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Risk factors for first and recurrent venous thrombosis : new insights from old concepts

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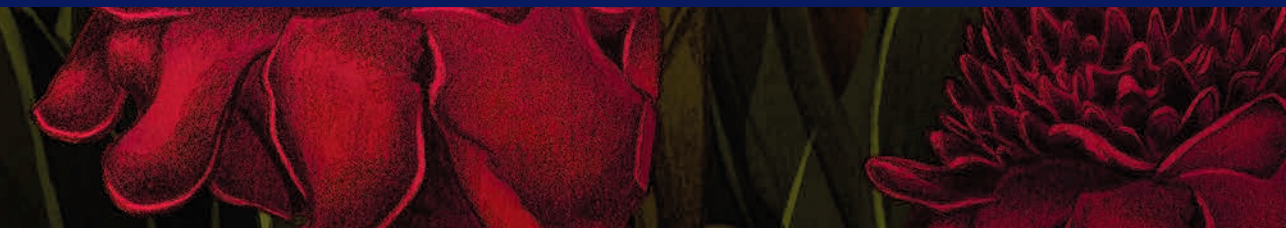


Risk factors for first and recurrent venous thrombosis

New insights from old concepts



Daniel Dias Ribeiro



Stellingen behorend bij het proefschrift

Risk factors for first and recurrent venous thrombosis

New insights from old concepts

1. Pneumonia increases the risk of venous thrombosis. (this thesis)
2. Modifying the winter season to summer season will not substantially lower the frequency of venous thrombosis in the Netherlands or Norway. (this thesis)
3. Synergistic effects of common acquired and congenital risk factors for venous thrombosis have a major impact. (this thesis)
4. The identification of individuals with low free protein S or low total protein S levels will only rarely could at best rarely identify subjects at risk for venous thrombosis in a population based study. (this thesis)
5. Information on exposure to risk factors for venous thrombosis long before the first event does not provide useful information to classify patients at reduced risk for recurrence of venous thrombosis. (this thesis)
6. Thrombophilic abnormalities are present in many patients with venous thrombosis. However, thrombophilia testing in all patients with a first venous thrombosis is neither clinically feasible nor useful. (this thesis)
7. Venous thrombosis is a multicausal disease. (Frits Rosendaal. Lancet 1999)
8. Accuracy of prevention strategies for recurrent venous thrombosis may be improved by including venous thrombosis associated with a transient infection in the current definition of “provoked” event. (Jasmijn F. Timp. British Journal of Haematology 2017)
9. Despite the increasing knowledge about the etiology of venous thrombosis, testing for thrombophilia serves only a limited purpose and should not be performed on a routine basis. (Saskia Middeldorp. Hematology 2011)
10. Epidemiology makes headlines more often than most other sciences. Every time you hear that something is good for you or bad for you, either there is an epidemiological study involved, or there is an epidemiological study being planned to check whether the claim is true. (If neither of these is the case, you should get your news somewhere else.) Yet many people have never heard of epidemiology. (Alex Broadbent. Philosophy of Epidemiology 2013)

Daniel Dias Ribeiro

Leiden 7 June 2017

**Risk factors for first and recurrent venous
thrombosis**

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**Risk factors for first and recurrent venous
thrombosis**

New insights from old concepts

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TABLE OF CONTENTS

Chapter 1	General introduction and outline of the thesis	7
Part I:	<i>Risk factors for first venous thrombosis</i>	
Chapter 2	Pneumonia and risk of venous thrombosis: results from the MEGA study <i>Journal of Thrombosis and Haemostasis 2012;10:1179-1182.</i>	23
Chapter 3	Seasonal variation of venous thrombosis: a consecutive case series within studies from Leiden, Milan and Tromsø <i>Journal of Thrombosis and Haemostasis 2012;10:1704-1707.</i>	33
Chapter 4	Risk of venous thrombosis in persons with increased body mass index and interactions with other genetic and acquired risk factors <i>Journal of Thrombosis and Haemostasis 2016;14:1572-1578.</i>	43
Chapter 5	Protein S levels and the risk of venous thrombosis: results from the MEGA case-control study <i>Blood 2013;122:3210-3219.</i>	59
Part II:	<i>Risk factors for recurrent venous thrombosis</i>	
Chapter 6	Epidemiology of recurrent venous thrombosis <i>Brazilian Journal of Medical and Biological Research. 2012;45:1-7.</i>	91
Chapter 7	Past provoking venous thrombosis risk situations on the risk of a recurrent thrombotic event: a cohort study <i>Thrombosis Research. 2011;128:227-232.</i>	111
Chapter 8	Risk of recurrent venous thrombosis related to past provoking risk situations: follow-up of a cohort study <i>Blood Coagulation and Fibrinolysis. 2013;24:562-566.</i>	129
Chapter 9	The influence of prothrombotic laboratory abnormalities on the risk of recurrent venous thrombosis <i>Thrombosis Research. 2012;130:974-976.</i>	143
Chapter 10	Summary and Discussion	153
Addendum	Nederlandse samenvatting	169
	List of publications	173
	Curriculum Vitae	174
	Acknowledgements	175

Chapter **1**

*General introduction
and outline of the thesis*

GENERAL INTRODUCTION

The overall incidence of venous thrombosis is 1-2 per 1000 persons each year and rises exponentially from $< 0.005\%$ in children to 1% per year in the elderly [1]. Most venous thrombosis start in the calf veins, from where they may extend and cause proximal deep vein thrombosis, and subsequently pulmonary embolism [2]. Patients with deep vein thrombosis or pulmonary embolism remain at risk for recurrent venous thrombosis. This risk is most pronounced in the first months after the acute episode and declines slowly over subsequent years [3]. Known risk factors for venous thrombosis are presented in Table 1. Although the existence of this long list of risk factors may seem reassuring, it does not come close to give us all the answers: many people have several of these risk factors but never develop thrombosis; others suffer from thrombosis but have none. Therefore, the challenge that we are facing today is not to just add more risk factors to this list but rather to integrate them all in a causal model that allows us to understand how and when thrombotic disease develops in an individual. Such a causal model for venous thrombosis was first described in 1999 by Rosendaal [4], and was coined as “the thrombosis potential model”. The idea behind this model is that an individual is at risk for venous thrombosis throughout life, which is reflected in the ‘thrombosis potential’ and that each risk factor contributes to increase the potential. Only when the combination of thrombosis risk factors reach a certain potential, venous thrombosis will occur (crossing of the thrombosis threshold). A worked out example is given in Figure 1. The black lines in the figure represent the thrombosis potential of each separate risk factor for a particular individual, and the grey line the thrombosis threshold. The horizontal axis shows time (i.e. the age of the individual). As the thrombosis potential is age-dependent, based on the observation that the incidence of thrombosis increases with age [5], the black line increases through time. Consider a person who has factor V Leiden (person A) and a person with blood group non-O (person B). Factor V Leiden carriers have a 5-7 fold increased risk of venous thrombosis [6], while blood group non-O is associated with a 1.5-fold increased risk [7]. So, person A has a larger inherited thrombosis potential than person B. They are of similar age, i.e., 55 years old. At this age, person A encounters a short period of immobilisation due to a strained ankle. In this example, this short period of a high thrombosis potential exceeds the thrombosis threshold and consequently leads to deep vein thrombosis (in the absence of anticoagulant prophylaxis, which would substantially decrease the potential). For person B, however, a similar acquired risk factor has not led to a thrombosis potential exceeding the thrombosis threshold. Therefore, this person does not develop the disease at this age.

In this thesis, the thrombosis potential model will be applied to several known risk factors for venous thrombosis to better understand why first and recurrent venous thrombosis can develop in an individual patient.

Old concepts: risk factors for first venous thrombosis

Pneumonia

Although several studies have focused on infectious diseases as possible causes of arterial thrombosis [8,9], less is known about their role in the development of venous thrombosis. According to Virchow's triad, infections could affect blood flow, damage the vessel wall or increase coagulability of the blood [10]. It is therefore interesting to note that a prospective population-based cohort study showed that elevated C-reactive protein levels, a marker of inflammation, were associated with an increased risk of venous thrombosis [11]. This risk was highest when C-reactive protein levels were elevated during the period directly prior to the onset of venous thrombosis. This finding supports the hypothesis that the coagulation system can be stimulated by transient inflammatory processes such as infections. Although two retrospective cohort studies showed that patients with pneumonia had a 2- to 3-fold elevated risk of acute venous thrombosis [12,13], there is uncertainty over the extent to which this risk association is causal. Pneumonia is often accompanied by transient periods of immobilization or hospitalization, leading to venous stasis that could explain the association. Alternatively, lifestyle could confound the association, when habits such as smoking and physical activity are related to both risk of pneumonia and risk of venous thrombosis. In **Chapter 2**, we elaborate whether the risk of venous thrombosis in patients with pneumonia is influenced or explained by immobility or unhealthy lifestyle factors, or that transient pneumonia can be seen as the final trigger that leads to the crossing of the thrombosis threshold.

Seasons

Seasonal variation in the incidence of various diseases, mainly characterised by a winter peak, has been reported for acute myocardial infarction and sudden cardiac death [14], and for ischemic and hemorrhagic stroke [15]. Although circannual variability of venous thrombosis has been reported since 1939, results of the reported studies are conflicting [16,17]. Nevertheless, the reported association between the seasons and the incidence of venous thrombosis is supported by studies showing that cold temperature is associated with increased levels of coagulation factors [18]. Also, transient

infections (such as pneumonia or influenza) are more common in the colder seasons of temperate climates, which may also increase the risk of venous thrombosis [13]. Furthermore, air pollutants are more common in winter months than in spring, specially in larger city areas [19]. Finally, vitamin D, synthesized in the skin after sun exposure, has been related to fibrinolytic and procoagulant activity [20]. Therefore sun exposure during the summer months may consequently lower the risk of thrombotic events. So, although the seasons should not be seen here as final triggers of crossing of the thrombosis threshold, there may be information available within a specific season that could explain a higher chance of developing venous thrombosis. In **Chapter 3**, we describe a consecutive case series of patients with venous thrombosis from three different regions; i.e. Italy, The Netherlands and Norway in which we study the incidence of venous thrombosis within the four seasons.

Overweight/Obesity

Overweight or obesity is associated with a 2- to 3-fold increased risk of first venous thrombosis compared with individuals with a normal weight [21,22]. It is not completely understood how obesity predisposes to venous thrombosis, but a recent observation provided evidence that it is at least partly mediated by factor VIII induced Activated Protein C-resistance [23]. In the same study it was shown that the combination of blood group non-O with a high body mass index or factor V Leiden led to higher venous thrombosis risks than expected when these prothrombotic factors were analyzed separately [23]. However, due to small numbers, the authors could not sufficiently study the risk of venous thrombosis for the combination of a high body mass index with factor V Leiden and blood group non-O in subgroups in sufficient detail, like separately for individuals at risk of provoked or unprovoked venous thrombosis. In terms of personalized prevention measures, it is of interest to identify high and low-risk groups. Hypothetically, these individuals might have such a high thrombosis potential, that additional provoking risk factors, such as oral contraception or long-haul travel, could easily lead to the crossing of their thrombosis threshold. If so, it could be justifiable to target these individuals for future preventative thromboprophylaxis decisions. For this reason, we set out to determine whether the presence of factor V Leiden with blood group non-O modifies the risk for venous thrombosis in various body mass index strata (**Chapter 4**) and evaluated the presence of gene-environment interactions in specific environmental situations to this risk.

Protein S levels

Protein S is a vitamin K-dependent plasma glycoprotein that functions as a nonenzymatic cofactor of activated protein C in the inactivation of the procoagulant factors Va and VIIIa, and plays an important role in regulating thrombin generation [24]. Based on antigen levels and Activated Protein C-cofactor activity, protein S deficiency can be classified as type I (decreased levels of both total and free protein S antigen), type II (total and free protein S antigen levels within their normal ranges, but decreased Activated Protein C-cofactor protein S activity), and type III (decreased levels of free protein S antigen levels only) [25]. Most studies that showed that protein S deficiency was related with an increased risk for venous thrombosis have been performed in thrombophilic families [26,27]. In population-based studies, the risk of thrombosis associated with protein S deficiency is less well defined. Some studies find an up to 2.5 times increased risk, others find no increase at all [28-30]. A difference in risk between subjects with type I and type III deficiency, together with variation in the distribution of hereditary protein S deficiency among the studied populations may explain this discrepancy, although these types were not distinguished in the published reports. Nevertheless, the clinical relevance for the individual patient could be substantial as individuals with inherited protein S deficiency seem to easily cross the thrombosis threshold for both first venous thrombosis (absolute risk of 1.5-2% per year) and recurrent venous thrombosis (annual recurrence risk of 6-10%) [26]. In **Chapter 5** we therefore set out to determine if in a large population-based case-control study (MEGA study) low levels of free protein S or total protein S were associated with an increased risk of venous thrombosis. The molecular basis for protein S deficiency was additionally investigated by analysis of copy number variation of *PROS1* and sequencing of individuals with the lowest levels of protein S in an attempt to explain the different findings in risk estimates between families and population studies.

Old concepts: risk factors for recurrent venous thrombosis

Treatment with oral anticoagulants successfully reduces the risk of recurrent venous thrombosis, albeit at the cost of an increased risk of severe or fatal bleeding. The optimal duration of anticoagulant treatment therefore depends on the balance between risk of recurrent venous thrombosis and risk of bleeding. According to the American College of Chest Physicians 2012 guidelines, primary factors for estimating the risk of recurrence are the presence of a reversible provoking risk factor, unprovoked venous thrombosis and the presence of active cancer [31]. Yet, only 50% of patients can be classified at such low risk of recurrence that anticoagulation can be discontinued after 3-6 months (provoked venous thrombosis) or at such high risk of recurrence that anticoagulation should be continued for indefinite time (active malignancy). For the other 50% of patients prediction of recurrent venous thrombosis remains a clinical dilemma [32]. **Chapter 6** briefly outlines what is currently known about the epidemiology of recurrent venous thrombosis, and also focuses in more detail on potential new risk factors for venous thrombosis recurrence.

Past provoking risk factors

Although it has been recognized that the presence of a transient or reversible provoked risk factor at the time of venous thrombosis is associated with a decreased risk of recurrence after anticoagulant therapy is stopped [33], a substantial number of recurrent events occur without a clear provoking risk factor. The challenge therefore is to identify other patient groups that may also have a low risk of recurrence and could receive anticoagulants for a fixed (short) time. The thrombosis potential model takes into consideration that the thrombosis potential will drop sharply after the occurrence of a provoked event and that such an individual will not readily cross the thrombosis threshold again, consequently leading to a reduced risk for recurrence. Considering this model we hypothesized in **Chapter 7** that patients at risk for recurrent venous thrombosis who had experienced transient provoked risk factors previously, yet not developed thrombosis at that time, would have a lower risk of recurrence than those whose first (provoked or unprovoked) event was not preceded by provoked risk factors. In **Chapter 8** we re-evaluated the same cohort with a longer follow-up.

Thrombophilia

Since 1965, an increasing number of abnormalities of coagulation and fibrinolysis have been identified as risk factors for venous thrombosis. These thrombophilic defects include hereditary deficiencies of antithrombin, protein C and protein S, factor V Leiden, prothrombin G20210A, elevated levels of factors VIII, and hyperhomocysteinemia [34]. Meanwhile thrombophilic alterations can be demonstrated in at least 50% of patients with venous thrombosis [34], testing patients with a first venous thrombosis for thrombophilia has gained great interest. However, whether patients with venous thrombosis should be tested for thrombophilic defects is still a matter of debate [35]. Potential advances of testing patients might be the opportunity to elucidate the cause of the thrombosis and to track unaffected family members. However, there are potential disadvantages of testing for thrombophilia. For instance, the negative results of thrombophilia for the prediction of a recurrent event does not support wide spread testing [35,36]. However, these negative results were mostly obtained in persons with any first venous thrombosis. Whether thrombophilia has predictive value for recurrence in persons who had a first unprovoked event is less well studied. In order to assess these issues, we performed a prospective cohort study, described in **Chapter 9** to assess the risk of recurrence in patients with provoked and unprovoked first venous thrombosis, related to the presence or absence of thrombophilia.

