
CC/AA	CA/CA	94	123	22	108	122	24
CT/AA	CA/TA	291	123	23	443	123	20
TT/AA	TA/TA	233	123	22	362	121	20
CC/AG	CA/CG	360	117	21	550	118	20
CT/AG	TA/CG*	625	118	22	840	117	22
TT/AG	TA/TG	2	129	30	5	112	11
CC/GG	CG/CG	437	113	21	549	112	18
CT/GG	CG/TG	1	129	--	0		
Total		2043			2857		

SD, standard deviation
* Most likely haplotype combination since the TG haplotype of the alternative CA/TG combination is very rare
Note: Individuals with oral anticoagulation therapy or protein C deficiency were excluded

The genotype distributions of the 2405C/T and 2418A/G polymorphisms in the overall group are presented in table 1. The distribution of both polymorphisms did not differ from Hardy-Weinberg equilibrium in control subjects. The T allele was present in 34% of patients and 35% of control subjects. Compared to the CC genotype, the TT genotype had a reduced risk of venous thrombosis (OR 0.85, CI95 0.74-0.97). This protective effect disappeared after adjustment for the 2418A/G polymorphism (OR 1.03, CI95 0.87-1.23). For the 2418A/G polymorphism, the G allele was slightly more frequent in patients (47%) than in control subjects (43%). The GG genotype had a small increase in risk of venous thrombosis compared

to the AA genotype (OR 1.29, CI95 1.14-1.45). After adjustment for the 2405C/T polymorphism the risk remained 1.3-fold increased (OR 1.31, CI95 1.13-1.53).

In table 2 protein C levels for the various combinations of the two polymorphisms are presented. Of the nine possible genotypes, six were frequently observed, two were rare and one was not observed. Within the six frequent genotypes, the CC/GG genotype was associated with low mean protein C level in control subjects (112%), the CC/AG and CT/AG genotypes had intermediate protein C levels (respectively, 118 and 117%) and the CC/AA, CT/AA, TT/AA genotypes were associated with high protein C levels (121 to 123%). A similar pattern was found in patients.

Table 3 presents the association of the six frequent genotypes with the risk of venous thrombosis. We chose one of the homozygous genotypes with a high protein C level (TT/AA) as the reference group. Compared to the TT/AA genotype the CC/GG genotype was associated with the highest increase in risk (OR 1.27, CI95 1.09-1.48). Factors that are known to influence protein C levels did not account for this increased risk; after additional adjustment for body mass index, smoking and oral contraceptive use the risk estimate remained unchanged (OR 1.33, CI95 1.13-1.56).

To verify whether the effect of the CC/GG genotype on the risk of venous thrombosis was in fact mediated via protein C levels, we also investigated whether a decrease in protein C levels was associated with an increased risk of venous thrombosis. Variations of the PC levels within the normal range were however not clearly associated with the risk of venous thrombosis. With protein C as a continuous variable in the logistic model no increased risk was observed (OR 0.99, CI95 0.99-0.99). With protein C as a categorical variable only protein C levels below 81% (compared to protein C levels between 111 and 120%) appeared to be associated

Table 3. Relative risk of venous thrombosis according to genotype

Genotype	Patients	Control subjects	OR (CI95)*
TT/AA	482	612	1 (ref.)†
CT/AA	561	764	0.93 (0.79-1.10)
CC/AA	187	199	1.19 (0.95-1.51)
CT/AG	1342	1438	1.19 (1.03-1.36)
CC/AG	777	914	1.08 (0.93-1.26)
CC/GG	923	924	1.27 (1.09-1.48)

OR, odds ratio; CI95, 95% confidence interval; ref., reference category

*adjusted for age and sex

† The TT/AA genotype was chosen as reference category, because it was associated with high protein C levels, was highly prevalent and was reported as reference category in previous studies thereby facilitating comparison between study results

Table 4. Combined effect of two protein C genotypes with factor V Leiden (FVL) or oral contraceptive (OC) use[1] on the risk of venous thrombosis

Genotype	FVL	Patients	Control subjects	OR (CI95)*
TT/AA	-	403	583	1 (ref.)
CC/GG	-	781	877	1.29 (1.10-1.51)
TT/AA	+	79	29	3.96 (2.54-6.18)
CC/GG	+	140	44	4.65 (3.24-6.68)
	OC use			
TT/AA	-	43	100	1 (ref.)
CC/GG	-	107	163	1.53 (0.99-2.35)
TT/AA	+	110	59	4.33 (2.67-7.01)
CC/GG	+	202	91	5.16 (3.32-8.00)

[1] in women aged 18 to 49

OR, odds ratio; CI95, 95% confidence interval; ref., reference category; -, absent; +, present

* adjusted for age and sex (not adjusted for sex in OC analysis)

with a moderately, but not significant increased risk of venous thrombosis ($OR_{<75\%}$ 1.52, CI95 0.75-3.07; $OR_{76\text{-}80\%}$ 1.24, CI95 0.71-2.17).

In table 4 the joint effect of factor V Leiden and the CC/GG protein C genotype is presented. The TT/AA genotype combined with factor V Leiden resulted in a 4.0-fold increased risk (OR 3.96 CI95 2.54-6.18) compared to TT/AA carriers without the mutation. Relative to TT/AA carriers without the factor V Leiden mutation, the joint effect of factor V Leiden and the GG/CC genotype led to a 4.7-fold increased risk (OR 4.65, CI95 3.24-6.68).

The joint effect of the CC/GG genotype together with oral contraceptive use in women younger than 50 is also presented in table 4. The TT/AA genotype combined with oral contraceptive use resulted in a 4.3-fold increased risk of venous thrombosis, compared to non-users with the TT/AA genotype (OR 4.33, CI95 2.67-7.01). The CC/GG genotype together with oral contraceptive use was associated with a 5.2-fold increased risk (OR 5.16, CI95 3.32-8.00) compared to TT/AA carriers without oral contraceptive use.

In addition to these analyses, we also investigated the combined effect of the CC/GG genotype with body mass index, since obesity is also related to activated protein C resistance. We found no synergistic effect for the combination of the CC/GG genotype and obesity (data not shown).

DISCUSSION

In a large population-based case-control study, we investigated two polymorphisms, 2405C/T and 2418A/G, within the protein C gene as risk factors for venous thrombosis. The T allele at position 2405 was associated with a protective effect and the G allele at position 2418 was associated with a small increased risk of venous thrombosis compared to the C and A alleles at these positions. Combining these alleles into different genotypes revealed that the CC/GG genotype was associated with low protein C levels compared to the other genotypes. The CC/GG genotype was associated with a 1.3-fold increased risk of venous thrombosis compared to the TT/AA genotype, which was a genotype with relatively high protein C levels. There were only minor synergistic effects of the CC/GG genotype with the factor V Leiden mutation or oral contraceptive use.

An increased risk for the C and G alleles compared to the T and A alleles is in accordance with the findings of previous studies[10,11]. The finding of low protein C levels in carriers of the homozygous CC/GG genotype is also in agreement with these studies[10,11]. The G allele at 2418 in the genotype seemed to be the most important determinant of protein C levels. Carriers of genotypes without a G allele presented relatively high protein C levels, carriers of genotypes with one G allele had intermediate levels and carriers of the genotype with two G alleles had the lowest protein C level. Homozygosity or heterozygosity for the 2405C/T polymorphism was less important in the determination of protein C levels, indicating that the effect on protein C levels was mainly mediated by the 2418A/G polymorphism. To verify this, we calculated the relative risk of the 2405C/T polymorphism adjusted for the 2418A/G polymorphism. As was expected the effect of the 2405C/T polymorphism disappeared. In contrast, the relative risks of the 2418A/G polymorphism remained elevated after adjustment for the 2405C/T polymorphism. In this study 19.1% of the control subjects, who represent the general population, had the GG genotype. This suggests that 5% of all venous thrombotic disease is associated with this genotype (population attributable risk: 5.1%).