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Table 1. Risk factors for first venous thrombosis

	Relative risk*	Reference with risk estimates†	Discussed in this thesis
Genetic			
- Antithrombin deficiency	15-20	Heijboer et al, 1990; Simioni et al, 1999	No
- Protein C deficiency	15-20	Heijboer et al, 1990; Simioni et al, 1999	No
- Protein S type I deficiency	15-20	Heijboer et al, 1990; Simioni et al, 1999	Yes, chapter 5
- Factor V Leiden	5-7	Rosendaal et al, 1995	Yes, chapters 1,4
- Prothrombin G20210A	2-3	Poort et al, 1996	Yes, chapters 1,4
- Non-O blood group	1.5-1.8	Jick et al, 1969	Yes, chapters 1,4
- Factor XIII val34leu	1.2-1.5	Franco et al, 1999	No
- Recently discovered SNPs	1.1-1.4	Smith et al, 2007; Bezemer et al, 2008	No
Acquired			
- Increasing age	1-∞	Naess et al, 2007	Yes, chapter 6
- Malignancy	7-20	Blom et al, 2005	Yes, chapters 4,6,7
- Lupus anticoagulant	3-10	Ginsberg et al, 1995	No
- Systemic lupus erythematosus	3-8	Brouwer et al, 2004	No
- Inflammatory bowel disease	3-4	Grainge et al, 2010	No
- Hyperthyroid disease	1.5-3	Cannegieter et al, 2009	No
- HIV	3-10	Sullivan et al, 2000	No
- Nephrotic syndrome	3-10	Mahmoodi et al, 2008b	No
- Renal transplant recipients	3-8	Poli et al, 2006	No
- Chronic kidney disease	1.3-1.7	Wattanakit et al, 2008	No
- Microalbuminuria	1.5-2.5	Mahmoodi et al, 2009	No
- Overweight and obesity	2-3	Kakkar et al, 1970; Pomp et al, 2007	Yes, chapters 4,5,6
Environmental			
- Surgery, trauma, immobilization	5-50	Nicolaides et al, 1973	Yes, chapter 7
- Pregnancy and puerperium	3-5	Kierkegaard, 1983; McColl et al, 1997	Yes, chapters 4,7
- Oral contraceptives	4-7	Sartwell et al, 1969	Yes, chapters 4,7
- Hormone replacement therapy	2-5	Grodstein et al, 1996	Yes, chapters 4,7
- Air travel	1.5-3	Sarvesvaran, 1986; Cannegieter et al, 2006	No
- Transient infectious disease	1.0-3.0	Smeeth et al, 2006	Yes, chapters 1,8
Mixed			
- Low (free) protein S levels	5-10	Faioni et al, 1997	Yes, chapter 5
- High factor VIII	3-5	Koster et al, 1995	Yes, chapter 8
- APC-resistance	3-5	Koster et al, 1993	Yes, chapter 4
- High factor IX	2-3	van Hylckama Vlieg et al, 2000	No
- High factor XI	1.5-2.5	Meijers et al, 2000	No
- High TAFI	1.5-2.5	van Tilburg et al, 2000	No
- Hyperhomocysteinaemia	1.5-2.5	Den Heijer et al, 1996	No
- Hypofibrinolysis	1.5-2.5	Lisman et al, 2005	No
- Enhanced thrombin generation	1.5-2.5	Brummel-Ziedins et al, 2005	No
Not well established			
- Air pollution	1.1-2.0	Baccarelli et al, 2009	Yes, chapter 7
- High CRP	1.2-1.8	Quist-Paulsen et al, 2009	No
- Abnormal interleukin levels	1.0-2.5	van Aken et al, 2002	No
- Seasonal variance	1.0-2.5	Bounameaux et al, 1996	Yes, chapter 3
- Hypertension	0.7-2.0	Agno et al, 2008	No
- Diabetes mellitus	0.7-2.0	Tsai et al, 2002; Heit et al, 2009	No
- Male sex	0.8-1.5	Naess et al, 2007; Andreou et al, 2008	Yes, chapter 6,7
- Smoking	0.8-1.5	Pomp et al, 2008	No
- Dyslipidemia	0.8-1.5	Glynn & Rosner, 2005	No

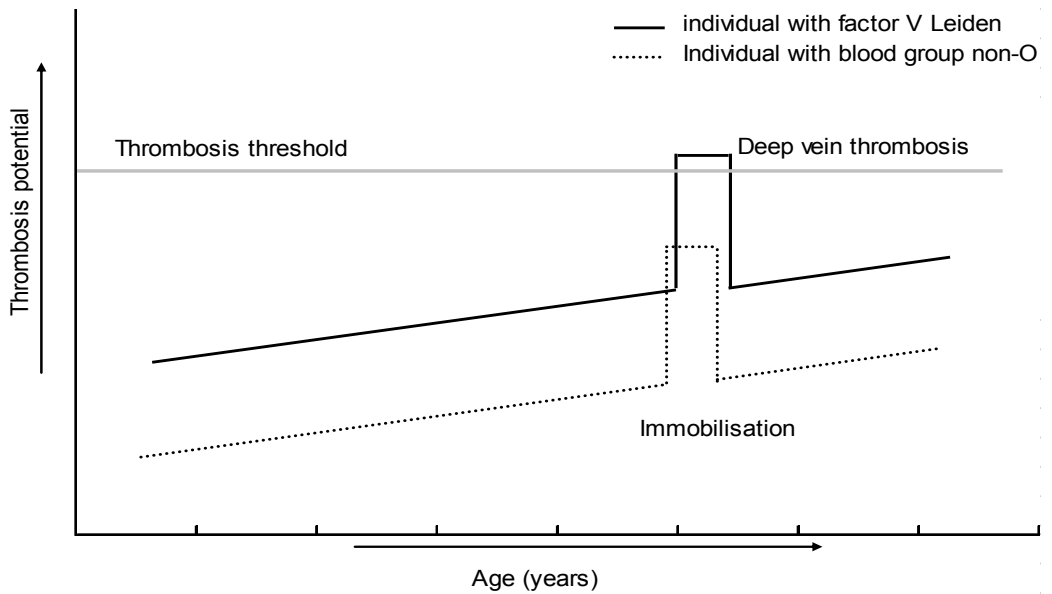
* Risk for first venous thrombosis compared to the general population.

† We have attempted to give a reference to the first paper from which a risk estimate could be inferred.

In some cases, these were such early papers with unstable estimates, that we also included references to more recent papers with precise estimates.

SNPs denote single nucleotide polymorphisms; TAFI, thrombin activatable fibrinolysis inhibitor.

Figure 1. A way to think about causality for venous thrombosis: the thrombosis potential model



The figure shows the thrombosis potential of an individual with factor V Leiden and of an individual with blood group non-O. Both experience a short period of immobilisation at a similar age. This leads to an excess of the thrombosis threshold level in the individual with factor V Leiden which consequently leads to deep vein thrombosis, while the individual with blood group non-O does not develop thrombosis.

Part **I**

Risk factors for first venous thrombosis

Chapter 2

Pneumonia and risk of venous thrombosis: results from the MEGA study

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A recent prospective population-based cohort study showed that elevated C-reactive protein (CRP) levels were associated with an increased risk of venous thrombosis [1]. Interestingly, it was shown that this risk was highest when CRP levels were elevated during the period directly prior to the onset of venous thrombosis. This finding supports the hypothesis that the coagulation system can be stimulated by transient inflammatory processes [2], which has also been suggested by experimental studies [3]. Two retrospective cohort studies showed that patients with pneumonia had a 2- to 3-fold elevated risk of acute venous thrombosis [4,5]. However, there is uncertainty on the extent to which this risk is causal. Pneumonia is often accompanied by transient periods of immobilization or hospitalization leading to venous stasis that could explain the association. Alternatively, life style could confound the association, when habits such as smoking and physical activity are related to both risk of pneumonia and risk of venous thrombosis. Furthermore, it is uncertain whether the degree to which the risk is increased justifies thromboprophylaxis in these patients. For this discussion it may be important to have knowledge on risk groups in whom the risk is additionally increased.

To study this, we set out to determine if pneumonia was associated with an increased risk of venous thrombosis in a large population-based case-control study (MEGA study), and whether this risk was influenced by underlying relative immobility or unhealthy lifestyle factors. Furthermore, we assessed gene-environment interactions, i.e., the combined effect between pneumonia and prothrombotic genes that may be clinically relevant for targeted thromboprophylaxis.

The MEGA study is a population-based case-control study that has been described in detail elsewhere [6]. Approval for this study was obtained from the Medical Ethics Committee of the Leiden University Medical Center, and all participants provided written informed consent according to the Helsinki Declaration. Participants were aged 18 to 70 years. 4956 consecutive patients with deep-vein thrombosis or pulmonary embolism (both objectively confirmed with radiologic techniques) were enrolled, together with 6297 age- and sex-matched controls. Participants were asked to fill in a standardized questionnaire within a few weeks after inclusion in the study. The questionnaire provided information on weight, height, smoking status, physical activity, and risk factors for venous thrombosis, including recent (within three months prior to the index date) trauma, plaster cast use, surgery, pregnancy/puerperium, or immobilization (bedridden at home for at least 4 days, hospitalization), > 4 hours travelling by air, train, bus, or car in the 8 weeks before the index date, estrogen/progestagen use (oral contraceptives or hormonal replacement therapy) at time of the index date, and diagnosis of malignancy within five years before the index date. In addition, questions were asked about pneumonia in the year before the index date. All answers were self-reported and the exact dates of these events were not available. Body mass index (BMI) was calculated by dividing weight (kg) by height squared (m^2). Smokers were divided in current, previous, 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. Participants who participated in sports activities at least once a week were considered to be physically active [9]. The index date was the date of the thrombotic event for patients and their partners and the date of filling in the questionnaire for the random controls. In addition, participants provided a blood or buccal swab sample for DNA. Common genetic risk factors were assessed, i.e., the factor V Leiden mutation and prothrombin G20210A. These were determined by polymerase chain reactions using the TaqMan assay. Technicians were blinded to whether the samples came from patients or controls. For the present analysis, questionnaire data on pneumonia were available from 4281 patients and 5752 controls.

Odds ratios (ORs) with 95% confidence intervals (95% CIs) were calculated as estimates of the relative risk, and were adjusted for age and sex by logistic regression methods to take the matching into account. Subgroup analysis involved stratification of patients with deep vein thrombosis of the leg only, or with pulmonary embolism +/- deep vein thrombosis. We adjusted our findings for classical venous thrombosis risk factors (defined as trauma, surgery, > 4 hours travelling, malignancy and, for women, pregnancy/puerperium or the use of hormone therapy) [6] and for unhealthy life style factors (overweight/obesity, smoking habit and physical activity) with logistic regression methods to determine whether the associations could be explained by confounding through these factors. To quantify the effect of immobilization as a mediator of the risk of venous thrombosis in pneumonia participants (pneumonia leads to immobilization which in its turn leads to venous thrombosis), we further adjusted the analysis for immobilization. The combined effect of prothrombotic genes and pneumonia was evaluated through a stratification analysis (4 strata with the different combinations of presence and absence of either factor). All statistical analyses were performed with SPSS for Windows, release 17.0 (SPSS Inc, Chicago, IL).

Our study included 2887 patients with deep-vein thrombosis only and 2069 patients with pulmonary embolism +/- deep-vein thrombosis. Median age (interquartile range) was 50 years (39-59) for patients and 48 years (37-57) for controls. 2274 (46%) of patients were male and 2914 (46%) of controls were male. Patients were slightly older than control subjects (median 50 vs. 48 years), they were more often overweight/obese (63% vs. 51%), smoked more often (66% vs. 60%) and were more often physically inactive (69% vs. 62%). Furthermore, classical venous thrombosis risk factors were more often present in patients than in control subjects (72% vs. 40%). 891 Patients (18%) were hospitalized in the previous three months before the index date, as compared with 176 (3%) controls.

A period of pneumonia in the year before the thrombotic event was present in 7.2% (N=307) in patients and in 1.5% (N=87) in controls (Table 1). Participants with prior pneumonia were 5 times more likely to have venous

thrombosis (odds ratio 5.0; 95% CI, 3.9-6.3), than participants without pneumonia. This risk was higher when pulmonary embolism was considered (odds ratio 7.9; 95% CI, 6.1-10.3, for pulmonary embolism plus or minus deep vein thrombosis and [8.1; 95% CI, 6.2-10.6 for pulmonary embolism only] than when deep vein thrombosis only was considered ([odds ratio 3.0; 95% CI, 2.2-4.0]). To investigate whether the study results in Table 1 could be due to the presence of confounding, we adjusted our findings for age, sex, classical risk factors for venous thrombosis and healthy lifestyle, which led to minimal attenuation of the effect estimates (Table 2). Additional adjustment for immobilization (as a mediator) led to a slight decrease in relative risk for venous thrombosis with an odds ratio of 3.8 (95% CI, 2.9-5.1). When patients with deep-vein thrombosis only were compared with controls, this odds ratio was 1.9 (95% CI, 1.3-2.7). Further exclusion of vitamin K antagonist users at time of the index date (11 patients and 34 controls) did not affect the risk estimates.

Next, we analyzed whether a combined effect of prothrombotic genes and pneumonia would lead to an increased venous thrombosis risk (Table 3). Among factor V Leiden carriers without pneumonia the risk of venous thrombosis was 3.4-fold (95% CI, 2.9-4.0) increased. Participants with pneumonia who were non-carriers of factor V Leiden had a 5.5-fold (95% CI, 4.1-7.3) increased risk, while those who had factor V Leiden and pneumonia had a 17.8-fold (95% CI, 5.5-57.7) increased risk compared to non-carriers without pneumonia. A similar joint effect was observed for prothrombin G20210A and pneumonia on venous thrombosis risk (Table 3).

In this large population based case-control study, recent pneumonia was a moderate to strong risk factor for venous thrombosis. Participants who had pneumonia had a 5-fold increased risk of venous thrombosis within one year. Pneumonia resulted in a more pronounced relative risk of pulmonary embolism than of deep vein thrombosis. This latter finding may reflect local inflammatory effects in the lungs [7], but it may also be due to misclassification, which is possible as signs and symptoms of pneumonia can be similar to pulmonary embolism. To determine whether the associations could be explained by classical venous thrombosis risk factors or unhealthy life style, we adjusted for these factors. The presence of classical venous thrombosis risk factors did not affect our outcomes, nor did adjustment for unhealthy lifestyle factors. These results provide evidence that the association between infectious or inflammatory disease and venous thrombosis cannot be explained by unhealthy life style. Immobilization, however, attenuated the effect from 5.0 to 3.8, indicating that immobilization could play a role in the mechanism of pneumonia leading to venous thrombosis. Although the relation that remained could possibly be explained by residual confounding, as unhealthy life style could not be measured in great detail [8,9], it is not likely that we missed a confounder that can fully explain the remaining 4-fold increased risk. Alternatively, the association could point towards a causal association between pneumonia and venous thrombosis. Inflammation

influences endothelial function through protease-activated receptors (PARs) signaling [10]. Our finding that pneumonia is associated with venous thrombosis is biologically plausible if pneumonia increases venous thrombosis risk by PAR signaling leading to a procoagulant response [11], and ultimately, to venous thrombosis. Other aspects that are in favor for the supposed causal association between pneumonia and venous thrombosis include the strength of the association, consistency with studies that also analyzed the association [2,4,5], and a temporal relationship wherein pneumonia occurred before the event took place.

From Table 3 it can be noted that the ‘factor V Leiden paradox’ [12] is again present in patients with factor V Leiden without pneumonia: in these patients the risk of deep-vein thrombosis is higher than that of pulmonary embolism. For patients with just pneumonia in the absence of factor V Leiden the opposite is the case, i.e. risk of pulmonary embolism is clearly higher than that of deep-vein thrombosis. Interestingly, in patients who have both, both odds ratios are first of all much higher and secondly, the factor V Leiden paradox does not play a role anymore. Apparently the effect of the pneumonia is stronger with a stronger effect on pulmonary embolism than on deep vein thrombosis. The clear joint effects for both factor V Leiden and prothrombin G20210A in combination with pneumonia on venous thrombosis risk (18 to 32-fold increased risks compared to non factor V Leiden/prothrombin G20210A carriers without pneumonia) was high enough to deliberate on the need for a different thromboprophylactic approach in these subjects. Of note, such a strategy should be supported by clinical trials before a strong clinical recommendation on whether persons with abovementioned thrombophilia should or should not receive thromboprophylaxis at time of pneumonia.

The strengths of this study include the large patient sample, the detailed information about venous thrombosis risk factors in both patients and controls, and the combination with data on prothrombotic genes. A limitation of this study is that pneumonia was assessed via self-report in our study. However, we expect that both patients and control persons reported pneumonia without much misclassification, since pneumonia is a major disease with a large impact. Another limitation is that we could not study the exact temporal relationship between pneumonia and venous thrombosis risk as our questionnaire did not provide the exact dates of pneumonia, but only whether these diseases occurred in the year before the index date. This may have diluted our risk estimates.