In accordance with the finding of the lowest protein C levels in the CC/GG genotype we found the highest risk of venous thrombosis for carriers of this genotype. To verify whether the effect of the CC/GG genotype on the risk of venous thrombosis was truly mediated via protein C levels, low protein C levels themselves had to be associated with an increased risk of venous thrombosis. We found a moderately increase in risk, only for protein C levels below 81%. It seems that the risk of venous thrombosis is only influenced by protein C levels in the very low range. This is supported by the findings of a case-control study in women aged 45 to 64 years that reported a 2.9-fold increased risk for levels below 81 iu/dl[19]. In addition, we found

a 2.9-fold increased risk of venous thrombosis (CI95 1.25-6.73) in individuals with protein C levels below 66%. Previous studies on the 2405 C/T and 2418A/G polymorphisms[10,11] did not evaluate the relationship between protein C levels and the risk of venous thrombosis. In our analysis, the exclusion of individuals with oral anticoagulation therapy could have resulted in a small underestimation of risk estimates in the lower range. It is very likely that protein C levels of the excluded group were not evenly distributed throughout the categories; individuals with relatively low protein C levels were probably overrepresented in the excluded group.

We also assessed the combined effect of the CC/GG genotype with the factor V Leiden mutation or oral contraceptive use, which both lead to resistance to activated protein C[12,13]. Previously, a family study showed that a higher percentage of family members with both protein C deficiency and the factor V Leiden mutation had developed thrombosis (73%), compared with family members with either protein C deficiency (36%) or the factor V Leiden mutation (10%) ($P < 0.001$ for both groups). Of the subjects lacking both the mutations, only 7% had experienced a thrombotic episode[14]. In our study however, factor V Leiden together with the CC/GG genotype resulted in a 4.7-fold increased risk, which was only slightly higher than the sum of the separate effect of the CC/GG genotype and factor V Leiden. Also for the combination with oral contraceptive use or obesity no substantial synergistic effects were found.

In conclusion, the MEGA study confirmed the link between the CC/GG genotype, low protein C levels and an elevated risk of venous thrombosis. The increase in risk for the CC/GG genotype was mainly mediated by the presence of the G allele for the A/G 2418 polymorphism. CC/GG carriers, who were also factor V Leiden carriers or oral contraceptive users, did not have a substantial higher risk than expected based on the effect of each factor separately.

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CHAPTER 7

The experience of multiple control groups in a large case-control study on gene-environment interaction

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ABSTRACT

In a large case-control study on risk factors for venous thrombosis (MEGA study) we enrolled two different control groups; partners of patients and a random digit dialing group (RDD). This presented unexpected challenges in the analysis of three different types of research questions. For the evaluation of body mass index, a general life style factor, partners had to be analyzed with a matched analysis, RDD controls with an unmatched analysis. We developed a statistical approach which enabled us to pool the results of both analyses. For the analysis of pregnancy as risk factor for venous thrombosis only in women, simple pooling of both control groups was possible. However, lower pregnancy rates than expected were encountered in the partner group and higher rates in the RDD group. After combining both control groups, pregnancy frequencies were comparable with data from the general Dutch population. Frequencies of the factor V Leiden mutation, an example of a genetic risk factor, were identical in both control groups and in line with published data, indicating that for the analyses of this genetic risk factor both control groups were equally suitable. Our experience with the inclusion of two different control groups might be useful to others for choosing the most optimal research design and statistical approach.

INTRODUCTION

When designing a case-control study a very important decision is the choice of the appropriate control group. The purpose of a control group in a case-control study is to indicate the expected frequency of an exposure in patients under the null-hypothesis that there is no relation between exposure and disease. Therefore a uniform requirement for control selection is that the control group should be selected from the same source population as the cases independently of their exposure status[1;2].

This general aim nevertheless leads to several options in practice. Control subjects can be selected from the general population, such as random population control subjects, partners, friends or neighbors. Another potential source of control subjects is the hospital in which cases are hospitalized. Usually, there will be advantages for one group that are missing in the other, and vice versa. For example, random population controls may be more difficult to locate and less motivated to take part in the study than partners, friends or neighbors[3]. Situations arise in which the investigator may face a choice between two or more possible control groups to use. When different types of research questions are addressed and adjustment for different variables is required, multiple control groups can be useful. However, it has been suggested that the value of multiple control groups is limited[1], since it can lead to inconsistent results and proper analysis may become complex.

In the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA study), a very large population-based case-control study, we believed to have good reasons to include two different control groups, a partner control group and a random population control group. We included partners because the main focus of the study was on genetic risk factors for venous thrombosis and their interaction with environmental and lifestyle factors. It seemed unlikely that partners would select each other based on genetic differences in coagulation parameters. We also wanted to study environmental factors that are closely linked to lifestyle and for which partner controls might control for unmeasured confounding. In addition we assumed that asking partners would make it easier to recruit control subjects with malignancies or chronic diseases, which was a requisite if we wanted to study these diseases in relation to the risk of venous thrombosis. For the analysis with partner controls we had envisaged either a matched analysis of partners or an unmatched analysis with the opposite sex partners of cases becoming controls for same-sex cases. However, the proportion of men and women in the patient and control group was different in specific age categories; in particular there were very few young men with venous thrombosis (cases), resulting in few young female partners (controls), while there were many young female cases of

venous thrombosis. A population control group was added, that would be useful for certain analyses (such as pregnancy in young women), and might increase the overall numbers for the genetic analyses – as we did not expect differences in genetic make-up between partner controls and population controls. In the analysis phase, however, we learned that we had to distinguish quite carefully which analyses would be done with what control groups, as there were some unexpected differences, predominantly in environmental and lifestyle factors. In the process, we also had to devise a method for statistically combining the control groups if the analysis with one control group had to be matched and the other not. This process, as well as our solutions, might be useful to others who embark on large-scale gene environment interaction studies.

MEGA STUDY

Patients and partners

Between March 1999 and September 2004, we included consecutive patients with a first diagnosis of venous thrombosis. Patients were selected from the files of six large anticoagulation clinics in the Netherlands, which monitor anticoagulation treatment in all patients in a geographically well-defined area. Patients between the age of 18 and 70 with deep venous thrombosis of the leg, pulmonary embolism or a combination of these diagnoses were included. Patients with severe psychiatric problems or those unable to speak Dutch were considered as ineligible for practical reasons.

During the inclusion period partners of patients were asked to participate as control subjects. Only partner control subjects between the age of 18 and 70 with no history of deep venous thrombosis were included and the same exclusion criteria were applied as for patients.

Random digit dialing control subjects

From January 2002 until September 2004, another control group was recruited by using the random digit dialing (RDD) method according to Waksberg[4]. Only RDD control subjects between the age of 18 and 70 with no recent history of deep venous thrombosis were included and the same exclusion criteria were applied as for patients. The RDD method has proved to be a constructive method to collect a nearly random sample of all individuals in the population[5]. This method employs a two stage design which increases the likelihood of contacting households. Within

the geographical inclusion area, area codes and prefix numbers (first three digits of personal telephone number) combinations were obtained. For efficiency reasons, the prefixes were not generated completely at random in our study but were generated from the prefix numbers of the patients. To these prefixes, different random combinations of the next two digits were added. These eight digits formed the first stage of the sampling unit, i.e. the Primary Sampling Units (PSUs). To each PSU again two digits were added which were randomly generated by the computer. This number was dialed to determine whether or not it reached a household. If it did not reach a household because the telephone number was not in use or was used by a business or institution, the PSU was dropped from further consideration. If it did reach a household, 19 new numbers with the same PSU were randomly generated by the computer. Per household a maximum of seven attempts were made at different time points of the day and at different week days, with once at least three weeks between two attempts.