In summary, in this large population based case-control study pneumonia substantially increased the risk of venous thrombosis. This association could only partially be explained by a concurrent period of immobilization or life style. The 18 to 32-fold increased risk of venous thrombosis in participants with pneumonia and factor V Leiden or prothrombin G20210A should be considered when people known to have these prothrombotic genes present themselves with pneumonia.

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Table 1. Risk of venous thrombosis for various infectious or inflammatory diseases

	Patients, n	Controls, n	Adjusted odds ratio (95% CI)*		
			Overall	DVT only	PE+/-DVT
No pneumonia	3974	5665	Reference	Reference	Reference
Pneumonia	307	87	5.0 (3.9-6.3)	3.0 (2.2-4.0)	7.9 (6.1-10.3)

* Adjusted for age and sex

CI denotes confidence interval; DVT, deep vein thrombosis; PE, pulmonary embolism

Table 2. Risk of venous thrombosis in participants with pneumonia

Patients, n		Controls, n	Odds ratio* (95% CI)	Odds ratio (95% CI)* further adjusted for	
				Classical risk factors	+ Unhealthy lifestyle
Without immobilization adjustment					
No pneumonia	3974	5665	Reference	Reference	Reference
Pneumonia	307	87	5.0 (3.9-6.3)	4.9 (3.8-6.3)	4.8 (3.6-6.2)
With immobilization adjustment					
No pneumonia	3974	5665	Reference	Reference	Reference
Pneumonia	307	87	5.0 (3.9-6.3)	3.9 (3.0-5.1)	3.8 (2.9-5.1)

Classical risk factors include trauma, surgery, malignancy, > 4 hours travelling, plaster cast and, for women, pregnancy/puerperium or the use of hormone therapy

Unhealthy lifestyle include overweight/obesity, current or former smoking status and physical inactivity

CI denotes confidence interval

* Adjusted for sex and age

Table 3. Interactions of pneumonia with prothrombotic genes on venous thrombosis risk

		Patients, n	Controls, n	Adjusted odds ratio (95% CI)*		
				Overall	DVT only	PE+/-DVT
	Factor V Leiden -	3067	4356	Reference	Reference	Reference
Pneumonia -	Factor V Leiden +	590	248	3.4 (2.9-4.0)	4.4 (3.7-5.2)	2.1 (1.7-2.6)
Pneumonia -	Factor V Leiden -	233	60	5.5 (4.1-7.3)	3.2 (2.3-4.5)	8.6 (6.3-11.6)
Pneumonia +	Factor V Leiden +	37	3	17.8 (5.5-57.7)	15.0 (4.4-50.9)	21.3 (6.3-72.2)
				Adjusted odds ratio (95% CI)*		
	Prothrombin mutation -	3466	4514	Reference	Reference	Reference
Pneumonia -	Prothrombin mutation +	194	91	2.8 (2.2-3.6)	3.0 (2.3-4.0)	2.4 (1.8-3.3)
Pneumonia -	Prothrombin mutation -	254	62	5.3 (4.0-7.0)	3.1 (2.3-4.4)	8.5 (6.3-11.5)
Pneumonia +	Prothrombin mutation +	16	1	20.7 (2.7-155.9)	13.1 (1.6-109.0)	31.5 (4.0-246.7)

* Adjusted for age and sex

CI denotes confidence interval; DVT, deep vein thrombosis; PE, pulmonary embolism

Chapter 3

Seasonal variation of venous thrombosis: a consecutive case series within studies from Leiden, Milan and Tromsø

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Circannual variability of venous thrombosis has been reported since 1939, but there is no definitive determination of whether it really exists. Although a recent meta-analysis on this topic, including 35 000 patients with venous thrombosis, has recently been published [1], and showed a similar existence of a seasonal variability in venous thrombosis as for arterial cardiovascular disease [2-6], there are some issues that preclude whether this meta-analysis found a true association between seasonal variability in the incidence of venous thrombosis. First, the meta-analysis did not rule out the possibility of publication bias. Second, the meta-analysis did not include a large observational study of seven million individuals from the United States that failed to find a seasonal variability on incidence of (ICD-9-CM diagnosed) venous thrombosis [7]. Third, the meta-analysis was dominated by one study (n=20.000, 70% of the included cases) that found a strong effect on the incidence of venous thrombosis (lowest in spring, highest in winter) [8]. Therefore, it could be argued that the inclusion of the other studies in the meta-analysis added very little, and that the meta-analysis showed no more than the one large study published by Gallerani et al [8].

Nevertheless, the reported association between the seasons and the incidence of venous thrombosis is supported by studies showing that cold temperature was associated with increased levels of coagulation factors [9,10]. Also, transient infections (like pneumonia or influenza) are more common in the colder seasons of temperate climates, which may also increase the risk of venous thrombosis [11]. Furthermore, air pollutants are more common in Winter months as compared to Spring, especially in larger city areas [12]. Interestingly, some studies [13], but not all [14], suggest that air pollution increases the risk of venous thrombosis. Finally, vitamin D, synthesized in the skin after sun exposure, has been related to fibrinolytic and procoagulant activity [15,16]. Therefore sun exposure during the Summer months may consequently lower the risk of thrombotic events.

We had the opportunity to study circannual variability of venous thrombosis in three regions (i.e. Milan/ Italy, Leiden/the Netherlands and Tromsø/Norway). These three regions have a similar climate, and are similarly situated around sea level, but differ in the annual temperature, which is highest in Milan (mean 13.5°C) (<http://www.climatetemp.info/italy/milan.html>) lower in Leiden (mean 9.6°C) (<http://www.climatetemp.info/netherlands/>) and lowest in Tromsø (mean 2.9°C) (<http://www.climatetemp.info/norway/tromso.html>). Regions are demographically comparable in terms of ageing and health status (<http://en.istat.it/>, www.cbs.nl and www.ssb.no), but differ in terms of air pollution (high in Milan and Leiden, lower in Tromsø) [17]. They also differ in terms of sunlight (with the irregular fall in the hours of sunlight and the steady fall in the length of daylight over the seasons that can be extreme in Tromsø, and which is approximately similar in Milan as compared to Leiden). Therefore, regions are matched on demographic and climate variables, but differ in terms of temperature, sunlight and air pollution.

This study was performed in the Multiple Environmental and Genetic Assessment (MEGA) study (from Leiden), the Tromsø study (from Tromsø)

and in the Lombardy region (from Milan). The MEGA study is a large population-based case-control study. Between March 1, 1999 and August 31, 2004, all consecutive patients between the age of 18 and 70 with a first deep-vein thrombosis (DVT) of the leg or pulmonary embolism (PE) have been identified at six regional anticoagulation clinics in the Netherlands [18]. Four thousand nine hundred and fifty-six patients were included in whom the diagnosis of incident venous thrombosis was confirmed by objective methods.

In the Tromsø study, participants were recruited from the fourth survey of the Tromsø study conducted in 1994-95, a single-center, prospective, population based study, with repeated health surveys of inhabitants in the city of Tromsø, Norway [15]. All inhabitants aged 25 years or older were invited, and 27158 participated (77% of the population invited). Incident venous thrombosis (N=661) events among the study participants were recorded from the date of enrollment through the end of follow-up, September 1, 2007.

The study from Milan is a population based case-control study where 2081 patients had been diagnosed between 21 December 1993 through 20 December 2010 as having incident DVT or PE [16]. Patients were referred to the Thrombosis Center, Fondazione IRCCS Ca' Granda - Ospedale Maggiore Policlinico, Milan, Italy, for a thrombophilia screening. All three studies were approved by the regional committee for research ethics, and all participants gave their informed, written consent.

In the MEGA study (Leiden), we evaluated in 4 completed seasonal cycles (21 December 2000 to 20 December 2003), if consecutive cases with venous thrombosis (N=2666) had more events in winter compared to other seasons. Similarly, in the Tromsø study, we evaluated the frequency of venous events (N=596) in 12 completed seasonal cycles (21 March 1994 to 20 March 2006). In Milan, the same figure was evaluated in 17 completed seasonal cycles (21 December 1993 to 20 December 2010). Two thousand two hundred and ninety patients from MEGA, 65 patients of Tromsø and no patient from Milan were excluded in order to let the cycles of seasons be complete. We labeled Winter as the period of time between December 21 - March 20; Spring March 21 - June 20; Summer June 21 - September 20; and Autumn September 21 - December 20.

Clinical characteristics of the Leiden, Tromsø, and the study from Milan are provided in Table 1. Approximately half of the patients were men, and the mean age at time of thrombosis was 49 years (interquartile range 39-61). In all three studies, the prevalence of venous thrombosis was lowest in spring as compared with the other seasons (absolute risk difference - 2.3%, 95% CI, -4.0 to -0.7) (Figure 1). The absolute risk difference (taking Spring as a reference group) was -2.6% (95% CI, -4.3 to -1.0) to Winter, -2.2% (95% CI -3.8 to -0.6) to Summer and -2.2% (95% CI, -3.8 to -0.5) to Autumn, respectively. When unprovoked events, or incident PE, or men only were analyzed separately, a similar pattern was observed, that is: a lower prevalence of thrombosis in Spring than in the other seasons (Table 1).

In this study we observed a difference in the distribution of venous thrombosis over the seasons in all three studies, wherein the prevalence was lowest in Spring, and somewhat similar over the other seasons. Because our numbers were fairly large ($n=5343$), the absolute difference of venous thrombosis between Spring and the other seasons was statistically significant. However, the clinical relevance of a 2% seasonal variation in incidence of venous thrombosis is questionable. Still, these results were consistent within all three studies. Therefore, our finding (lower prevalence of venous thrombosis in Spring) at least provides a suggestion that the association that we found is true. But even if the association is true, a causal interpretation of this finding is hard to provide. To proof causality, one would like to know an intervention strategy [19], and the intervention “Spring” seems too ambiguous to use as an intervention tool. Nevertheless, some things can be inferred from our results. First, our findings cannot be explained by a sole linear increase of temperature, because the prevalence of venous thrombosis was higher in the hottest season (Summer) as compared to Spring in all three studies. Second, transient infections are also not very likely to explain our results. Although transient infections (pneumonia, influenza) are more common in the colder seasons of temperate climates [20], there was a small difference in venous thrombosis prevalence between the Summer season and Winter/Autumn in all three studies. Third, our results are also probably not explained by sunlight exposure. Although in all three regions, sunlight is mostly present from about 21 May to 21 July, sunshine hours are extreme in Tromsø (midnight sun around those dates), while the prevalence of venous thrombosis in Tromsø was only slightly different over the colder seasons compared to Spring and Summer, especially when compared to the Leiden or Milan findings. A fourth explanation may be air pollution, which is lowest in Spring and highest in Autumn/Winter and Summer months in the Milan region [8]. However, in Norway air pollution is rather high during Spring (explained probably by dry asphalt and studded tyres) [19]. Still, in Tromsø the prevalence of venous thrombosis was also lowest in Spring. Another explanation for seasonal variations of venous thrombosis is a referral bias (e.g. with more patients referred to diagnostic centers during the warm season due to more edema in that season). We only included confirmed venous thrombotic events and therefore we cannot rule out that this is the reason for an excess of patients in Summer. However, in a previous study, the distribution of suspected events has been scrutinized and shown not to be influenced by the season or calendar month [20].

In summary, in our study the risk of venous thrombosis was different between the seasons, with the lowest prevalence noted in Spring. The absolute difference in risk in Spring as compared with the other seasons was small (2%) is without any clinical relevance. This difference may well have to do with the weather, although other causes cannot be ruled out.

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Table 1. Clinical characteristics by seasons

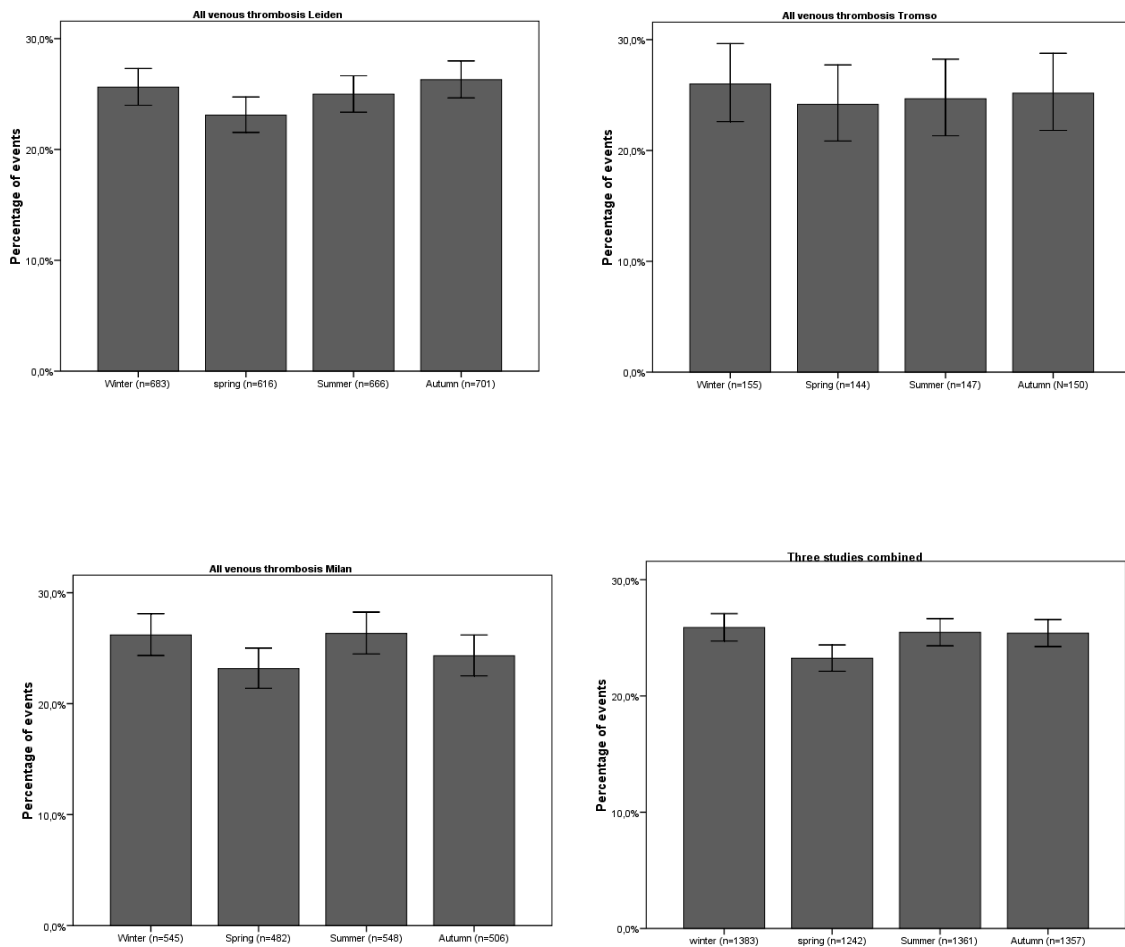
	Total	Winter	Spring	Summer	Autumn
Leiden study					
Any venous thrombosis	2666 (100)	683 (25.6)	616 (23.1)	666 (25.0)	701 (26.3)
Male	1219 (46)	334 (49)	269 (44)	294 (44)	322 (46)
Age at enrollment	49 (39-59)	49 (39-59)	50 (41-60)	48 (38-58)	49 (38-60)
DVT only	1567 (59)	418 (61)	361 (59)	391 (59)	397 (57)
PE +/- DVT	1099 (41)	265 (39)	255 (41)	275 (41)	304 (43)
Provoked*	1595 (62)	471 (72)	460 (77)	468 (73)	196 (29)
Unprovoked*	989 (38)	186 (28)	140 (23)	173 (26)	490 (71)
Tromsø study					
Any venous thrombosis	596 (100)	155 (26.0)	144 (24.2)	147 (24.7)	150 (25.2)
Male	290 (49)	71 (46)	73 (51)	78 (53)	68 (45)
Age at enrollment	62 (51-77)	62 (51-78)	66 (55-79)	60 (48-75)	60 (50-75)
DVT only	388 (65)	92 (59)	102 (71)	100 (67)	94 (64)
PE +/- DVT	208 (35)	63 (41)	42 (29)	50 (33)	53 (36)
Provoked	329 (55)	100 (65)	96 (67)	88 (60)	45 (30)
Unprovoked	267 (45)	55 (35)	48 (33)	59 (40)	105 (70)
Milan study					
Any venous thrombosis	2081 (100)	545 (26.2)	482 (23.2)	548 (26.3)	506 (24.3)
Male	986 (47)	265 (49)	227 (47)	265 (48)	229 (45)
Age at enrollment	45 (31-58)	46 (32-60)	45 (31-58)	44 (31-56)	45 (31-59)
DVT only	1675 (80)	437 (80)	397 (82)	435 (79)	406 (80)
PE +/- DVT	406 (20)	108 (20)	85 (18)	113 (21)	100 (20)
Provoked	1383 (67)	348 (36)	328 (32)	374 (68)	333 (66)
Unprovoked	698 (33)	197 (64)	154 (68)	174 (32)	173 (34)
Three studies combined					
Any venous thrombosis	5343 (100)	1383 (25.9)	1242 (23.2)	1361 (25.5)	1357 (25.4)
Male	2495 (47)	670 (48)	569 (46)	637 (47)	619 (46)
Age at enrollment	49 (36-61)	49 (36-61)	50 (38-61)	48 (35-59)	48 (36-61)
DVT only	3630 (67)	947 (69)	860 (69)	923 (68)	900 (66)
PE +/- DVT	1713 (32)	436 (31)	382 (31)	438 (32)	457 (34)
Provoked	3661 (70)	919 (68)	884 (72)	930 (70)	928 (69)
Unprovoked	1600 (30)	438 (32)	342 (28)	406 (30)	414 (31)

* Unknown for 82 cases.

Continuous variables denoted as mean (IQR), categorical data as count (%).

DVT, deep vein thrombosis; PE, pulmonary embolism.

Figure 1. Seasonal differences in the prevalence of first venous thrombosis in Leiden, Tromsø and Milan



Whiskers denote 95% confidence interval

Chapter 4

Risk of venous thrombosis in persons with increased body mass index and interactions with other genetic and acquired risk factors

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ABSTRACT

Background: Overweight/obesity has a substantial effect on the occurrence of venous thrombosis (VT). Blood group non-O has a high prevalence in Western populations and factor (F)V Leiden mutation could be present in 5% of Caucasians. These frequent prothrombotic risk factors will have a considerable impact on the incidence of VT, especially when combined.

Objectives: We investigated if FV Leiden with blood group non-O modifies VT risk in individuals with different body mass index (BMI) strata in a case-control study (n=11253).

Results: We observed a progressively increasing risk of VT for higher BMI with an odds ratio of 1.9 (95%CI, 1.6-2.3) for those in the upper tertile (BMI>26.7 kg/m²), as compared with the first BMI tertile (BMI< 23.5 kg/m², blood group O, and no FV Leiden (reference group). The addition of FV Leiden and blood group non-O to the model increased the risk in all BMI tertiles; odds ratios were 3.8 (95%CI, 3.2-4.6) in the third BMI tertile of individuals with blood group non-O, and 5.4 (95%CI, 3.5-8.5) in the third BMI tertile of individuals with FV Leiden, respectively. When both FV Leiden and blood group non-O were present, odds ratios were 9.1 (95%CI, 5.9-14.0) in the first BMI tertile, 9.4 (95%CI, 6.6-13.5) in the second BMI tertile, and 12.5 (95%CI, 8.9-17.6) in the third BMI tertile.

Conclusion: Individuals with a high BMI, blood group non-O and/or FV Leiden are at high VT risk. The high VT risks in some subgroups may justify targeted screening and thromboprophylaxis decisions in these patients.

INTRODUCTION

Obesity and overweight have considerable effects on the occurrence of a variety of disorders such as coronary artery disease, hypertension, type II diabetes and venous thrombosis (VT) [1,2]. A high and still increasing prevalence of obesity in developed countries (prevalence of 20-25% in Western populations) leads to substantial impact on disease occurrence. Several studies have shown an association between blood group non-O and factor (F)V Leiden with a 23-fold increase in risk for VT [3]. The prevalence of blood group non-O is about 50% in Western populations and FV Leiden is present in 5% of Caucasians [4,5]. These frequent prothrombotic risk factors will have a considerable impact on the overall incidence of VT, especially since the frequent combined presence of these mutations has a positive joint effect on venous thrombotic risk [4]. We previously reported that the increased risk of VT in individuals with a high body mass index (BMI) is mediated by FVIII induced APC-resistance, and that having blood group non-O *or* factor V Leiden plus a high BMI leads to higher VT risks than expected when these prothrombotic factors are analyzed separately [6]. However, due to small numbers we were not able to study the risk of VT for the combination of a high BMI with FV Leiden *and* blood group non-O, or to study whether these effects differ for example for provoked or unprovoked VT.

For this reason, we set out to determine whether the presence of FV Leiden alone or with blood group non-O modifies the risk of VT in various BMI strata. This study was performed in a different and larger population (i.e., Multiple Environmental and Genetic Assessment of risk factors for VT [MEGA] study) [5,7], than previously reported [6]. In addition, we evaluated the presence of gene-environment effect modification in specific subgroups.

METHODS

The MEGA study is a population-based case-control study that has been described in detail elsewhere [5,7]. Participants were aged 18-70 years. 4956 Consecutive patients with deep-vein thrombosis or pulmonary embolism were enrolled, together with 6297 age- and sex-matched controls. A questionnaire was filled in to assess VT risk factors. The questionnaires included items on surgery, injury, plaster cast, immobilization, malignancies, pregnancy, use of oral contraceptives, and hormone replacement therapy. BMI was calculated by dividing body weight (kg) by squared height (m²). Overweight was defined as a BMI between 25-30 kg/m² and obesity as BMI $\geq$ 30 kg/m². In addition, participants provided a blood or buccal swab sample for DNA. FV Leiden and ABO-blood group were determined by polymerase chain reactions using the TaqMan assay. Technicians were blinded to whether the samples came from patients or controls. For the present analysis, data on BMI, blood group and factor V Leiden was available in 4062 patients and 4659 controls.

Odds ratios with 95% confidence intervals (95%CI) for the risk of BMI tertiles (obtained from the control group) on VT were calculated by using logistic regression models, and were adjusted for age and sex. The combined effect of BMI, factor V Leiden, blood group non-O and immobility (defined as bedridden for more than 4 days, surgery, or hospitalization within 3 months prior to the index date) were evaluated by means of stratification. Subgroup analyses involved stratification by VT type (provoked or unprovoked event), VT location (deep-vein thrombosis or pulmonary embolism), and sex. For a transparent presentation of effect modification, we report the separate effect of each exposure as well as the joint effect compared to the unexposed group as a joint reference category to permit evaluation of interaction on both an additive and multiplicative scale [8].

RESULTS AND DISCUSSION

Our study included 2363 patients with deep-vein thrombosis only, and 1699 patients with pulmonary embolism with or without a diagnosed deep-vein thrombosis. Median age (interquartile range) was 50 years (39-59) for patients and 49 years (39-58) for controls. Prevalence of FV Leiden and blood group non-O in patients was 16% (n=651) and 72% (n=2907), respectively. In controls, these prevalences were 5% (n=243) and 54% (n=2513), respectively. Patients were more often overweight (43% vs 37% in controls) or obese (21% vs 14%).

Table 1 shows the combined effects of ABO blood group and FV Leiden within increasing BMI categories on the risk of VT. A progressive increase in BMI was associated with an increased risk for VT, odds ratios 1.1 (95%CI, 0.9-1.3) for those with a BMI in the median tertile, and 1.9 (95%CI, 1.6-2.3) for those in the upper tertile, as compared with participants in the first BMI tertile, blood group O, and no factor V Leiden (i.e. the reference group). The addition of factor V Leiden and blood group non-O increased the risk in all BMI tertiles; odds ratios for VT were 3.8 (95%CI, 3.2-4.6) in the third BMI tertile of participants with blood group non-O, and 5.4 (95%CI, 3.5-8.5) in the third BMI tertile of participants with factor V Leiden, respectively. When both factor V Leiden and blood group non-O were present, odds ratios for VT were 9.1 (95%CI, 5.9-14.0) in the first BMI tertile, 9.4 (95%CI, 6.6-13.5) in the second BMI tertile, and 12.5 (95%CI, 8.9-17.6) in the third BMI tertile as compared with the reference group. Subgroup analyses, that involved stratification by VT location (i.e. deep-vein thrombosis or pulmonary embolism), sex, and presence or absence of acquired VT risk factors, maintained positive joint effects of BMI with blood group non-O and FV Leiden on VT risk, with the exception for pulmonary embolism. Effect modification on at least the additive scale between the risk factors involved was observed for most groups. For instance, if blood group non-O, and FV Leiden were combined in those with a BMI in the lowest tertile, there was effect modification on the multiplicative scale ($1 \times 2.1 \times 3.1 < 9.1$). There was also effect modification on the additive scale of blood group non-O with FV Leiden and BMI in the upper tertile ($1.9 + 3.8 + 5.4 - 1 < 12.5$). The only group where effect modification was not observed was in patients with PE. For the latter, addition of several risk factors increased the risk of PE without synergy between these factors.

We next focused on participants who were exposed to hormone use (N=1869), recent travel (n=1452), or immobility (n=1613) (Table 2). Again, a dose-response relationship was observed when BMI level increased, with an effect for blood group non-O. Participants who were FV Leiden and blood group non-O carrier appeared to be at highest risk of VT, independent

of BMI, although no longer in a dose-response fashion. Similar results as shown in Table 1 and 2 were observed when exposure categories were based on WHO classification for overweight/obesity (Tables 3 and 4).

This study shows that individuals with high BMI, blood group non-O and/or FV Leiden are at high VT risk. This confirms the results from our previous study [6]. In addition, we showed that the risk increased with the degree of obesity and was also increased for unprovoked or provoked VT, for deep-vein thrombosis and in men or women. We observed little effect of FV Leiden on the risk of pulmonary embolism, (also known as the FV Leiden paradox) when combined with increasing BMI and/or blood group non-O [9,10]. This results adds credence to the hypothesis that APC resistance (present in FV Leiden carriers and in individuals with high BMI) [6,11], preferentially affects the risk of deep-vein thrombosis and not of pulmonary embolism. Furthermore, we found that the combination of high BMI, and blood group non-O in non-FV Leiden carriers increased the risk of VT in a dose-response fashion in individuals who were exposed to oral contraceptives/hormone replacement therapy (odds ratios up to 10-fold increased), recent travel (odds ratios up to 11-fold increased), or immobility (odds ratios more than 3.5-fold increased).

Apart from factor VIII induced APC resistance, another factor that could explain our observed increased risk estimates is the presence of microparticles. Recent evidence points towards microparticles as a potential promoter of a hypercoagulable state both in obese individuals, and in FV Leiden carriers [12,13]. Interestingly, microparticle activity is reduced in obese individuals after weight loss [14]. Therefore, our study, combined with other studies that looked into the etiological aspects of hypercoagulability in obesity [1,4,6,12-14], suggests that weight loss, especially in obese individuals with FV Leiden, could contribute to a decrease in risk of venous thrombosis.

A limitation of our study is that we cannot directly estimate absolute risk estimates from case-control data, which hampers clinical decision making with respect to thromboprophylaxis. As in persons with immobility the absolute risk of VT is estimated to be as high as 3.5% within three months after immobilization [15], while thromboprophylaxis with anticoagulant drugs reduces this risk with approximately 60% [16], the number needed to treat would be 13-20 in this group of individuals (i.e. immobilized individuals with a high BMI, blood group non-O and/or factor V Leiden). Because the absolute risk of VT in oral contraceptive users (estimated 6 per 10.000 per year) [17], or recent travel (estimated 1 per 4500 passengers) [18], is much lower, a 10 or 11-fold increased risk probably does not justify screening for blood group non-O in overweight/obese persons willing to travel or take oral contraceptives. It is difficult at this stage to propose a balance of benefits and risks for the combination of factor V Leiden with

blood group non-O in overweight/obese women who use hormones or individuals who travel (which revealed 15 to 45-fold increased risks of VT). Although it might be tempting to screen, such an undertaking would likely not be cost-effective since the population frequency of these combined genetic variations is 2% while nearly half of the Western population is overweight/obese. Therefore, we would not consider thromboprophylaxis decisions in overweight/obese women such as advice to use non-oral contraceptives and the same applies for air travel.

Strengths of this study include the large patient sample, the detailed information about VT risk factors in both patients and controls, and the combination with data on prothrombotic genes. A limitation of this study is that we did not have full information available for all patients and controls as some of them had not reported their BMI (9%) or did not supply DNA (19%). Although this may have led to reduced statistical power, it did not introduce bias since the frequencies of both factor V Leiden and blood group non-O (in patients and controls) were similar to those that have been reported previously in unselected populations [19]. Also, the prevalence of overweight and obesity in control subjects was similar to that observed in the Dutch population (35% overweight and 11% obesity) [20]. Second, height and weight were self-reported. As in general persons with underweight tend to over report their body weight, while individuals with overweight tend to underreport their body weight [21], actual risks will be somewhat higher if this phenomenon has occurred. Third, we analyzed the combination of oral contraceptives and hormone replacement therapy, while it would have been preferable to analyze these two risk factors separately. Unfortunately, small numbers in the control subjects with FV Leiden and/or blood group non-O did not enable to look at these two risk factors separately.

We conclude that individuals with a high BMI, blood group non-O and/or factor V Leiden are at high venous thrombosis risk. The high risks of venous thrombosis in immobilized individuals with a high BMI, blood group non-O and/or factor V Leiden suggests that these individuals are candidates to receive (extended) thromboprophylaxis with anticoagulant drugs.

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Authorship Contributions

DDR had full access to the database, performed statistical analysis, interpreted the data and drafted the manuscript. WML had full access to the database, supervised statistical analysis and revised the manuscript. FRR designed the MEGA study and revised the manuscript. SCC interpreted the data, supervised statistical analysis, and revised the manuscript.

Conflict of Interest Disclosures

The authors declare no competing financial interests.

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Table 1. Risk of venous thrombosis according to the combinations of BMI tertiles, factor V Leiden and blood group*

Case n (%)	Control n (%)	Overall OR [†] (CI95)	Unprovoked OR [†] (CI95)	Provoked OR [†] (CI95)	DVT+PE OR [†] (CI95)	PE only OR [†] (CI95)	Men OR [†] (CI95)	Women OR [†] (CI95)
241 (6)	674 (15)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
275 (7)	678 (15)	1.1 (0.9-1.3)	1.2 (0.8-1.7)	1.1 (0.8-1.3)	1.2 (0.9-1.5)	1.0 (0.7-1.3)	1.1 (0.8-1.5)	1.1 (0.8-1.5)
482 (12)	682 (15)	1.9 (1.6-2.3)	1.7 (1.3-2.5)	1.9 (1.6-2.4)	2.1 (1.7-2.7)	1.7 (1.3-2.2)	1.5 (1.1-2.1)	2.3 (1.8-2.9)
567 (14)	754 (16)	2.1 (1.8-2.5)	2.1 (1.5-2.9)	2.1 (1.7-2.6)	2.4 (1.9-3.0)	1.8 (1.4-2.3)	2.2 (1.6-3.0)	2.1 (1.7-2.7)
748 (18)	806 (17)	2.5 (2.1-3.0)	2.0 (1.5-2.8)	2.7 (2.2-3.3)	3.2 (2.6-4.0)	1.7 (1.3-2.2)	2.7 (2.0-3.6)	2.5 (2.0-3.2)
1098 (27)	822 (18)	3.8 (3.2-4.6)	3.4 (2.5-4.6)	3.9 (3.2-4.8)	4.9 (3.9-6.1)	2.7 (2.1-3.4)	3.4 (2.5-4.5)	4.2 (3.4-5.3)
41 (10)	31 (1)	3.7 (2.3-6.1)	3.5 (1.6-7.7)	3.5 (2.1-6.0)	5.1 (3.0-8.8)	1.9 (0.9-3.9)	7.1 (2.9-17.5)	2.7 (1.5-4.9)
52 (1)	47 (1)	3.1 (2.0-4.7)	4.4 (2.4-8.1)	2.6 (1.6-4.2)	4.4 (2.7-6.9)	1.4 (0.7-2.9)	3.5 (1.9-6.3)	2.8 (1.5-5.2)
67 (2)	34 (1)	5.4 (3.5-8.5)	7.2 (3.9-13.3)	4.5 (2.7-7.3)	7.9 (4.9-12.6)	2.3 (1.2-4.7)	5.6 (3.0-10.4)	5.5 (2.8-10.7)
99 (2)	32 (1)	9.1 (5.9-14.0)	5.3 (2.6-10.7)	9.8 (6.3-15.3)	13.4 (8.5-22.1)	4.0 (2.1-7.3)	8.6 (4.3-17.3)	10.1 (5.7-17.7)
164 (4)	48 (1)	9.4 (6.6-13.5)	9.1 (5.5-15.0)	8.9 (6.1-13.1)	14.2 (9.7-20.9)	3.3 (2.0-5.7)	9.0 (5.4-15.0)	9.7 (5.8-16.3)
228 (6)	51 (1)	12.5 (8.9-17.6)	12.4 (7.8-19.7)	11.1 (7.7-15.9)	18.8 (13.1-27.1)	4.7 (2.9-7.5)	14.7 (8.6-25.3)	10.5 (6.8-16.3)

BMI denotes body mass index (kg/m²); CI 95, 95% confidence interval; DVT, deep vein thrombosis; PE, pulmonary embolism; OR odds ratio.