This procedure of control sampling was expensive and time-consuming; on average only three persons per hour were included. The response rate is hereby dependent on demographic characteristics of the target population and telephone skill of the interviewers[5]. In addition the RDD method is only useful if the vast majority of individuals live in households with a fixed telephone. In December 2005 fixed telephone coverage in the Netherlands was still very high (96%)[6], indicating that telephone coverage was more than enough for our RDD method. However in the nearby future, increasing use of mobile phones will decrease the ability for the RDD method to target specific areas within a country and achieve complete coverage.

An important consideration in random digit dialing surveys is bias introduced by non-responders. Non-response bias can be a problem if responders differ from non-responders for the measured variables[7;8]. Most studies have found that reluctant respondents are older and less educated than respondents who readily agree. Differences with respect to income, occupation, race and marital status have been inconsistent[8].

For efficiency reasons, we frequency matched the random control subjects to the patients who provided a blood sample according to age and sex. With each telephone call we asked a specific person within a household to participate (e.g. youngest woman between 20 and 50) and therefore avoided that the first person who picked up the phone, who maybe more mobile and healthier, was constantly included as control subject.

Data collection

Within a few weeks after diagnosis and registration at the anticoagulation clinics patients received a letter with information about the study and were subsequently contacted by phone. Partners of patients were also invited to participate. If patients or partners refused to participate the reason for refusal was asked for. Patients, partners and random digit dialing control subjects received a questionnaire shortly after inclusion by phone. The questionnaires included items on potential risk factors for venous thrombosis e.g. body weight, body height and pregnancies. Most questions referred to a period of 12 months prior to the index date, i.e. the date of diagnosis of thrombosis of the patient or the date of filling in the questionnaire for the random control subjects. For partners the date of diagnosis of thrombosis of the patient was used as index date in the body mass index analyses and in the pregnancy analyses the date of filling in the questionnaire was used.

From March 1999 till June 2002, patients and their partners were asked to visit the anticoagulation clinic where after an overnight fast a blood sample was drawn at least three months after withdrawal of anticoagulation. Only in case of continuous use for more than one year a blood sample was taken during anticoagulation therapy. From December 1999 onwards, self-administered buccal swabs were obtained by mail when participants were unable or unwilling to provide a blood sample. From June 2002 onwards, blood draws were no longer performed in patients and their partners, and the study was restricted to DNA collection by buccal swabs sent by mail. The RDD controls were invited for a blood draw within a few weeks after the questionnaire was sent. Within this group buccal swabs were sent when someone refused the blood draw. In the blood samples and buccal swabs prothrombotic mutations including the Factor V Leiden (G1691A) mutation were determined. A detailed description of blood collection and DNA analysis for factor V Leiden in the MEGA study has been published previously[9].

RESPONSE RATES AND GENERAL CHARACTERISTICS

During the inclusion period, 5961 eligible patients, 3586 eligible partners and 4346 eligible RDD control subjects were approached to participate. In the patient group, 4957 patients (83%) were willing to participate, partners had a similar response rate (n=2917, 81%), and 3000 (69%) RDD control subjects participated (figure 1).

Of the participating patients 92% returned a questionnaire compared to 95% and 93% in partners and RDD control subjects. During the first part of the MEGA study (March 1999-June 2002) a blood sample was provided by 73% of

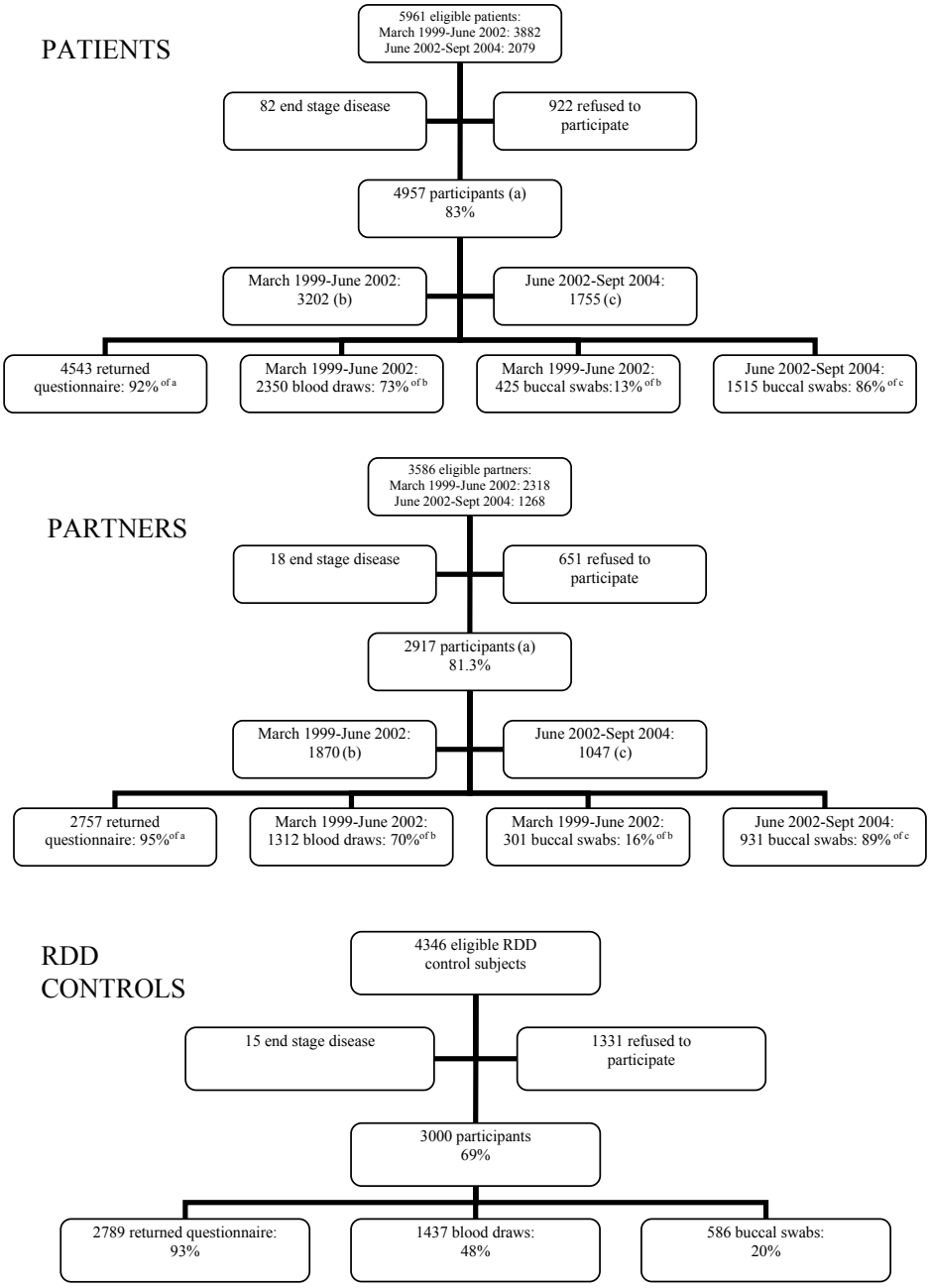

Figure 1. Response rates of patients, partners and RDD control subjects

participating patients and 70% of partners. Forty-eight percent of eligible RDD control subjects provided a blood sample. During the second part of the study (June 2002-September 2004), a buccal swab was obtained from 86% of patients

Table 1. Reasons for non-response in patients, partners and RDD control subjects

			Control subjects			
	Patients		Partners of participating patients		RDD controls	
	N	%	N	%	N	%
Refused to participate	922	100	651	100	1331	
No willingness	514	55.7	628	96.5	1243	93.4
Too many hospitals	93	10.1	–		–	
Not mobile	17	1.8	1	0.2	–	
Untraceable	271	29.3	14	2.2	88	6.6
Filled in questionnaire about recurrent VT	5	0.5	–		–	
Reason unknown	22	2.4	8	1.2	–	

– = not specified

and 89% of partners. Reasons why persons refused to participate are presented in more detail in table 1.