* Tertiles derived from MEGA control population.

Upper tertile: BMI: ≥ 26.7 kg/m². Median tertile: BMI: 23.5 -26.7 kg/m². Lowest tertile: BMI: < 23.5 kg/m².

† Odds ratio adjusted for age and sex, when appropriate.

Table 2. Risk of venous thrombosis according to the combinations of BMI tertiles, factor V Leiden and blood group: subgroup analysis

BMI	Factor V Leiden	Blood group	Acquired risk factor	Case n (%)	Control n (%)	Overall OR [†] (CI95)
Lowest tertile	-	O	Hormone use	78 (7)	149 (23)	Reference
Median tertile	-	O	Hormone use	50 (4)	83 (13)	1.2 (0.7-1.8)
Upper tertile	-	O	Hormone use	129 (11)	68 (10)	3.7 (2.4-5.5)
Lowest tertile	-	non-O	Hormone use	229 (19)	160 (24)	2.7 (1.9-3.8)
Median tertile	-	non-O	Hormone use	191 (16)	108 (16)	3.3 (2.3-4.8)
Upper tertile	-	non-O	Hormone use	312 (26)	61 (9)	10.1 (6.8-15.0)
Lowest tertile	+	O	Hormone use	16 (1.3)	3 (0.5)	10.1 (2.9-35.7)
Median tertile	+	O	Hormone use	15 (1.2)	7 (1.1)	4.1 (1.6-10.5)
Upper tertile	+	O	Hormone use	18 (1.5)	5 (0.8)	6.9 (2.5-19.7)
Lowest tertile	+	non-O	Hormone use	46 (4)	6 (0.9)	14.6 (6.0-35.8)
Median tertile	+	non-O	Hormone use	47 (4)	3 (0.5)	30.0 (9.1-99.6)
Upper tertile	+	non-O	Hormone use	78 (7)	7 (1.1)	21.4 (9.4-48.5)
Lowest tertile	-	O	Travel	22 (3)	134 (17)	Reference
Median tertile	-	O	Travel	31 (5)	119 (15)	1.8 (1.0-3.4)
Upper tertile	-	O	Travel	73 (11)	116 (15)	4.6 (2.6-8.1)
Lowest tertile	-	non-O	Travel	97 (14)	138 (18)	4.7 (2.7-7.9)
Median tertile	-	non-O	Travel	133 (20)	136 (18)	5.9 (3.5-10.0)
Upper tertile	-	non-O	Travel	197 (29)	125 (16)	11.3 (6.6-19.3)
Lowest tertile	+	O	Travel	9 (1.3)	4 (0.5)	13.9 (3.9-49.6)
Median tertile	+	O	Travel	6 (0.9)	11 (1.4)	3.8 (1.2-11.9)
Upper tertile	+	O	Travel	10 (1.5)	4 (0.5)	18.5 (4.8-71.4)
Lowest tertile	+	non-O	Travel	22 (3)	9 (1.2)	19.7 (7.4-52.8)
Median tertile	+	non-O	Travel	33 (5)	13 (1.7)	20.7 (8.5-50.3)
Upper tertile	+	non-O	Travel	44 (7)	7 (0.9)	44.7 (17.0-117.0)
Lowest tertile	-	O	Immobile*	98 (8)	54 (17)	Reference
Median tertile	-	O	Immobile	104 (8)	44 (14)	1.2 (0.7-2.0)
Upper tertile	-	O	Immobile	162 (12)	37 (12)	2.3 (1.4-3.8)
Lowest tertile	-	non-O	Immobile	172 (13)	51 (16)	1.9 (1.2-3.0)
Median tertile	-	non-O	Immobile	247 (19)	66 (21)	1.9 (1.2-2.9)
Upper tertile	-	non-O	Immobile	334 (26)	48 (15)	3.8 (2.4-6.0)
Lowest tertile	+	O	Immobile	12 (0.9)	2 (0.6)	3.5 (0.-16.1)
Median tertile	+	O	Immobile	13 (0.9)	1 (0.3)	7.0 (0.9-55.2)
Upper tertile	+	O	Immobile	21 (1.6)	3 (0.9)	3.6 (1.0-12.6)
Lowest tertile	+	non-O	Immobile	27 (2)	2 (0.6)	8.6 (1.9-38.1)
Median tertile	+	non-O	Immobile	47 (4)	2 (0.6)	12.5 (2.9-53.8)
Upper tertile	+	non-O	Immobile	60 (5)	6 (2)	5.5 (2.2-13.5)

BMI denotes body mass index (kg/m^2); CI 95, 95% confidence interval; OR odds ratio.

* Defined as bedridden for more than 4 days, surgery, or hospitalization within 3 months prior to the index date

† Odds ratio adjusted for age and sex when appropriate.

Table 3. Risk of venous thrombosis according to the combinations of overweight/obesity, factor V Leiden and blood group*

BMI	Factor V Leiden	Blood group	Case n (%)	Control n (%)	Overall OR [†] (CI95)	Unprovoked OR [†] (CI95)	Provoked OR [†] (CI95)	DVT+PE OR [†] (CI95)	PE only OR [†] (CI95)	Men OR [†] (CI95)	Women OR [†] (CI95)
Normal weight	-	O	344 (9)	982 (21)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Overweight	-	O	425 (11)	749 (16)	1.6 (1.3-1.9)	1.9 (1.4-2.6)	1.4 (1.2-1.7)	1.5 (1.2-1.9)	1.6 (1.2-2.0)	1.5 (1.2-2.0)	1.7 (1.3-2.1)
Obesity	-	O	212 (5)	279 (6)	2.1 (1.7-2.6)	2.1 (1.4-3.1)	2.0 (1.6-2.6)	2.4 (1.8-3.0)	1.7 (1.3-2.4)	1.6 (1.1-2.3)	2.5 (1.9-3.3)
Normal weight	-	non-O	889 (23)	1129 (25)	2.3 (1.9-2.6)	2.3 (1.8-3.1)	2.2 (1.9-2.6)	2.5 (2.0-2.9)	2.0 (1.5-2.5)	2.4 (1.9-3.1)	2.2 (1.8-2.7)
Overweight	-	non-O	1010 (26)	894 (20)	3.1 (2.7-3.7)	3.2 (2.5-4.2)	3.1 (2.6-3.6)	3.7 (3.1-4.4)	2.4 (1.9-3.0)	3.4 (2.7-4.3)	3.1 (2.5-3.8)
Obesity	-	non-O	484 (13)	329 (7)	4.2 (3.5-5.1)	4.2 (3.1-5.8)	4.1 (3.3-5.0)	5.2 (4.2-6.5)	2.9 (2.3-3.8)	3.3 (2.4-4.4)	5.0 (3.9-6.4)
Normal weight	+	O	63 (2)	52 (1)	3.5 (2.4-5.2)	4.3 (2.3-7.9)	3.1 (2.0-4.8)	4.6 (3.0-7.0)	2.0 (1.1-3.6)	5.3 (2.8-9.9)	2.6 (1.6-4.4)
Overweight	+	O	64 (2)	42 (1)	4.4 (2.9-6.6)	6.8 (3.9-11.9)	3.4 (2.1-5.5)	5.6 (3.6-8.7)	2.5 (1.3-4.7)	4.5 (2.7-7.7)	4.5 (2.3-8.9)
Obesity	+	O	32 (1)	16 (0.3)	5.6 (3.0-10.4)	8.2 (3.6-18.9)	4.3 (2.2-8.7)	8.7 (4.6-16.3)	1.3 (0.4-4.6)	6.4 (2.5-16.4)	5.1 (2.3-11.7)
Normal weight	+	non-O	99 (3)	32 (1)	9.1 (5.9-14.0)	7.9 (4.8-13.0)	9.1 (6.5-12.9)	13.0 (9.2-18.4)	4.0 (2.5-6.5)	8.7 (5.2-14.4)	9.9 (6.4-15.4)
Overweight	+	non-O	210 (5)	56 (1)	10.7 (7.7-14.8)	12.3 (8.0-18.8)	9.3 (6.6-13.1)	15.3 (10.9-21.5)	3.8 (2.3-6.1)	11.1 (7.1-17.3)	9.9 (6.2-15.8)
Obesity	+	non-O	100 (3)	19 (0.4)	15.7 (9.4-25.9)	18.3 (9.5-35.5)	13.4 (7.9-22.8)	21.7 (12.8-36.7)	7.3 (3.8-14.0)	26.5 (10.1-69.7)	11.6 (6.3-21.1)

BMI denotes body mass index (kg/m²); CI 95, 95% confidence interval; DVT, deep vein thrombosis; PE, pulmonary embolism; OR odds ratio.* Obesity: BMI: ≥ 30 kg/m². Overweight: BMI: 25-30 kg/m². Normal weight: BMI: 18.5-25 kg/m².

† Odds ratio adjusted for age and sex, when appropriate. |

Table 4. Risk of venous thrombosis according to the combinations combinations of overweight/obesity, factor V Leiden and blood group: subgroup analysis

BMI	Factor V Leiden	Blood group	Acquired risk factor	Case n (%)	Control n (%)	Overall OR [†] (CI95)
Normal weight	-	O	Hormone use	102 (10)	184 (29)	Reference
Overweight	-	O	Hormone use	76 (7)	75 (12)	1.8 (1.2-2.7)
Obesity	-	O	Hormone use	76 (7)	31 (5)	4.4 (2.7-7.1)
Normal weight	-	non-O	Hormone use	313 (31)	209 (33)	2.7 (2.0-3.6)
Overweight	-	non-O	Hormone use	243 (24)	89 (14)	5.1 (3.6-7.2)
Obesity	-	non-O	Hormone use	163 (16)	23 (4)	12.6 (7.7-20.8)
Normal weight	+	O	Hormone use	23 (2)	8 (1)	5.1 (2.2-11.9)
Overweight	+	O	Hormone use	16 (2)	5 (0.8)	5.7 (2.0-16.1)
Obesity	+	O	Hormone use	9 (1)	2 (0.3)	7.7 (1.6-36.4)
Normal weight	+	non-O	Hormone use	75 (7)	7 (1)	19.5 (8.6-43.8)
Overweight	+	non-O	Hormone use	58 (6)	5 (0.8)	21.0 (8.2-54.0)
Obesity	+	non-O	Hormone use	37 (4)	4 (0.6)	16.8 (5.8-48.5)
Normal weight	-	O	Travel	36 (5)	186 (23)	Reference
Overweight	-	O	Travel	61 (9)	139 (17)	2.6 (1.6-4.3)
Obesity	-	O	Travel	27 (4)	39 (5)	3.6 (1.9-6.8)
Normal weight	-	non-O	Travel	162 (24)	202 (25)	4.3 (2.8-6.5)
Overweight	-	non-O	Travel	174 (26)	155 (19)	5.9 (3.9-9.1)
Obesity	-	non-O	Travel	84 (13)	39 (5)	11.8 (6.9-20.4)
Normal weight	+	O	Travel	14 (2)	13 (2)	5.6 (2.4-12.9)
Overweight	+	O	Travel	6 (0.8)	3 (0.4)	13.6 (3.1-60.9)
Obesity	+	O	Travel	5 (0.7)	3 (0.4)	9.2 (2.0-42.2)
Normal weight	+	non-O	Travel	35 (5)	14 (2)	16.3 (7.5-35.4)
Overweight	+	non-O	Travel	43 (6)	12 (1)	21.7 (10.0-47.1)
Obesity	+	non-O	Travel	21 (3)	3 (0.4)	37.5 (10.5-133)
Normal weight	-	O	Immobile*	144 (11)	75 (24)	Reference
Overweight	-	O	Immobile	143 (11)	44 (14)	1.6 (1.0-2.5)
Obesity	-	O	Immobile	69 (5)	14 (4)	2.5 (1.3-4.7)
Normal weight	-	non-O	Immobile	287 (22)	87 (28)	1.7 (1.2-2.5)
Overweight	-	non-O	Immobile	312 (24)	51 (16)	3.1 (2.0-4.6)
Obesity	-	non-O	Immobile	147 (11)	25 (8)	3.0 (1.8-5.0)
Normal weight	+	O	Immobile	19 (1)	2 (0.6)	5.5 (1.2-24.5)
Overweight	+	O	Immobile	14 (1)	3 (0.9)	2.0 (0.5-7.3)
Obesity	+	O	Immobile	13 (1)	1 (0.3)	7.1 (0.9-55.4)
Normal weight	+	non-O	Immobile	48 (4)	2 (0.6)	14.4 (3.4-61.6)
Overweight	+	non-O	Immobile	58 (5)	6 (2)	4.9 (2.0-11.9)
Obesity	+	non-O	Immobile	26 (2)	2 (0.6)	7.2 (1.7-31.2)

BMI denotes body mass index (kg/m²); CI 95, 95% confidence interval; OR odds ratio.

* Defined as bedridden for more than 4 days, surgery, or hospitalization within 3 months prior to the index date

† Odds ratio adjusted for age and sex when appropriate.

Chapter 5

Protein S levels and the risk of venous thrombosis: results from the MEGA case-control study

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ABSTRACT

In thrombophilic families, protein S deficiency is clearly associated with venous thrombosis. We aimed to determine whether the same holds true in a population-based case-control study (n=5317). Subjects were regarded protein S deficient when protein S levels were <2.5th percentile of the controls. Free- and total protein S deficiency was not associated with venous thrombosis: free protein S <53 U/dL, odds ratio [OR] 0.82 (95%CI, 0.56-1.21) and total protein S <68 U/dL, OR 0.90 (95%CI, 0.62-1.31). When lower cut-off values were applied, it appeared that subjects at risk of venous thrombosis could be identified at levels <0.10th percentile of free protein S (<33 U/dL, OR 5.4; 95% CI, 0.61-48.8). In contrast, even extremely low total protein S levels were not associated with venous thrombosis. *PROS1* was sequenced in 48 subjects with free protein S level <1st percentile (<46 U/dL), and copy number variations were investigated in 2718 subjects, including all subjects with protein S (free or total) <2.5th percentile. Mutations in *PROS1* were detected in five patients and five controls reinforcing the observation that inherited protein S deficiency is rare in the general population. Protein S testing and *PROS1* testing should not be considered in unselected patients with venous thrombosis.

INTRODUCTION

Protein S is a vitamin K-dependent glycoprotein encoded by *PROS1* and synthesized mainly in hepatocytes and endothelial cells [1]. Protein S circulates at a plasma concentration of ~350 nM, of which 40% is free and 60% is bound to C4b-binding protein [2]. Protein S is a major anticoagulant protein that assists in the down-regulation of thrombin formation by stimulating the activity of both activated protein C and tissue factor pathway inhibitor [3, 4].

Hereditary protein S deficiency is a haploinsufficiency disorder, associated with an increased risk of venous thrombosis [5]. Protein S deficiency is associated with decreased activated protein C (APC)-cofactor activity and can be classified as type I (decreased levels of both total and free protein S antigen), type II (decreased APC-cofactor activity but total and free protein S antigen levels within their normal ranges), and type III (decreased levels of free protein S antigen levels only) [6]. The genetic basis of protein S deficiency is heterogeneous. Currently more than 200 mutations in *PROS1* have been described, the vast majority missense or nonsense mutations [7-9]. Sequencing exons and splicing junctions of *PROS1* has been successful in the identification of a mutation in only around 50% of families with type I or mixed type I and type III protein S deficiency [8]. Recently, we and others have shown that gross deletions and insertions of *PROS1* are present in around 30% of the point mutation-negative families with protein S deficiency [5, 10, 11].

Most of the insights regarding the association between protein S deficiency and venous thrombosis have been derived from studies of thrombophilic families in which there was evidence for inherited protein S deficiency. This has led to the notion that the risk of venous thrombosis in protein S deficient subjects is five to ten times increased in comparison to non-protein S deficient relatives [5, 12-14]. The clinical relevance for the individual patient is substantial as subjects with familial protein S deficiency have a first venous thrombosis incidence of 0.7% per year [15] and an annual venous recurrence risk of 6-10% [14, 16, 17]. It is not clear to what extent the conclusions from these family studies can be extrapolated to the general population. The co-inheritance of other defects may contribute to or explain this effect in families, as was shown for factor V Leiden, and protein C deficiency [18, 19]. Evidence obtained from relatively small population-based studies (maximum number of participants 327) [19] point towards a much lower risk of thrombosis associated with protein S deficiency. Although some studies reported an up to 2.5 times increased risk, others found no increase at all [19-21].

Due to these uncertainties, we set out to determine in a large population-based case-control study whether low levels of free or total protein S were associated with an increased risk of venous thrombosis. The molecular basis for protein S deficiency was also investigated by analysis of copy number variation of *PROS1* and resequencing of subjects with the lowest levels of protein S in an attempt to support the risk estimates from a genetic viewpoint.

METHODS

Subjects

The MEGA case-control study has been described in detail previously [22]. Consecutive patients aged 18 to 70 years with a first episode of deep venous thrombosis or pulmonary embolism between March 1, 1999 and August 31, 2004 were included, to a total of 4956 patients. Age- and sex-matched controls were partners of patients (n=3297) or subjects recruited by random digit dialing (RDD) between January 1, 2002 and December 1, 2004 (n=3000). The participants filled in a questionnaire on potential risk factors for venous thrombosis. For logistic reasons, blood sampling was performed for participants included up to June 2002.

All subjects provided informed consent. The study was approved by the Medical Ethics Committee of the Leiden University Medical Center, Leiden, the Netherlands.

Blood samples

Patients in the first half of the study, diagnosed before June 1, 2002 were invited, together with their partners as controls, to the anticoagulation clinic for a blood sample at least three months after discontinuation of the oral anticoagulant therapy. In those patients who continued to take oral anticoagulant therapy for more than one year after the event, blood was drawn during therapy. Blood was collected into tubes containing trisodium citrate 0.106 mol/L as previously described [22]. An additional control group was recruited from the population by RDD. DNA was obtained by standard methods and was available for 4485 patients and 4889 control subjects.

Total and free protein S were measured in plasma on a STA-R automated coagulation analyzer (Diagnostica Stago, Asnières, France). Total protein S was measured by an enzyme-linked immunosorbent assay (ELISA, Diagnostica Stago, Asnières, France) and free protein S was measured by an immune-turbidimetric method (Diagnostica Stago, Asnières, France), both accordingly to the manufacturer instructions.

Cut off points

The range from 2.5th-97.5th percentile of both total and free protein S in control subjects who did not use vitamin K antagonists at time of blood sampling were considered as reference values. We also classified cut off points of protein S into different percentiles (i.e. 97.5-75th percentile as reference group compared with the 50th-75th percentile, 50th-25th percentile, 10th-25th percentile and <10th percentile of the control population), to see if this would change our results. Furthermore, a sensitivity analysis was performed in which very low protein S levels (i.e. less than 0.5th percentile) were compared with the 2.5th-97.5th percentile of (free) protein S. We did not include the 97.5-100th percentile in the reference population as other studies suggest that high levels protein S may be indicative of underlying comorbidity [23].

We also constructed cumulative distribution functions to visualize a possible difference between free and total protein S levels in patients versus controls. For these analyses we excluded patients and controls who used vitamin K antagonists at blood draw, were pregnant or used oral contraceptives and made a distinction between men, women, and between patients that had provoked or unprovoked venous thrombosis.

Genetic analysis of *PROS1*

Copy number variations were analyzed by multiplex ligation-dependent probe amplification (MLPA) [24] using the SALSA MLPA KIT P112 *PROS1* (MRC-Holland, Amsterdam, The Netherlands) as previously described [11].

Exons and their flanking regions, and the 5' and 3' prime untranslated regions were re-sequenced using primers designed to avoid the amplification of the highly similar *PROSP*. After amplification, the PCR product was sequenced using an ABI Prism[®] 3730 DNA Analyzer (Applied Biosystems, Carlsbad, California, USA). Primers and PCR conditions are available on request. Results were analyzed using vector NTI[®] software version 10 (Invitrogen, Paisle, UK).

Statistical analysis

With linear regression methods we estimated the effect of vitamin K antagonist use, estrogen use, pregnancy and puerperium on protein S levels. Based on previously reported associations [25], we also estimated the effect of obesity on protein S levels. Mean differences were adjusted for age and sex, as both age and sex influence protein S levels [26], and for each other in a multivariate analysis. Because there is no reason to assume that low levels of protein S are more common among partners of patients than in the general population, both RDD subjects and partners of patients were considered as control subjects and all analyses were unmatched.

Since protein S levels were collected after venous thrombosis, it is conceivable that the event itself influenced protein S levels ('reverse causation'). To analyze the possibility of such a post-hoc phenomenon, we performed a sensitivity analysis: scatter diagrams were constructed with regression lines, wherein protein S level was plotted against time between venous thrombosis and blood sampling (in patients not on vitamin K antagonists).

Odds ratios with 95% confidence intervals (CIs) were calculated, and were adjusted for age and sex by logistic regression methods. Since in the MEGA study cases were patients with a first venous thrombosis who were sampled from a stable, dynamic population, and since partner controls were matched on time to cases and since random controls were selected from the same population as the cases, these odds ratios can be interpreted as relative risks [27]. Subjects who used vitamin K antagonists at the time of the blood draw were excluded when obtaining relative risk estimates. A preplanned sensitivity analysis was performed in which we excluded estrogen users and pregnant women at the time of the blood draw (as estrogen use and pregnancy decrease protein S levels) [28, 29]. To further diminish the influence of estrogen related hormones to protein S levels, we restricted an analysis to men only. In addition, we assessed the risk of free and total protein S deficiency in individuals with a positive family history [30]. Stratified analyses were performed in which the common thrombophilias (i.e. blood group non-O, factor V Leiden and prothrombin G20210A) were compared with normal and low (free) protein S levels. By doing so, one can identify possible interaction or confounding of low free and total protein S levels to the risk of venous thrombosis by the common thrombophilias.

All statistical analyses were performed with SPSS for Windows, release 20.0 (SPSS Inc, Chicago, Ill).

RESULTS

General characteristics

Approximately 50% of patients and controls who were included in MEGA were tested for free and total protein S levels. No differences in clinical characteristics between tested participants and non-tested participants was observed, indicating that the tested individuals were representative of the whole of MEGA (Table 1). Of note, only 2331 patients were tested on free protein S while 2377 patients were tested on total protein S. Of controls, 2872 were tested on free protein S (n= 1479 partners and n= 1393 RDD controls) and 2940 on total protein S (n= 1481 partners and n=1459 controls). That some patients or controls were tested on total protein S only and not on free protein S was due to technical failures. Both total and free protein S levels were as expected reduced in vitamin K antagonist users, in pregnant women, and in women using estrogens (Table 2). Protein S levels were higher in overweight/ obese control subjects than in those with normal weight.

Risk estimates

Subjects with low free protein S levels (<2.5th percentile, 53 U/dl) or low total protein S levels (<2.5th percentile, < 68 U/dl) were not at increased risk of venous thrombosis as compared with subjects with protein S levels in the 2.5th-97.5th percentile; odds ratio 0.82 (95% CI, 0.56-1.21) and 0.92 (95% CI, 0.63-1.33), respectively. Acquired conditions such as estrogen use, pregnancy or puerperium may transiently decrease protein S levels, which may have influenced these findings. We therefore repeated the analysis by excluding all women who used estrogens, were pregnant or in puerperium at time of venous thrombosis or at time of blood sampling. This adjustment increased the odds ratios to 1.51 (95% CI, 0.82-2.78) for subjects with low free protein S levels and to 1.34 (95% CI, 0.74-2.44) in subjects with low total protein S levels as compared with subjects with protein S levels in the 2.5th-97.5th percentile (Table 3). If protein S levels are somehow correlated with lifestyle, patients and partners may have been overmatched. To analyze if this occurred, we added analyses where patients were only compared with RDD controls. Risk estimates for the higher levels of free protein S were somewhat higher than when RDD and partner controls were combined. For the lower (free) protein S levels, however, risk estimates remained close to 1.0.

When we classified subjects into different percentiles (Table 4), again no increases in risks for venous thrombosis were observed for lower levels of free and total protein S.

When we restricted the analysis to subjects who did not change hormone use status between thrombosis and blood draw, i.e., either consistently used hormones at both time points, or did not use hormones at either time point, these odds ratios were 1.12 (95% CI, 0.69-1.81), and 0.83 (95% CI, 0.48-1.44), respectively. In men, the odds ratio for venous thrombosis in those with low free protein S was 2.15 (95% CI, 0.74-6.29). For total protein S levels, this odds ratio was 1.57 (95% CI, 0.70-3.49). Interestingly, subjects with free protein S levels who were >97.5th percentile appeared to be at increased risk of venous thrombosis (odds ratio 1.55; 95% CI, 1.14-2.11), which could only partly be attributed to overweight or obesity, as further adjustments of overweight or obesity attenuated this odds ratio only marginally to 1.48 (95% CI 1.07-2.04) (data not shown).

To analyze if our results were sensitive to the cut-off level of free or total protein S to identify inherited protein S deficiency, we subsequently compared decreasing cut-off values of free and total protein S levels, respectively, on the risk of venous thrombosis as compared to the reference group (subjects with protein S levels that were between the 2.5th-97.5th percentile). Although numbers became small, it appeared that a free protein S cut off level of $\leq 0.10^{\text{th}}$ (free protein S < 33 U/dL) - 0.20^{th} (free protein S < 34 U/dL) percentile could identify subjects at high risk of venous thrombosis (Figure 1A). Low, or even extremely low (< 0.20^{th} percentile or total protein S < 53 U/dL) total protein S levels were not associated with venous thrombosis (Figure 1B). Due to small numbers, the latter analysis (performed in Figure 1) could not be performed in subjects that were not pregnant or were not using hormones.

As shown in the cumulative distribution functions, patients had as compared with controls, slightly higher free protein S levels (mean 100 U/dL in patients versus 94 U/dL in controls) and total protein S levels (mean 107 U/dL in patients versus 105 U/dL in controls) (Figure 2).

Increased risks for venous thrombosis in protein S deficient subjects are mainly reported from thrombophilic family studies. We therefore selected patients with a positive family history and repeated the analysis (Table 5). Again, no effects were apparent. These null findings could also not be explained by confounding of the common thrombophilias or by an interaction between the common thrombophilias and low levels of free and total protein S (Table 6).

Because vitamin K antagonist use was self-reported (and therefore we might have included subjects with low protein S levels that was due to unreported

vitamin K antagonist treatment), we performed an extra sensitivity analysis by looking at the distribution of the vitamin K dependent coagulant factors (i.e. factors II, VII, IX and X) in cases and control subjects that did not report to be on vitamin K antagonist treatment. All factors were normally distributed, and there were no apparent outliers in the lower range, suggesting that we did not include subjects with low protein S levels that was due to vitamin K antagonist treatment.

Genetic analysis

Copy number variation

First, we tested samples from 2270 consecutive MEGA subjects (1395 patients and 875 controls). An abnormal MLPA pattern was detected in only one individual and the test was repeated confirming the result. This individual was a female patient of 66 years old who was not using estrogens or vitamin K antagonists at the moment of thrombosis or sample collection. She had an unprovoked first venous thrombosis and her family history was not available. The patient was heterozygous for a complete deletion of *PROS1*. Her total protein S was 64 U/dL ($< 2.5^{\text{th}}$ percentile of the control group), and her free protein S was 22 U/dL ($< 2.5^{\text{th}}$ percentile of the control group).

The presence of only one copy number variation in 2270 samples shows that copy number variation of *PROS1* is at most a rare cause of venous thrombosis, and a rare finding in a normal population. To verify this, we zoomed in on potentially protein S deficient subjects by selecting DNA from all subjects with low (i.e. lower than the 2.5^{th} percentile of the control group) total or low free protein S. Patients who used vitamin K-antagonists at the time of the blood draw were also included to guarantee that protein S deficient subjects were not missed. In total, 191 new samples were selected (84 patients and 107 controls), in which no new copy number variation of *PROS1* was identified.

Sequencing of PROS1

To further investigate the molecular basis of protein S deficiency, we selected the first 48 subjects with the lowest levels of free protein S. All these subjects were below the 1st percentile from the controls (cutoff < 46 U/dL); of the 48 selected subjects 24 were patients and 24 were controls. For this analysis, patients using vitamin K antagonists were excluded.

Eleven non-synonymous variations were identified in ten subjects (5 patients and 5 controls); one of these subjects (subject 25) is carrier of two different variations. The variations identified included: 1 complete deletion, 1 indel, 7 missense and 1 nucleotide substitution in the regulatory 3'-untranslated region. Table 7 summarizes the results and gives references for variations previously described. Subjects are numbered according to increasing levels of free protein S. *PROS1* coding DNA reference sequence is NM_000313.3 and protein reference sequence is NP_000304.2.

Four variations were novel; subject 2 (patient) carried an indel mutation (Phe323fs) in the SHBG-like domain that is predicted to result in a truncated protein; subject 17 (patient) carried a 684C>G (Cys228Trp) substitution in the EGF-domain 3; subject 12 (control) carried a nucleotide substitution in the 3'UTR; and subject 45 (control) carried a 1095T>G (Asn365Lys) substitution in the SHBG-like domain.

A complete deletion of *PROS1* was detected in subject 4 (patient). Subject 6 is a patient who carried a 431C>A (Thr144Asn) substitution in the EFG-domain 1. Protein S Heerlen (Ser501Ala) was detected in two control subjects (subjects 25 and 32), and one carrier of protein S Heerlen (subject 25) carried also a second variation in the SHBG-like domain (13012T>C; Val434Ala). This variation is known as dbSNP (rs6803112) with a reported minor allele frequency (MAF) of 0.097. Subject 43 (control) carried a 698G>A (Arg233Lys) substitution, which is also listed in dbSNP (rs41267007, MAF 0.002). Finally, control subject 44 carried an 814G>A (Gly272Arg) variation in the EGF-domain 4. This variation is listed in dbSNP (rs41267005, MAF 0.002) but no information regarding protein S levels is available.

DISCUSSION

Conflicting data have been reported regarding venous thrombotic risk in subjects with protein S deficiency within the community [2]. In the present study, subjects with low levels of free protein S had an increased risk of unprovoked venous thrombosis (odds ratio 2.31; 95% CI, 1.06-5.05). However, the prevalence of patients with such levels was low (n=8, or 0.4% of the total patient population), making it unpractical to use free protein S levels to help identify patients at risk in a clinical setting. The present study, confirms the results of a previous population based case-control study (Leiden Thrombophilia Study) [20], where only very low levels of free protein S (i.e. < 0.10th percentile or < 33 U/dL), but not of total protein S, were associated with an increased risk for venous thrombosis (odds ratio 5.44; 95% CI, 0.61-48.78). This increased risk at very low free protein S levels corresponds to some extent with findings from family studies of protein S deficiency on venous thrombosis risk (although relative risk estimates in family studies for first venous thrombosis in subjects with very low free protein S levels are even higher than 5) [12, 31], as well as the finding from family studies that showed that the cut off level of free protein S to identify true inherited protein S deficiency lies far below the commonly used normal range [12, 14]. Total protein S levels were not able to identify subjects at risk of venous thrombosis even when these levels were very low. This is in agreement with studies where free protein S was a better indicator of venous thrombosis risk than total protein S [32]. Nevertheless, as shown by its broad confidence interval, this analysis suffers from small sample size (n=7 of the patient population), indicating that even if very low free protein S levels are associated with venous thrombosis, large numbers of patients with venous thrombosis will need to be tested to identify only a few with a true free protein S deficiency. Such a strategy is unlikely to become clinically useful.

Many patients with venous thrombosis who are on anticoagulant treatment at the time of the blood sampling are identified with a protein S deficiency, which is thought to have clinical consequences [17, 33]. However, both our study and previous studies show reduced protein S levels in vitamin K antagonist and oral contraceptive users in the absence of a known true (genetic) deficiency [34, 35]. This reinforces the importance of avoiding the collection of blood for protein S measurement at moments when acquired deficiency can be present.

Molecular analysis of *PROS1* in 48 subjects with very low levels of protein S (below the 1st percentile) identified mutations in only ten subjects. This finding suggests that inherited protein S deficiency is rare in the general population and in patients with thrombosis. It is important to note, however,

that our analysis was limited to copy number variations, exons and their flanking regions, and the 5'- and 3'-UT regions. We cannot exclude that genetic variations, for example in introns or in promoter regions not covered in the present experiments, could play a role in protein S deficiency.