Mean age of 4957 patients was 48.6 (5^{th}-95^{th} percentiles, 25.7-67.9), the 2917 partners were on average 48.3 years (5^{th}-95^{th} percentiles, 28.0-66.1) and the 3000 RDD control subjects had a mean age of 45.3 (5^{th}-95^{th} percentiles, 23.5-66.9). Fifty four percent (n=2680) of patients, 50% (n=1463) of partners and 57% (n=1719) of RDD control subjects were women.

DIFFERENT RESEARCH QUESTIONS, DIFFERENT USE OF CONTROLS

In the MEGA study we investigated genetic or acquired factors and their interaction as possible risk factors for venous thrombosis. As genetic risk factors several prothrombotic mutations, such as the factor V Leiden mutation, were measured in blood samples or buccal swabs collected from the participants. Included were a wide range of acquired risk factors like malignancies, surgery, injuries and various lifestyle related risk factors as pregnancy, oral contraceptive use, overweight, smoking, physical activity, alcohol use and (air) travel. When analyzing lifestyle factors as possible risk factors for venous thrombosis different considerations concerning the choice of a control group have to be made compared to the analysis of genetic risk factors. It is challenging to use both control groups in such a way that statistical power is maintained and bias is reduced to a minimum.

For set forth the analytic considerations of two different control groups we will describe the association of a general lifestyle risk factor (body mass index), a lifestyle risk factor in women (pregnancy) and an example of a genetic risk factor (factor V Leiden mutation) with the risk of venous thrombosis. We will present

a statistical method that allowed us to use both control groups in the analyses of body mass index as risk factor for venous thrombosis.

Body mass index- General lifestyle risk factor

For the analyses of body mass index (BMI) as risk factor for venous thrombosis[10] the most obvious control group seems to be the RDD control group because one instinctively would say that partners are too much alike. When we investigated the BMI distribution in patients, partners and the RDD controls, frequencies of overweight (BMI: 25-29 kg/m^2) and obesity (BMI: ≥ 30 kg/m^2) were indeed more similar in patients and their partners than in patients and the RDD controls, resulting in lower risk estimates when using the partner control group compared to the RDD control group (table 2). These results were obtained with an unconditional logistical regression analysis, which is not correct because it uses partners of cases as control subjects for other cases. Partners are matched with patients and this matching has to be considered in the statistical analysis since ignoring matching generally introduces bias, even if the matched variable is not a confounder[1]. Performing an unmatched analysis with matched data will result in an underestimation of the true effect. Matching was accounted for with a conditional logistic regression analysis[11], i.e. matched analysis, which adjusts for similar lifestyle factors between patients and their partners by including only discordant pairs. In table 3 the results of the matched analysis with patient-partner pairs is presented. Risk estimates appeared to be still somewhat lower compared to the analysis with the RDD control subjects (overweight$_{partners}$ OR 1.45, CI95 1.26-1.67; overweight$_{RDD}$ OR 1.83, CI95 1.63-2.05; obesity$_{partners}$ OR 1.81, CI95 1.49-2.20; obesity$_{RDD}$ OR 2.87, CI95 2.45-3.35). A possible explanation for this difference is that adjustment for similar lifestyle factors in the matched analysis may include some unknown, unmeasured confounders, which will lead to risk estimates closer to the real estimates compared to the risk estimates obtained from the analysis with the RDD controls. It is important to

Table 2. Body mass index (BMI) distribution in patients, partners and RDD control subjects – Unmatched analyses

BMI (kg/m^2)	Patients		Partners		RDD		OR$_{partner}$*	OR$_{RDD}$*
	N	%	N	%	N	%	(CI95)	(CI95)
<25	1369	36.5	1306	44.8	1409	55.7	1	1
25-29	1593	42.4	1172	40.2	848	33.5	1.33 (1.20-1.49)	1.83 (1.63-2.05)
≥30	794	21.1	438	15.0	274	10.8	1.75 (1.52-2.01)	2.87 (2.45-3.35)
Total	3756		2916		2531			

*adjusted for age and sex

Odds ratios (ORs) calculated with unconditional logistic regression

Note: BMI analyses were performed in non-pregnant individuals without malignancies

Table 3. BMI as risk factor for venous thrombosis - Matched analyses

BMI (kg/m^2)	Patients	Partners	$OR_{matched}$* (CI95)
<25	739	925	1
25-29	949	860	1.45 (1.26-1.67)
≥30	415	318	1.81 (1.49-2.20)
Total	2103	2103	

*adjusted for age and sex

Table 4. BMI as risk factor for venous thrombosis - Combined analyses with patients, partners and RDD control subjects

BMI (kg/m^2)	Patients	Partners	RDD	$OR_{combined}$* (CI95)
<25	1369	925	1409	1
25-30	1593	860	848	1.71 (1.54-1.89)
≥30	794	318	274	2.45 (2.14-2.80)
Total	3756	2103	2531	

*adjusted for age and sex

Table 2-4: Adapted from British Journal of Haemaology 2007;139:289-269

realize that in the matched analysis only patient-partner pairs can be included, resulting in less power than the analysis with the RDD control subjects. Besides this, both the patient and the partner of a pair must have valid data for the required variable, otherwise the whole pair cannot be included in the analysis. Finally, the matched analysis itself only uses pairs who are discordant for the variable of interest, resulting in further reduced power.

Using the RDD control subjects in the analyses of BMI as risk factor for venous thrombosis may result in a slightly overestimation of the true risk estimates because there were somewhat fewer RDD controls with overweight compared to the general Dutch population. According to data of the Central Bureau of Statistics in the Netherlands the prevalence of overweight and obesity was respectively 36% and 11% during the study period[12], compared to a 33% and 11% found in the RDD group.

Both partner and RDD analyses showed consistent results in terms of clearly increased risks. In a combined analysis the most powerful estimate was obtained. We used a simple approach in which the estimates of the odds ratios of the two analyses were pooled[13]. In this combined analysis we accounted for the correlation between the estimated odds ratios since most patients were included both in the matched and the unmatched analysis. Table 4 presents the odds ratios of the combined analysis ($OR_{overweight}$ 1.71, CI95 1.54-1.89, $OR_{obesity}$ 2.45, CI95 2.14-2.80), which were of course in between partner and RDD odds ratios.

When analyzing the risks in men and women separately it was not possible to perform a matched analysis with the partner controls, as control individuals were nearly always of the opposite sex to the cases.

Pregnancy- Lifestyle risk factor in women

In the MEGA questionnaire, participants were asked if they had been pregnant in the year before the index date or if they were still pregnant, and what the (expected) date of delivery was.

In the analysis of pregnancy as risk factor for venous thrombosis, only women were included. In addition, only participants with a partner were included in the analysis, since being in a relationship affects the probability of getting pregnant. During the invitation by phone, patients were asked if they had a partner, partner controls had a partner per definition, and civil status was asked for in the questionnaire, also allowing the inclusion of only RDD controls with a partner. However, we encountered a much higher frequency of pregnancies in the RDD control subjects with a partner than in the partner control subjects (table 5). The percentage of pregnant or postpartum women was 12.3% in the RDD control group and 3.9% in the partner control group compared to 8.8% in the general population. These

Table 5. Pregnancy and postpartum in patients, partners and RDD control subjects

Pregnancy status	Patients		Partners		RDD		$OR_{partner}$*	OR_{RDD}*	OR_{total}*
	N	%	N	%	N	%			
Neither	163	61.3	394	96.1	371	87.7	1	1	1
Pregnant	35	13.2	14	3.4	44	10.4	9.28 (4.37-19.70)	3.60 (2.01-6.44)	4.67 (2.72-8.00)
Postpartum†	68	25.5	2	0.5	8	1.9	198.07 (38.00-1032.29)	42.22 (17.38-102.60)	61.21 (27.06-138.48)
Total	266		410		423				

*adjusted for age, †three months after delivery

ORs calculated with unconditional logistic regression

Note: Pregnancy analyses were performed using women who were between 18 and 50 years of age, had a partner, did not use oral contraceptives or hormone replacement therapy and had no malignancies or a partner* with malignancies (*for patients and partner controls).