Mutations were equally distributed among patients and controls. However, three out of six patients with protein S levels below percentile 0.2 carried mutations that are likely to be detrimental (complete deletion of *PROS1*, 967delTinsGG and Thr144Asn). In contrast, mutations in controls were likely not to be detrimental. Protein S Heerlen (known to be associated with type III protein S deficiency) was detected in two controls. Another control carried a variation previously described as a neutral polymorphism (Arg233Lys) and a final control carried a variation in the 3'-UT region.

PROS1 deletion was present in only one patient, showing that gross copy number variation of *PROS1* is rare in the general population.

Some aspects of our study need additional comment. First, we have noted that high levels of free protein S were associated with an increased risk of venous thrombosis in our study. Interestingly, high levels of free protein S have been associated with an increased risk of coronary heart disease in another study [36]. Free protein S has been described as a semi-delayed acute phase reactant [19, 37] and protein S levels have been described to be higher, on average, in overweight/obese control subjects than in controls subjects with normal weight [25]. Obesity is associated with a chronic inflammatory status and with increased risk for venous thrombosis [38]. It is possible that high levels of free protein S are a consequence of the former. However, adjustment for overweight and obesity only attenuated the risk of venous thrombosis associated with high levels of free protein S, suggesting that other mechanisms could play a role.

Second, our study is a case-control study in which the blood sample was collected after the thrombotic event. Therefore, we cannot exclude the possibility that differences in plasma levels of protein S in patients and control subjects were the result of the thrombotic event itself. However, the blood draw was performed at least 3 months after the thrombotic event, and it is unlikely that the thrombotic event itself caused persistent abnormalities in protein S levels. Furthermore, no differences in protein S levels were observed between patients in whom blood was drawn 3-6 months after their thrombotic event compared with those in whom the blood draw took place later (e.g. more than 6-9, 9-12 or >12 months).

Third, we only had one measurement of free and total protein S. This may have led to misclassification of protein S deficiency when protein S levels fluctuate due to transient acquired conditions. However, only use of female hormones and vitamin K antagonists are known determinants of protein S

levels. Sensitivity analyses in which oral contraceptives, and pregnant or puerperic women were excluded did not materially affect our risk estimates for total protein S, but did slightly for free protein S. Moreover subjects who were still on vitamin K antagonists at time of blood draw were excluded from analyses where relative risk estimates were determined. Finally, qualitative analysis of protein S was not performed, as the laboratory analysis of protein S activity levels is known to produce spurious false low levels. Therefore, we may have missed individuals with type II protein S deficiency [39].

In summary, low free protein S and low total protein S levels could at best rarely identify subjects at risk for venous thrombosis in a population based study. Only when cut off levels for free protein S were far below the normal range or when unprovoked venous thrombosis was considered as outcome event, we found a 2- to 5-fold increased risk of venous thrombosis, which is far below the relative risk estimates that are obtained for free protein S in family based studies. Protein S testing and subsequent testing on *PROS1* mutations should therefore not be considered in unselected patients with venous thrombosis.

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Table 1. Clinical characteristics

	Patients in MEGA (N=4956)	Patients tested (N=2377)	Controls in MEGA (N=6297)	Controls tested (N=2940)
Male	2274 (46)	1083 (46)	2914 (46)	1393 (47)
Age at enrollment	49 (13)	48 (13)	47 (13)	48 (12)
Vitamin K antagonist use†	294 (6)	288 (12)	34 (0.5)	30 (1.0)
Estrogen use (women)	1384 (54)	739 (58)	934 (29)	412 (27)
Pregnancy/puerperium (women)	183 (7)	94 (7)	87 (3)	29 (2)
Normal weight (BMI 18.5-25 kg/m ²)	1634 (37)	818 (37)	2805 (49)	1354 (48)
Overweight (BMI 25-30 kg/m ²)	1834 (42)	965 (43)	2112 (37)	1109 (39)
Obesity (BMI ≥ 30 kg/m ²)	907 (21)	456 (20)	763 (13)	365 (13)

Continuous variables denoted as mean (SD), categorical data as count (%).

Data were missing for some participants in some subgroups

* There were slightly less patients (n=2331) and controls (n=2872) tested on free protein S due to sampling errors.

† At time of blood draw.

Table 2. Association of protein S with venous thrombotic risk factors in control subjects

	Free protein S			Total protein S		
	Subjects (N)	Mean level (U/dL)	Mean difference (95% CI)	Subjects (N)	Mean level (U/dL)	Mean difference (95% CI)
No vitamin K antagonists	2858	92	Reference	2910	102	Reference
Vitamin K antagonists	14	44	-50 (-61 to -39)	30	62	-37 (-48 to -28)
Non-estrogen users (women)	1067	88	Reference	1097	105	Reference
Estrogen users (women)	408	79	-4 (-6 to -1)	412	89	-9 (-12 to -7)
No pregnancy/puerperium (women)	1473	86	Reference	1505	101	Reference
Pregnancy/puerperium (women)	28	70	-6 (-13 to 2)	29	89	1 (-6 to 8)
Normal weight	1332	87	Reference	1354	98	Reference
Overweight	1072	96	6 (4 to 7)	1109	105	5 (3 to 6)
Obesity	358	97	8 (6 to 11)	365	108	8 (5 to 10)

* Adjusted for age and sex where applicable.

† Adjusted for age, sex (where applicable), and the effects of all other factors in the table.

Table 3. Risk of venous thrombosis for high or low levels of protein S

	Patients, n (%)	Controls, n (%)	Odds ratio (95% CI)*	
			Overall	Overall minust
Total				
Free protein S antigen				
> 97.5th percentile (> 139 U/dL)	88 (4.3)	83 (2.9)	1.55 (1.14-2.11)	1.81 (1.31-2.50)
97.5th-2.5th percentile (53 - 139 U/dL)	1937 (94)	2705 (95)	Reference	Reference
< 2.5th percentile (< 53 U/dL)	44 (2.1)	70 (2.4)	0.82 (0.56-1.21)	1.51 (0.82-2.78)
Total protein S antigen				
> 97.5th percentile (> 146 U/dL)	42 (2.0)	72 (2.5)	0.82 (0.56-1.21)	1.00 (0.66-1.53)
97.5th-2.5th percentile (68 - 146 U/dL)	1997 (96)	2766 (95)	Reference	Reference
< 2.5th percentile (< 68 U/dL)	50 (2.4)	72 (2.5)	0.92 (0.63-1.33)	1.34 (0.74-2.44)
Unprovoked†				
Free protein S antigen				
> 97.5th percentile (> 139 U/dL)	33 (6)	83 (2.9)	1.74 (1.13-2.67)	1.72 (1.12-2.65)
97.5th-2.5th percentile (53 - 139 U/dL)	504 (92)	2705 (95)	Reference	Reference
< 2.5th percentile (< 53 U/dL)	8 (1.5)	70 (2.4)	1.77 (0.84-3.71)	2.31 (1.06-5.05)
Total protein S antigen				
> 97.5th percentile (> 146 U/dL)	16 (2.9)	72 (2.5)	1.21 (0.70-2.10)	1.15 (0.66-2.00)
97.5th-2.5th percentile (68 - 146 U/dL)	524 (94)	2766 (95)	Reference	Reference
< 2.5th percentile (< 68 U/dL)	19 (3.4)	72 (2.5)	1.19 (0.55-2.57)	1.59 (0.71-3.58)
Provoked‡				
Free protein S antigen				
> 97.5th percentile (> 139 U/dL)	55 (3.8)	83 (2.9)	1.61 (1.12-2.30)	1.91 (1.30-2.80)
97.5th-2.5th percentile (53 - 139 U/dL)	1390 (94)	2705 (95)	Reference	Reference
< 2.5th percentile (< 53 U/dL)	35 (2.4)	70 (2.4)	0.69 (0.45-1.05)	1.17 (0.53-2.58)
Total protein S antigen				
> 97.5th percentile (> 146 U/dL)	26 (1.7)	72 (2.5)	0.74 (0.47-1.18)	0.95 (0.56-1.59)
97.5th-2.5th percentile (68 - 146 U/dL)	1431 (96)	2766 (95)	Reference	Reference
< 2.5th percentile (< 68 U/dL)	39 (2.6)	72 (2.5)	0.78 (0.52-1.17)	1.14 (0.54-2.43)

* Adjusted for age and sex

† Estrogen users or pregnancy/puerperium

‡ Minus estrogen users or pregnancy/puerperium. Control group confined to random digit dialing subjects only.

^ Provoked venous thrombosis classified as thrombosis due to trauma, plaster cast use, surgery,

pregnancy/puerperium, or immobilization, within three months prior to the index date,

> 4 hours travelling by air, train, bus, or car in the 8 weeks before the index date,

estrogen/progestagen use at time of the index date, and diagnosis of malignancy within five years

Table 4. Risk of venous thrombosis for strata of protein S levels

Measurement	Total		
	Patients, n (%)	Controls, n (%)	Odds ratio (95% CI)* Overall
Free protein S antigen			
97.5th-75th percentile (105 - 139 U/dL)	511 (25.8)	617 (22.2)	Reference
50th-75th percentile (91 - 105 U/dL)	528 (26.7)	692 (25.0)	0.85 (0.72-1.00)
25th-50th percentile (77 - 91 U/dL)	507 (25.6)	738 (26.6)	0.81 (0.69-0.96)
10-25th percentile (77 - 66 U/dL)	291 (14.7)	449 (16.1)	0.75 (0.61-0.92)
< 10th percentile (< 66 U/dL)	144 (7.2)	279 (10.0)	0.61 (0.45-0.85)
Total protein S antigen			
97.5th-75th percentile (115 - 146 U/dL)	498 (24.3)	653 (23.0)	Reference
50th-75th percentile (101 - 115 U/dL)	573 (28.0)	720 (25.4)	1.02 (0.86-1.19)
25th-50th percentile (89 - 101 U/dL)	500 (24.4)	723 (25.5)	0.85 (0.72-1.01)
10-25th percentile (78 - 89 U/dL)	296 (14.5)	470 (16.6)	0.79 (0.65-0.97)
< 10th percentile (< 78 U/dL)	180 (8.8)	272 (9.6)	0.86 (0.63-1.16)

* Adjusted for age and sex

† Estrogen users or pregnancy/puerperium

Table 5. Risk of venous thrombosis for high or low levels of protein S, according to family history

	Patients, n	Controls, n	Odds ratio (95% CI)*	
			Overall	Overall minust
Negative family history				
Free protein S antigen				
97.5th-2.5th percentile (53 - 139 U/dL)	936	2705	Reference	Reference
< 2.5th percentile (< 53 U/dL)	24	70	0.75 (0.46-1.21)	1.95 (0.93-4.05)
Total protein S antigen				
97.5th-2.5th percentile (68 - 146 U/dL)	964	2766	Reference	Reference
< 2.5th percentile (< 68 U/dL)	21	72	0.65 (0.40-1.07)	1.41 (0.66-3.00)
Positive family history				
Free protein S antigen				
97.5th-2.5th percentile (53 - 139 U/dL)	524	2705	Reference	Reference
< 2.5th percentile (< 53 U/dL)	10	70	0.86 (0.44-1.70)	0.92 (0.27-3.08)
Total protein S antigen				
97.5th-2.5th percentile (68 - 146 U/dL)	540	2766	Reference	Reference
< 2.5th percentile (< 68 U/dL)	12	72	1.00 (0.53-1.86)	1.08 (0.37-3.15)

* Adjusted for age and sex

† Estrogen users or pregnancy/puerperium

Table 6. Risk of venous thrombosis for low levels of protein S, stratified analysis with common thrombophilias

Free protein S antigen		Blood group non-O		Patients, n (%)	Controls, n (%)	Odds ratio (95% CI)*
97.5th-2.5th percentile (53 - 139 U/dL)	Absent	Absent	556 (28.1)	1270 (45.9)	Reference	
< 2.5th percentile (< 53 U/dL)	Absent	Absent	13 (0.7)	30 (1.1)	0.93 (0.48-1.79)	
97.5th-2.5th percentile (53 - 139 U/dL)	Present	Present	1378 (69.7)	1429 (51.6)	2.21 (1.95-2.50)	
< 2.5th percentile (< 53 U/dL)	Present	Present	31 (1.6)	39 (1.4)	1.71 (1.05-2.78)	
Total protein S antigen		Blood group non-O				
97.5th-2.5th percentile (68 - 146 U/dL)	Absent	Absent	575 (28.1)	1300 (45.9)	Reference	
< 2.5th percentile (< 68 U/dL)	Absent	Absent	13 (0.6)	32 (1.1)	0.86 (0.45-1.65)	
97.5th-2.5th percentile (68 - 146 U/dL)	Present	Present	1418 (69.4)	1459 (51.5)	2.20 (1.95-2.49)	
< 2.5th percentile (< 68 U/dL)	Present	Present	37 (1.8)	40 (1.4)	2.00 (1.27-3.18)	
Free protein S antigen		Factor V Leiden				
97.5th-2.5th percentile (53 - 139 U/dL)	Absent	Absent	1633 (82.5)	2578 (92.9)	Reference	
< 2.5th percentile (< 53 U/dL)	Absent	Absent	42 (2.1)	66 (2.4)	0.94 (0.63-1.40)	
97.5th-2.5th percentile (53 - 139 U/dL)	Present	Present	303 (15.3)	127 (4.6)	3.79 (3.05-4.71)	
< 2.5th percentile (< 53 U/dL)	Present	Present	2 (0.1)	4 (0.1)	0.79 (0.14-4.33)	
Total protein S antigen		Factor V Leiden				
97.5th-2.5th percentile (68 - 146 U/dL)	Absent	Absent	1689 (82.6)	2630 (92.7)	Reference	
< 2.5th percentile (< 68 U/dL)	Absent	Absent	44 (2.2)	69 (2.4)	0.94 (0.64-1.39)	
97.5th-2.5th percentile (68 - 146 U/dL)	Present	Present	307 (15.0)	136 (4.8)	3.35 (2.86-4.37)	
< 2.5th percentile (< 68 U/dL)	Present	Present	6 (0.3)	3 (0.1)	2.99 (0.75-12.0)	
Free protein S antigen		Prothrombin G20210A				
97.5th-2.5th percentile (53 - 139 U/dL)	Absent	Absent	1843 (93.0)	2658 (95.8)	Reference	
< 2.5th percentile (< 53 U/dL)	Absent	Absent	42 (2.2)	69 (2.5)	0.84 (0.57-1.24)	
97.5th-2.5th percentile (53 - 139 U/dL)	Present	Present	94 (4.7)	47 (1.7)	2.87 (2.01-4.09)	
< 2.5th percentile (< 53 U/dL)	Present	Present	1 (0.1)	1 (0.0)	1.46 (0.09-23.4)	
Total protein S antigen		Prothrombin G20210A				
97.5th-2.5th percentile (68 - 146 U/dL)	Absent	Absent	1905 (93.1)	2718 (95.8)	Reference	
< 2.5th percentile (< 68 U/dL)	Absent	Absent	48 (2.3)	70 (2.5)	0.93 (0.61-1.35)	
97.5th-2.5th percentile (68 - 146 U/dL)	Present	Present	92 (4.5)	48 (1.7)	2.73 (1.92-3.89)	
< 2.5th percentile (< 68 U/dL)	Present	Present	2 (0.1)	2 (0.1)	1.37 (0.19-9.78)	

* Adjusted for age and sex

Table 7. Molecular analysis of PROS1 in subjects with free protein S level below 1st percentile, as determined in MEGA controls

Subject number*	Venous thrombosis	fPS (U/dL)	tPS (U/dL)	Sequence change†	Predicted protein change‡	Reference	Comments
1	NO	16	123	-	-	-	-
2	YES	18	63	967delTinsGG	Phe323fs	-	NOVEL
3	YES	18	68	-	-	-	-
4	YES	22	64	(?-133)_(+1_?)del	Complete deletion	5,10,11	-
5	YES	30	74	-	-	-	-
6	YES	30	58	431C>A	Thr144Asn	40	Associated with quantitative deficiency of protein S
7	NO	31	54	-	-	-	-
8	NO	32	50	-	-	-	-
9	YES	33	78	-	-	-	-
10	YES	33	88	-	-	-	-
11	NO	34	63	-	-	-	-
12	NO	34	90	*370G>A	-	-	3'UTR. NOVEL
13	NO	34	66	-	-	-	-
14	YES	34	53	-	-	-	-
15	NO	34	99	-	-	-	-
16	YES	36	63	-	-	-	-
17	YES	37	66	684C>G	Cys228Trp	-	NOVEL
18	NO	37	57	-	-	-	-
19	YES	38	60	-	-	-	-
20	NO	39	96	-	-	-	-
21	YES	39	81	-	-	-	-
22	NO	39	65	-	-	-	-
23	YES	40	70	-	-	-	-
24	YES	40	58	-	-	-	-
25	NO	40	74	1301T>C	Val434Ala	-	-
25	NO	40	74	1501T>G	Ser501Ala	23	rs6803112 Protein S Heerlen
26	YES	40	76	-	-	-	-
27	NO	40	81	-	-	-	-