Table 6. Different stages of pregnancy and postpartum in patients, partners and RDD control subjects

Pregnancy status	Patients		Partners		RDD	
	N	%	N	%	N	%
Neither	161	60.5	378	92.2	347	82.0
1st and 2nd trimester	8	3.0	6	1.5	30	7.1
3rd trimester	27	10.2	8	2.0	14	3.3
Puerperium (1-6 weeks)	65	24.4	1	0.2	5	1.2
7 weeks to 3rd month postpartum	3	1.1	1	0.2	3	0.7
4th month to 1 year postpartum	2	0.8	16	3.9	24	5.7

Table 5-6: Adapted from Journal of Thrombosis and Haemostasis 2008;6:632-637

frequencies in the control groups were unexpected; before the start of our study we assumed that including partners would make it easier to recruit pregnant individuals because pregnant women in general would be less motivated to participate in a study. However, the opposite appeared to be true. The high frequency in the RDD group may be due to more awareness of health issues in pregnant women and therefore more willingness to participate than non-pregnant women. The low frequency in the partner control group remains difficult to explain.

It was possible to combine the two separate control groups into one large group. The prevalence of pregnant or postpartum control women (8.1%) then became similar to that of the general population (8.8%). Not only for the overall analysis but also for the stratified analysis of different stages of pregnancy and the postpartum period it was important that the proportion of control subjects in each time frame during and after pregnancy was a good reflection of the general population (table 6). To verify this, we calculated the expected number of controls in each period, using data from the general population[14]. During pregnancy the number of controls in the overall group was similar to what we would expect to find (6.9% compared to an expected 6.6%). In the first three months postpartum we observed a lower number of controls (1.2% compared to an expected 2.2%), possibly due to a reduced motivation to participate in our study after child delivery. In the period from four months up to one year postpartum the number of controls was still somewhat reduced (4.7% compared to an expected 6.6%). These lower proportions might have resulted in a slight overestimation of relative risks in the postpartum period.

These analyses illustrate that the inclusion of multiple control groups appeared to be very useful. A priori assumptions about control group characteristics were not in line with the collected data. If only a partner control group or only the RDD control group was collected, pregnancy associated risks were either over- or underestimated.

Factor V Leiden- Genetic risk factor

For genetic risk factors it is unlikely that their frequency is different in partners compared to RDD control subjects. However, the prevalence of factor V Leiden is related to ethnicity[15] so you could speculate that if partners chose their partner according to ethnicity the factor V Leiden distribution in partners would be dissimilar compared to RDD control subjects. In the MEGA study most participants were of Dutch origin, so differences in the distribution of factor V Leiden due to intra-racial partnerships were unlikely. For the RDD controls you could hypothesize that RDD controls with a positive family history of venous thrombosis will

Table 7. Factor V Leiden mutation (FVL) in patients, partners and RDD control subjects

FVL	Patients		Partners		RDD		$OR_{partner}$*	OR_{RDD}*	OR_{total}*
	N	%	N	%	N	%			
-	3612	84.3	2403	94.7	1914	94.6	1	1	1
+	675	15.7	134	5.3	109	5.4	3.38 (2.78-4.09)	3.36 (2.72-4.15)	3.36 (2.88-3.92)
Total	4287		2537		2023				

*adjusted for age and sex
ORs calculated with unconditional logistic regression

be more willing to give blood than RDD controls without a positive family history, leading to an overestimation of the prevalence of factor V Leiden in this group. However, we found the same percentage of individuals with factor V Leiden in the partner and the RDD group (partner controls, 5.3%; RDD controls, 5.4%) (table 7). Obviously, both percentages could be an overestimation of the true prevalence, but the percentages were equal to a previously recorded prevalence of factor V Leiden in Caucasians[16].

Since both control groups had the same percentage of factor V Leiden carriers and this percentage was supported by literature both control groups were combined as if they were one in an unconditional logistic regression analysis (table 7).

DISCUSSION

In the MEGA study, a large population-based case-control study, we evaluated the use of two different control groups, a partner control group and a RDD control group, in the analyses of three different types of research questions. We learned that we had to distinguish quite carefully which analyses would be done with what control groups, as there were some unexpected differences. For the evaluation of body mass index, we had to devise a method for statistically combining the control groups in the analysis. Using the partner control group asked for a matched analysis and for the RDD group an unmatched analysis was required. For pregnancy, simple pooling of both female control groups was possible. However, lower pregnancy rates than expected were encountered in the partner group and higher rates in the RDD group. After combining both control groups, pregnancy frequencies were comparable with data from the general Dutch population. Frequencies of the factor V Leiden mutation were identical in both control groups and in line with published data, indicating that for the analyses of genetic risk factor both control groups were equally suitable.

There are only a few studies reporting their experience with multiple control groups. In 1983, Savraky and Clarke wrote a paper that summarizes their experience in using hospital and neighborhood control subjects[17]. When testing the hypothesis if oxidative hair dyes were carcinogenic, they found to their surprise lower rates of hair dye use among 314 hospitals (40.5%) than among 470 neighborhood control subjects (52.8%). Several other striking differences were observed. Compared with hospital controls, neighborhood controls were older, ethnically more heterogeneous, less likely to be oral contraceptive users and more likely to be smokers. The investigators believed that most of these differences arose from different lifestyles in the relatively rural region from which the hospital controls were derived and in the urban region that provided the neighborhood group. These geographical differences demonstrate the importance of selection of patients and control subjects from the same source population[18]. A study investigating the association between machining fluid and laryngeal cancer risk used control subjects with oral cancer and a stratified random sample of all deaths in a distinct geographical area as control subjects[19]. When cases (n=888) were compared to oral cancer controls (n=752) high exposure to machining fluids resulted in a 1.5-fold increased of laryngeal cancer. However, when cases were compared with population controls (n=3594) no increased risk of exposure was found. A possible explanation, besides a chance finding, may be that exposure data quality for the cases and oral cancer controls may have differed from that of the population controls. These studies illustrate the problem of multiple results; at least one of the results is biased. Only further external information could help to evaluate the likely extent of bias in the estimates from different controls.

Not only characteristics may differ substantially between control groups, but also response rates may vary. In the MEGA study partner controls were more willing to participate than RDD controls (83% versus 69%). Especially for blood draws the difference was considerable; 70% percent of participating partners and 48% of participating RDD controls provided a blood sample. A possible explanation for this difference may be that partners motivated each other to participate and were able to join each other to the location of the blood draw. Another consideration which may explain differences between RDD and partner response rates is the fact that partners of non-participating patients were not included in the non-response; if a patient refused to participate, we did not ask the patients partner to participate. Thus beforehand a selection of more willing couples, with participating patients, was made which could have positively influenced the partner response.

In the analyses of BMI as risk factor for venous thrombosis partner controls were included in a conditional logistic regression analysis (matched analysis) since ignoring matching introduces bias. Aside from the complication of matching, the

fact that partners have a relationship may be associated with certain characteristics which make partners somewhat different from the source population[1].

It is important to realize that a priori assumptions about control group characteristics may not be confirmed by the data. We had the wrong assumption that including partners would make it easier to recruit pregnant women or individuals with severe diseases. Besides the low frequency of pregnancies in partners, frequencies of malignancies were also different from what we expected; both control groups had about the same percentage of malignancies (data not shown). These findings could indicate that health issues for RDD controls are an extra motivation to participate. The low frequency of pregnancies in partners is however difficult to understand. It may be due to the fact that partners were approached via the patient. It is possible that because of the pregnancy or disease of the partner, the patients decided on their own that their partner was not willing to participate. This illustrates the importance of asking in detail reasons for non-response.

In conclusion, when different types of research questions are addressed in a case-control study, it is important to think thoroughly about control group choice and the way controls are to be used in the statistical analyses. We hope the discussion of our experience in using multiple control groups can help others to create the most optimal study design and statistical approach for answering their research questions.

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CHAPTER 8

Discussion and Summary

In the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA study), a large population-based case-control study, we investigated lifestyle factors as risk factors for venous thrombosis. Overweight, smoking and alcohol consumption were addressed and pregnancy and the postpartum period were evaluated in women. Due to the large sample size of the study it was possible to investigate the joint effect of these risk factors with important genetic risk factors for venous thrombosis such as the factor V Leiden and the prothrombin 20210A mutation. In addition to these lifestyle related risk factors, two polymorphisms within the promoter region of the protein C gene were studied as risk factors for venous thrombosis and the influence of genotypic variation on plasma protein C levels was assessed. Finally, we described our experience with the inclusion of two different control groups in the MEGA study.

This discussion evaluates the main findings of this thesis and includes brief summaries of all chapters.

Recent studies indicate that obesity increases the risk of venous thrombosis[1-8]. In accordance with these studies we found that relative to those with a normal body mass index (BMI<25 kg/m^2), overweight (BMI≥25 and BMI<30 kg/m^2) increased the risk of venous thrombosis 1.7-fold and obesity (BMI≥30 kg/m^2) 2.4-fold. Body weight as a separate risk factor for venous thrombosis was also positively associated with thrombotic risk. Tall men had an increased risk of venous thrombosis, in short men a protective effect was found. This latter is remarkable, since body height is not associated with the relative amount of fat, in contrast body weight and body mass index both are. Biological support for the observed relationship between obesity and the risk of venous thrombosis arises from studies showing an increase of procoagulant factors, such as factor VII, factor VIII, factor XII and fibrinogen, with increasing body mass index[9-12]. Together with the fact that the association between body mass index and venous thrombosis is consistent over studies and shows a dose response relationship, the association is likely to be causal. The effect of obesity was more pronounced in women than men, with a 24-fold increased risk for women using oral contraceptives compared to normal weight women who did not use oral contraceptives. The joint effect of obesity with the factor V Leiden mutation or the prothrombin mutation appeared both slightly higher than the sum of the separate effects. The synergistic effect of both oral contraceptive use and factor V Leiden with obesity may be explained by the fact they all lead to APC-resistance[13;14] which is associated with a higher risk of venous thrombosis **(chapter 2)**.

The results of studies investigating the relationship of smoking with venous thrombosis are inconsistent[2;4;6;15;16]. In our study, smoking was associated with a moderately increased risk of venous thrombosis; in current smokers the risk was

1.4-fold increased and former smokers had a 1.2-fold increased risk compared to individuals who had never smoked. In current smokers the risk increased with the amount of smoking. No dose response relation was found for the number of smoking-years in either current or former smokers. In the youngest age category (<37.8 yrs) the risk of thrombosis increased with pack-years smoked, with a 4.3-fold increased risk for smokers with 20 or more pack-years. In those aged over 38, no association between pack-years and the risk of venous thrombosis was found. The presence of a dose response relationship for the amount but not the duration of smoking, the higher risk in current compared to former smokers and the finding of a dose response relationship with pack-years in young individuals only, suggests that the effect of smoking on venous thrombosis is largely an acute effect. The effect of smoking was more pronounced in women than men, which may be explained by our finding of a synergistic effect of smoking with oral contraceptive use; smoking together with oral contraceptive use resulted in an 8.8-fold increased risk compared to non-smokers who did not use oral contraceptives. This interaction between smoking and oral contraceptive use is in accordance with the results of studies on myocardial infarction[17]. To investigate a mechanism for the association between smoking and venous thrombosis we adjusted our analyses for fibrinogen levels, hypothesizing that the risk was mediated via elevated fibrinogen levels. This adjustment, however, resulted only in slightly decreased risk estimates for current smoking, and therefore fibrinogen levels are not a crucial part of the mechanism. Besides coagulation factors, inflammatory factors may be involved. Interleukin-6 has been shown to be elevated in smokers[18] and is also associated with the risk of recurrent venous thrombosis[19]. The involvement of inflammatory factors in the etiology of venous thrombosis would be an interesting topic for future research **(chapter 3)**.

Moderate alcohol consumption is an established protective factor for cardiovascular disease[20], however the effect on venous thrombosis is unknown. In the MEGA study, alcohol consumption was associated with a decreased risk of venous thrombosis, with two to four glasses per day resulting in the strongest effect compared to abstainers. The effect appeared to be more pronounced in women than men and for pulmonary embolism than for deep venous thrombosis of the leg. In the literature, an association between moderate alcohol intake and reduced levels of fibrinogen, factor VII and von Willebrand factor has been reported[21] which may explain the relationship between alcohol consumption and the reduced risk of venous thrombosis. In our study, fibrinogen levels were decreased in individuals who consumed alcohol compared to abstainers. Factor VII and von Willebrand levels were mildly decreased in these individuals but not consistently over the categories of alcohol consumption. Therefore, the effect of alcohol seems to be mainly medi-

ated by a decrease in fibrinogen. The difference between men and women in the alcohol-related risk of venous thrombosis may be explained by the differential effects of wine and beer[22], the latter of which is consumed more by men than women. The inverse relationship between alcohol consumption and fibrinogen was most marked with wine drinking. In our study we had no information about the kind of alcoholic drinks the participants consumed. It was striking that the protective effect of alcohol was still present at high alcohol intake for pulmonary embolism but not for deep venous thrombosis of the leg. We do not have an explanation for this finding **(chapter 4)**.

In addition to these common lifestyle factors we also studied a women-specific risk factor. In women of reproductive age, over half of all venous thrombotic events are related to pregnancy[23]. During pregnancy, we found a 4.6-fold increased risk of venous thrombosis, which is in accordance with the results of other studies[24;25]. While previous reports were conflicting about the risk per trimester of pregnancy[24;26-29], we found the highest risk during the third trimester, namely an 8.8-fold increased risk. The risk of venous thrombosis during the first six weeks after delivery was very high compared to the overall pregnancy-associated risk. Our finding of an 84.0-fold higher risk during this period is however within the range of findings from the majority of other studies[29-31]. During pregnancy venous thrombosis occurred far more often in the left than in the right leg. In factor V Leiden carriers the risk of pregnancy-associated venous thrombosis was 52.2-fold increased and 30.7-fold increased in carriers of the prothrombin 20210A mutation compared to non-pregnant women without the mutation.

A consideration with the pregnancy analysis is the inclusion of patients through anticoagulation clinics. Some women with venous thrombosis during pregnancy are initially treated without involvement of the anticoagulation clinic and receive low molecular weight heparin (LMWH). Women who had their venous thrombosis during the first or second trimester are more likely to be treated with LMWH only than women with a venous thrombosis during the third trimester, the latter who are referred to the anticoagulation clinic for additional treatment after child delivery. This might have led to an underestimate of the risk of thrombosis during early stages of pregnancy in our study **(chapter 5)**.

Besides acquired risk factors, genetic factors play an important role in the etiology of venous thrombosis. The factor V Leiden and the prothrombin 20210A mutation are important risk factors, but there are many polymorphisms with a relatively small contribution to the risk of venous thrombosis. Two polymorphisms within the protein C gene (2405C/T and 2418A/G) were investigated as risk factors for venous thrombosis. Out of the various combinations of these two polymorphisms, the CC/GG genotype was associated with lowest mean protein C levels and high-

est risk of venous thrombosis. Compared to carriers of the TT/AA genotype - a genotype associated with high protein C levels - the relative risk of venous thrombosis was 1.3-fold increased in CC/GG carriers. The effect of the CC/GG genotype was mainly mediated by the 2418A/G polymorphism; the effect of the 2405C/T polymorphisms disappeared after adjustment for the 2418A/G polymorphism. The finding of low protein C levels and an elevated risk of venous thrombosis in carriers of the homozygous CC/GG genotype is in agreement with other studies[32;33]. To verify if the effect of the CC/GG genotype on the risk of venous thrombosis was truly mediated via protein C levels, low protein C levels themselves had to be associated with an increased risk of venous thrombosis. Previous studies did not investigate this relationship. We found a small increase in thrombotic risk, only for protein C levels below 81%. It seems that the risk of venous thrombosis is only influenced by protein C levels in the very low range **(chapter 6)**.

In the MEGA study we included two different control groups; partners of patients were asked to participate as control subjects and a control group was recruited using a random digit dialing (RDD) method. Asking partners as control subjects was very practical. They could be approached together with the patient, which was very efficient. Another advantage was their high participation rate (81%). They were aware of the importance of the study since they had seen the consequences of the disease in the patient. Since not all patients had a partner an additional control group was recruited with the RDD method. This method has proved to be a constructive method to collect a nearly random sample of all individuals in the population, but it is expensive and time-consuming. In the MEGA study, sixty-nine percent of eligible RDD controls participated.

In **chapter 7** we evaluated the analytic possibilities of these two different control groups and described the association of a general lifestyle risk factor (body mass index), a lifestyle risk factor in women (pregnancy) and an example of a genetic risk factor (factor V Leiden mutation) with the risk of venous thrombosis.

When evaluating body mass index as risk factor for venous thrombosis, partners and patients have more similar body mass indices than patients compared to random digit dialing controls (chapter 2). This matching between patients and partners has to be considered in the statistical analysis since ignoring matching generally introduces bias. A conditional logistic regression analysis (i.e. matched analysis) takes these similarities into account. It is important to realize that in a matched analysis only patient-partner pairs can be included, resulting in less power than an ordinary unconditional logistic regression analysis. Besides this, both patient and partner of a pair must have valid data for the required variables, otherwise the complete pair cannot be included in the analysis. Finally, the matched analysis itself only uses pairs who are discordant for the variable of interest, resulting in further reduced

power. For the analysis of body mass index, both matched analyses with partners and unmatched analyses with RDD controls showed consistent results in terms of clearly increased risks. We performed a combined analysis to obtain the most powerful estimate. A simple approach was used in which the estimates of the odds ratios of the matched and unmatched analyses were pooled[34]. In this combined analysis we accounted for the correlation between the estimated odds ratios since most patients were included both in the matched and unmatched analysis.

We assumed that asking partners would make it easier to recruit control subjects with pregnancies, malignancies or chronic diseases, which was a prerequisite if we wanted to study these diseases in relation to the risk of venous thrombosis. However, our pregnancy analysis showed that the opposite was true (chapter 5); partner controls group had fewer pregnancies than the RDD group. These findings could indicate that being pregnant for RDD controls was an extra motivation to participate, which is plausible considering the common knowledge that pregnancy is a risk factor for venous thrombosis. Because only women were included in the pregnancy analysis, simple pooling of both control groups was possible. In the combined control group pregnancy frequencies were comparable with data from the general Dutch population. These analyses illustrate that the inclusion of multiple control groups appeared to be very useful. A priori assumptions about control group characteristics were not in line with the collected data. If only a partner control group or only the RDD control group was collected, pregnancy associated risks were either over- or underestimated.

In the analysis of a genetic risk factor, frequencies of the factor V Leiden mutation were identical in both control groups and in line with published data. For the analyses of factor V Leiden as risk factor for venous thrombosis both control groups were thus equally suitable and could be simply combined.

Concluding remark

In the past, lifestyle factors as obesity, smoking and alcohol use were only considered to be associated with the risk of arterial disease. In this thesis we show that these factors are also related to the risk of venous thrombosis. Nowadays an increasing number of 'arterial risk factors' are linked with venous thrombosis[35;36]. This sharing of common risk factors between arterial and venous thrombosis suggests that the link between these two diseases is stronger than previously thought.

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Samenvatting

'The Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis' (MEGA studie) is een groot patiënt-controle onderzoek naar risicofactoren voor veneuze trombose. In deze studie zijn tussen 1999 en 2004 uit zes grote trombosediensten opeenvolgende patiënten in de leeftijd van 18 tot 70 jaar geïncludeerd met een eerste veneuze trombose. Als controle personen zijn partners van patiënten gevraagd mee te doen en ook werden controles verzameld door middel van een 'random digit dialing' (RDD) methode.

In dit proefschrift zijn binnen de MEGA studie verscheidene 'lifestyle' factoren als risicofactoren voor veneuze trombose onderzocht. Overgewicht, roken en alcoholgebruik komen aan bod en ook het gezamenlijk effect van deze factoren met genetische risicofactoren voor veneuze trombose, zoals de factor V Leiden mutatie en de protrombine 20210A mutatie worden besproken. Daarnaast worden zwangerschap en de periode na de bevalling geëvalueerd als risicofactoren voor veneuze trombose. Tevens hebben we twee genetische variaties in het promoter deel van het proteïne C gen onderzocht als risicofactor voor veneuze trombose en de invloed bekeken van deze genetische variatie op de bloedspiegels van dit antistollingseiwit. Tot slot hebben we onze ervaringen met de inclusie van twee verschillende controlegroepen in de MEGA studie besproken.

Recente studies laten zien dat overgewicht leidt tot een verhoogd risico op veneuze trombose. In overeenstemming met deze studies vonden wij in de MEGA studie een bijna twee keer verhoogd risico voor individuen met overgewicht (BMI≥25 en BMI<30 kg/m^2) en een 2.5 keer verhoogd risico voor individuen met obesitas (BMI≥30 kg/m^2) ten opzichte van individuen met een normale 'body mass index' (BMI<25 kg/m^2). Biologische ondersteuning voor deze relatie volgt uit studies waarin een verhoging van stollingsfactoren is gevonden met een toename in body mass index. Wij vonden een groter effect in vrouwen dan mannen, met een 24-voudig risico in obese vrouwen die de pil gebruikten ten opzichte van vrouwen met een normaal gewicht die de pil niet slikten. Het gezamenlijk effect van obesitas met de factor V Leiden mutatie en de prothrombine 20210A mutatie was iets groter dan op basis van de afzonderlijke effecten verwacht zou worden **(hoofdstuk 2)**.

De resultaten van studies die ingaan op de relatie tussen roken en veneuze trombose verschillen van elkaar. Er wordt zowel melding gemaakt van een verhoging als een verlaging van het trombose risico ten gevolge van roken. In the MEGA studie bleek roken geassocieerd te zijn met een 1.4-maal verhoogd risico. Wanneer in het verleden was gerookt, leidde dit tot een 1.2-maal verhoogd risico ten opzichte van individuen die nooit hadden gerookt. In rokers resulteerde een verhoging van het aantal sigaretten per dag in een verhoging van het tromboserisico. Er werd geen dosis-respons relatie gevonden tussen het aantal rookjaren en het risico op veneuze

trombose. Een verhoging in pakjaren leidde alleen in de jongste leeftijdscategorie (<38 jaar) tot een verhoging van het risico. Het effect van roken was sterker in vrouwen dan mannen, wat mogelijk verklaard kan worden door het hoge risico in pilgebruiksters. Roken samen met de pil leidde tot een 8.8 keer verhoogd risico. Tot op heden is niet duidelijk wat het mechanisme achter de relatie tussen roken en veneuze trombose is. Correctie voor het stollingseiwit fibrinogeen leidde niet tot een duidelijke risicoverlaging, wat aangeeft dat er andere factoren betrokken zijn bij het mechanisme. Daar de ontstekingfactor interleukine-6 verhoogd is in rokers en interleukine-6 het risico op een recidief trombose verhoogt, is de betrokkenheid van ontstekingsfactoren in de etiologie van veneuze trombose interessant voor toekomstig onderzoek **(hoofdstuk 3)**.

Matig alcoholgebruik is een bekende beschermende factor voor arteriële cardiovasculaire ziekten. Er is echter weinig onderzoek gedaan naar het effect van alcohol op veneuze trombose. Alcoholgebruik is in de MEGA studie geassocieerd met een verlaagd tromboserisico waarbij het effect het grootst is voor 2 tot 4 glazen per dag. In de literatuur wordt matig alcoholgebruik geassocieerd met verlaagde niveaus van bepaalde stollingsfactoren, wat het beschermende effect van alcohol mogelijk kan verklaren. In onze studie waren met name de niveaus van het stollingseiwit fibrinogeen verlaagd in alcoholgebruikers ten op zichte van geheelonthouders **(hoofdstuk 4)**.

Vanuit de literatuur is bekend dat voor vrouwen in de vruchtbare leeftijd meer dan de helft van alle veneuze tromboses gerelateerd is aan zwangerschap. In de MEGA studie laten wij gedurende de zwangerschap een bijna vijf keer verhoogd risico op veneuze trombose zien, wat in overeenstemming is met andere studies. Tijdens de zwangerschap vonden we het hoogste risico in het derde trimester (bijna negen keer verhoogd risico). Postpartum was het risico veruit het hoogst in de eerste zes weken na de bevalling; namelijk een 84-voudig risico ten opzichte van individuen die niet zwanger of postpartum waren. Zwangerschap in combinatie met de factor V Leiden mutatie resulteerde in een ruim 50 keer verhoogd risico ten opzichte van niet zwangeren zonder de mutatie. Zwangerschap in combinatie met de prothrombine 20210A mutatie resulteerde in een ruim 30 keer verhoogd risico **(hoofdstuk 5)**.

Naast lifestyle factoren spelen genetische factoren een belangrijke rol in de etiologie van veneuze trombose. De factor V Leiden en de prothrombine 20210A mutatie zijn risicofactoren met respectievelijk een minimaal driemaal en tweemaal verhoogd risico op veneuze trombose. Er zijn ook vele polymorfismen met een kleinere bijdrage aan het tromboserisico. We hebben twee polymorfismen in het promoter deel van het protein C gen (2405C/T en 2418A/G) onderzocht. Het CC/GG genotype was geassocieerd met het hoogste risico op veneuze trombose en met

de laagste proteïne C levels. Ten opzichte van het TT/AA genotype – een genotype geassocieerd met hoge protein C levels – was het risico op veneuze trombose 1.3 keer verhoogd in dragers van het CC/GG genotype. Dit resultaat is in overeenstemming met andere studies. Dit effect van het CC/GG genotype bleek voornamelijk gemedieerd te worden door het 2418A/G polymorfisme. Om na te gaan of het effect van het CC/GG genotype op het tromboserisico werkelijk werd veroorzaakt door verandering in protein C levels, moesten lage protein C levels geassocieerd zijn met een verhoging van het tromboserisico. Protein C levels onder de 81% waren inderdaad geassocieerd met een kleine risicoverhoging **(hoofdstuk 6)**.

In het laatste hoofdstuk hebben we het gebruik van de twee verschillende controlegroepen (partners en RDD controles) geëvalueerd bij het beantwoorden van verschillende soorten onderzoeksvragen. Wanneer body mass index onderzocht werd als lifestyle risicofactor voor veneuze trombose leken de body mass indices van patiënten en partners meer op elkaar dan de body mass indices van patiënten en RDD controles. Voor deze gelijkenis tussen patiënten en partners moest gecorrigeerd worden met een zogenaamde gematchte analyse. De RDD controles konden geanalyseerd worden met een ongematchte analyse. We ontwikkelden een methode om de resultaten van de gematchte en ongematchte analyse te kunnen samenvoegen zodat de meest precieze odds ratio werd verkregen.

Bij de analyse van zwangerschap als vrouw-specifieke risicofactor voor veneuze trombose konden beide controlegroepen simpel samengevoegd worden. We gingen ervan uit dat het includeren van partners het makkelijker zou maken om zwangere controles te includeren. Het tegenovergestelde bleek echter waar te zijn. In de partner controlegroep was het percentage zwangeren lager dan in de RDD groep. Waarschijnlijk was voor random controles het zwanger-zijn een extra motivatie om mee te doen, daar het bij veel vrouwen bekend is dat zwangerschap een risicofactor is voor veneuze trombose. Bij samenvoeging van de twee controlegroepen was het percentage zwangerschappen gelijk aan dat van de algemene bevolking. Het includeren van meer dan één controlegroep was voor deze analyse dus erg nuttig; als alleen een partner of RDD groep was geincludeerd waren de risico's over- of onderschat.

Bij de analyse van de factor V Leiden mutatie als genetische factor voor veneuze trombose waren de frequencies van deze mutatie in beide controlegroepen gelijk. Deze frequentie kwam tevens overeen met de frequentie gerapporteerd in de literatuur. Het is ook niet plausibel dat deelname van controle individuen af zou hangen van dit genetische kenmerk. Ook in dit geval konden beide controlegroepen dus simpel worden samengevoegd en geanalyseerd met een ongematchte analyse **(hoofdstuk 7)**.

Tot slot

Tot op heden werden lifestyle factoren als obesitas, roken en alcoholgebruik alleen geassocieerd met het risico op arteriële ziekten. Tegenwoordig worden echter steeds meer 'arteriële risicofactoren' geassocieerd met het risico op veneuze trombose. Het bestaan van gezamenlijke risicofactoren voor arteriële en veneuze trombose suggereert dat de overeenkomst tussen deze twee ziekten groter is dan vooralsnog werd aangenomen.

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Curriculum Vitae

Elisabeth Pomp werd geboren op 10 juli 1980 te Hoogeveen. In 1992 begon zij aan haar middelbare school opleiding (Menso Alting College te Hoogeveen) waar zij in 1998 haar atheneum diploma behaalde. Vervolgens begon zij in dat zelfde jaar met haar studie Biologie aan de Rijks Universiteit te Groningen. Tijdens deze studie heeft zij stage gelopen op de afdeling Neuro-Endocrinologie en de afdeling Humane Chronobiologie van de Rijks Universiteit Groningen. Daarnaast schreef zij tijdens haar afstuderen een scriptie over de rol van infecties in het ontstaan van schizofrenie en hield zij een colloquium over genderverschillen in depressiviteit. In 2003 studeerde zij cum laude af met als specialisatie de Gedrags- en Neurowetenschappen. In dat zelfde jaar kreeg zij de Unilever Research prijs voor haar stage over de werking van verzadigingsmechanismen op de afdeling Neuro-Endocrinologie.

In september 2003 begon zij met haar promotieonderzoek naar risicofactoren voor veneuze trombose bij de afdeling Klinische Epidemiologie van het Leids Universitair Medisch Centrum. Tijdens haar promotie traject heeft zij verschillende cursussen gevolgd, onder andere de Boerhaave cursus Klinische Epidemiologie op Schiermonnikoog, Causal inference (Ass. Prof. M.A. Hernán) en Principles of genetic epidemiology (Prof. Dr. C.M. van Duijn). Tevens heeft zij verschillende (inter)nationale congressen en symposia bezocht waar zij een poster heeft gepresenteerd of een mondelinge presentatie heeft gehouden.

Sinds april 2008 werkt zij als wetenschappelijk medewerkster bij het Expertisecentrum Forensische Psychiatrie (EFP) in Utrecht. Zij schrijft teksten voor onder andere het zorgprogramma persoonlijkheidsstoornissen en het zorgprogramma seksueel grensoverschrijdend gedrag.