Subject number*	Venous thrombosis	fPS (U/dL)	tPS (U/dL)	Sequence change†	Predicted protein change‡	Reference	Comments
28	NO	40	57	-	-	-	-
29	NO	41	76	-	-	-	-
30	YES	41	61	-	-	-	-
31	YES	41	53	-	-	-	-
32	NO	41	81	1501T>G	Ser501Ala	23	Protein S Heerlen
33	NO	41	82	-	-	-	-
34	YES	42	69	-	-	-	-
35	YES	42	81	-	-	-	-
36	NO	42	59	-	-	-	-
37	YES	42	43	-	-	-	-
38	NO	42	96	-	-	-	-
39	YES	43	74	-	-	-	-
40	YES	43	77	-	-	-	-
41	NO	43	62	-	-	-	-
42	NO	43	132	-	-	-	-
43	NO	43	60	698G>A	Arg233Lys	7,41,42	rs41267007 Probably neutral polymorphism rs41267005 NOVEL
44	YES	43	60	814G>A	Gly272Arg	-	-
45	NO	44	61	1095T>G	Asn365Lys	-	-
46	NO	44	63	-	-	-	-
47	NO	45	88	-	-	-	-
48	YES	45	77	-	-	-	-

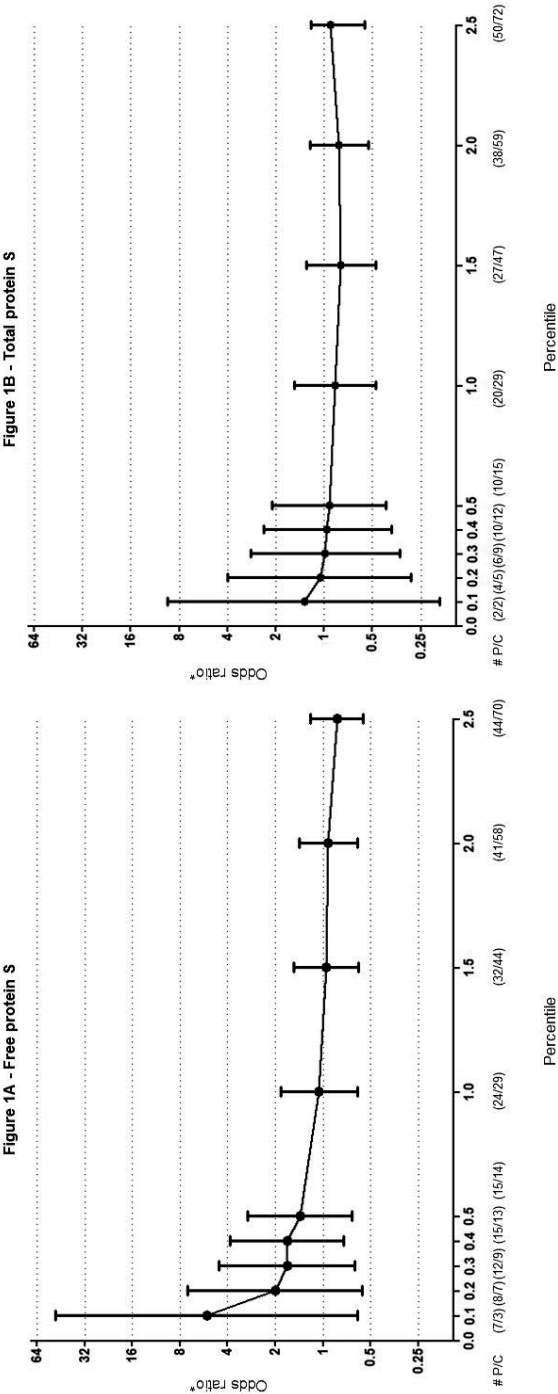
Mutations are described accordingly to the Human Genome Variation Society.⁴³

fPS indicates free protein S; tPS indicates total protein S; MAF indicates minor allele frequency.

*Numbers are accordingly to increasing levels of free protein S. †PROS1 coding DNA reference sequence is NM_000313.3.

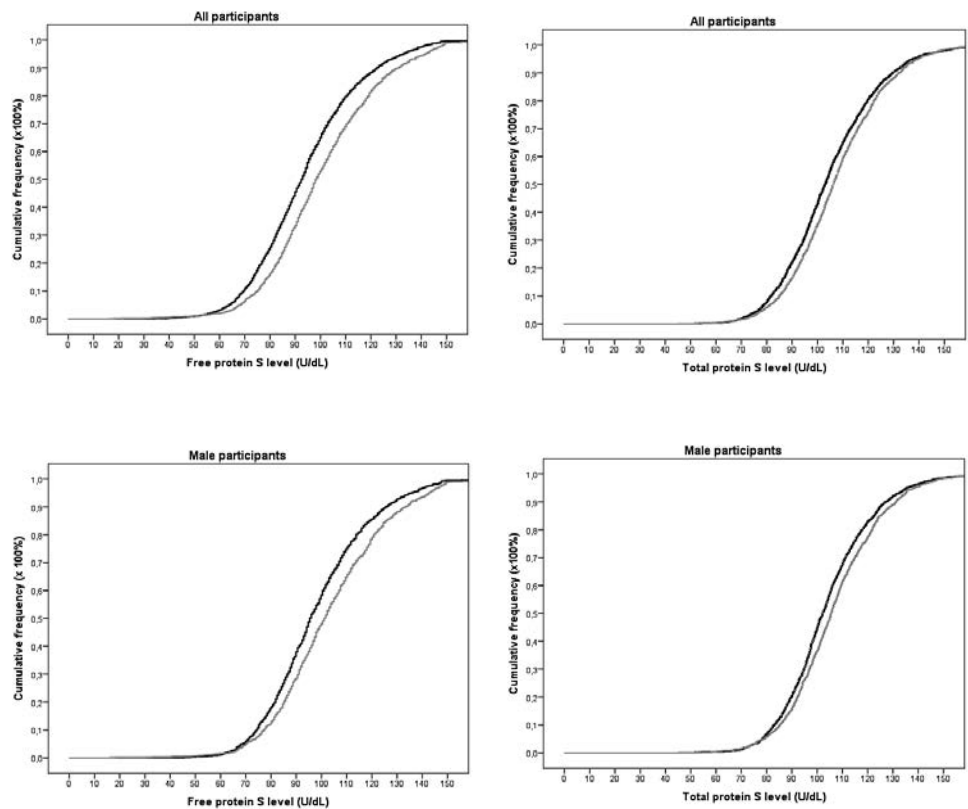
‡Protein reference sequence is NP_000304.2.

Figure 1. Risk of venous thrombosis for low free and total protein S levels

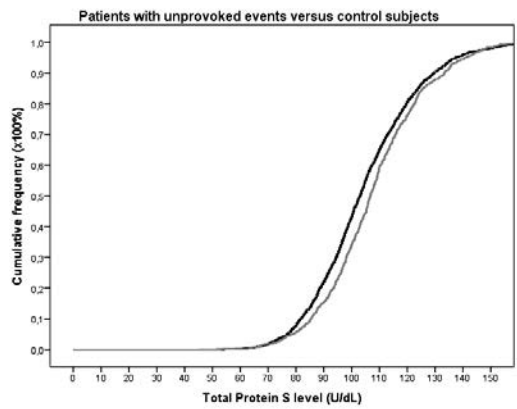
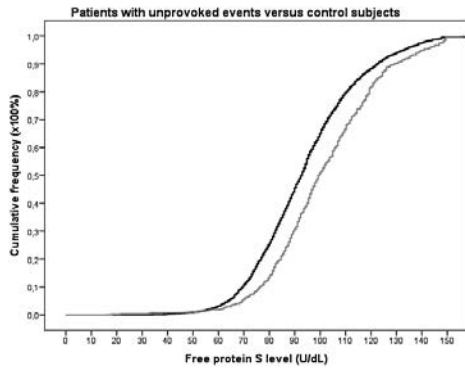
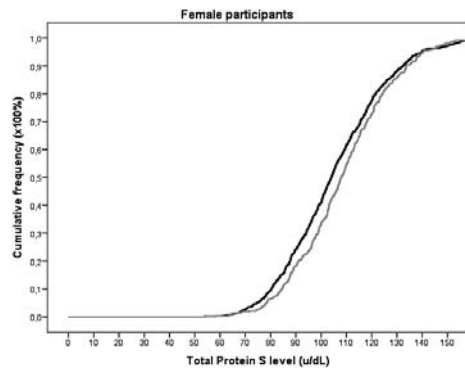
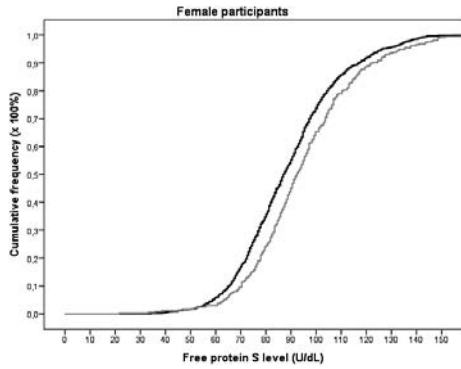


All odds ratios are compared to free or total protein S levels between 2.5th-97.5th percentile
I denotes 95% confidence interval.
* Adjusted for age and sex.
P denotes number of patients and C number of controls per percentile

Figure 2. Cumulative distribution functions of free and total protein S levels in patients and controls



*Protein S levels and the risk of venous thrombosis:
results from the MEGA case-control study*



Grey lines indicate patients, black lines controls.

Part II

*Risk factors for recurrent
venous thrombosis*

Chapter 6

Epidemiology of recurrent venous thrombosis

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ABSTRACT

Venous thrombosis, including deep vein thrombosis and pulmonary embolism, is a common disease that frequently recurs. Recurrence can be prevented by anticoagulants, but this comes at the risk of bleeding. Therefore, assessment of the risk of recurrence is important to balance the risks and benefits of anticoagulant treatment. This review briefly outlines what is currently known about the epidemiology of recurrent venous thrombosis, and focuses in more detail on potential new risk factors for venous recurrence. The general implications of these findings in patient management are discussed.

INTRODUCTION

Pulmonary embolism and deep vein thrombosis are part of the spectrum of a single disease defined as venous thrombosis, which occurs in approximately 1-2 per 1000 persons per year [1]. Approximately 30% of the apparently isolated episodes of pulmonary embolism are associated with silent deep vein thrombosis [2]. In patients presenting symptoms of deep vein thrombosis, the frequency of silent pulmonary embolism ranges between 40 and 50% [3]. The short-term death rate due to pulmonary embolism is 3-6% [1,4]. A common complication of deep vein thrombosis is the post-thrombotic syndrome, which occurs in up to 50% of patients [5].

Venous thrombosis can recur and its recurrence rate (after stopping anticoagulant treatment) is 25% within 5 years [6,7]. Although many clinical and laboratory risk factors for first venous thrombosis have been established [8], only a few of these risk factors are known to play a role in the prediction of a recurrent event (Table 1) [9,10]. Recurrent venous thrombosis usually leads to a decision for life-long anticoagulant treatment, which substantially increases long-term health care costs. Furthermore, anticoagulation is associated with serious potential side effects such as major bleeding. Therefore, efforts are required to reduce the risk of recurrent venous thrombosis while minimizing the use of anticoagulant treatment. This requires the identification of factors associated with recurrence.

This review briefly outlines what is currently known about the epidemiology of recurrent venous thrombosis, and also focuses in more detail on potential new risk factors for venous recurrence. The general implications of these findings in patient management are discussed.

CURRENTLY KNOWN RISK FACTORS FOR THE RECURRENCE OF VENOUS THROMBOSIS

The risk of thrombosis recurrence is especially high in patients in whom the initial venous thrombosis was unprovoked (i.e., the event occurred in the absence of transitory risk factors including surgery, hospitalization, immobilization, and oral contraceptive use or pregnancy/puerperium) [11-13]. It is important to keep in mind that, as long as the risk factor that caused the thrombosis is present the increased risk remains. This is especially important in patients with active cancer, who have a high risk of recurrence even during vitamin K antagonist treatment [14,15]. Women who had a first venous thrombotic event that was associated with concurrent oral contraceptive use are at high risk for recurrence when they do not refrain from oral contraceptives after the event occurred. This is illustrated by the LETS-follow-up study where 40% of women continued, started or restarted oral contraceptive use during follow-up. These women had a 4.6-fold (95%CI = 1.9-11.5) higher recurrence rate than women in the same age group (16-48 years) who stopped using oral contraceptives after their first event, and who were not pregnant during follow-up [recurrence rate of 48.8/1000 person/years (95%CI = 24.3-87.2) for oral contraceptive use versus 10.5/1000 person/years (95%CI = 4.5-20.7) in no hormonal contraception or pregnancy] [16].

After discontinuation of anticoagulant therapy the annual risk of recurrence among patients with antiphospholipid syndrome may be as high as 50 to 67%, especially during the first few months [17]. Most clinicians therefore opt to treat venous thrombosis patients with strict diagnostic criteria for antiphospholipid syndrome, [18] with long-term anticoagulant treatment [11,19].

POTENTIAL NEW RISK FACTORS FOR VENOUS RECURRENCE

General characteristics

Sex

Recent studies have shown that men have a 2-4-fold higher risk of recurrent venous thrombosis than women [16,20,21]. The reason why men would be at higher risk of recurrent venous thrombosis than women is unknown. It could be explained by an imbalance of environmental risk factors for venous thrombosis in men compared to women, for example, due to hormonal risk factors to which only women are exposed. As oral contraceptives are discouraged after first venous thrombosis and thromboprophylaxis is often recommended in women during pregnancy or puerperium after a first event [22,23], women may have a lower thrombosis potential level for recurrence than men in whom environmental risk factors are less often associated with risk for a first venous thrombosis [24]. Some studies indeed suggested that the lower risk of recurrent venous thrombosis in women could be explained by a reduced rate of recurrence after venous thrombosis associated with oral contraceptive use or pregnancy [24-26]. However, a recent meta-analysis concluded that the lower risk of recurrent venous thrombosis in women could not to be fully accounted for by a reduced rate of recurrence after venous thrombosis associated with oral contraceptive use or pregnancy [27]. Whether or not male sex in itself is a potential factor in the development for recurrent venous thrombosis is still unclarified; nevertheless, it is a clear predictor of an increased recurrence risk.

Age

Although age is a strong risk factor for first venous thrombosis, it seems to have no effect on the risk of recurrence. Some authors have reported a slightly increased risk (hazard ratio 1.36 per increase in each decade of age) [28,29], and others found no relationship [6,30,31], or even a decreased risk with ageing [32]. In routine clinical practice, age at first venous thrombosis is usually taken into consideration when a patient is counseled regarding duration of anticoagulant treatment. Some clinicians are reluctant to treat

elderly patients with anticoagulants for a long period of time because they consider risk of bleeding rather than risk of venous recurrence as the determinant for duration of anticoagulation. Others, in contrast, are hesitant to recommend extended anticoagulation to younger patients because they assume that these patients may have a low recurrence risk. Many younger individuals dislike long-term anticoagulation simply because of the prospect of a long-time medical treatment. According to previous findings, the risk of recurrence is similar for younger and older patients. Therefore, age at first venous thrombosis should not matter when determining how long patients with thrombosis should receive anticoagulation, provided their risk of bleeding is low [30].

Overweight/obesity

Obesity is a risk factor for first and recurrent venous thrombosis [33,34]. It is not completely understood why obesity predisposes to venous thrombosis. People with overweight or obesity tend to be more immobile, which may lead to clot formation through stasis. It is also possible that these individuals acquire a prothrombotic state. Indeed, studies have shown an association between increase in body mass index (BMI) and factor VIII levels [35], which is a risk factor for venous thrombosis [36]. Adipose tissue may contribute to enhanced coagulation by direct tissue factor production, but hypercoagulability could also be due to direct effects of adipose tissue on the hepatic synthesis of coagulation factors [37,38]. Another explanation is that estrogen levels are higher in obese men and women due to an increased conversion from androgen to estrogen in adipose tissue [39]. As estrogens and progestagens are prothrombotic [40], this may also be a possible pathway. A third explanation may be that obesity is considered to be a chronic low-grade inflammatory state, which may result in increases of clotting factors leading to venous thrombosis [38]. It seems plausible that weight loss can reduce the risk of recurrent venous thrombosis [30], although prospective follow-up studies on this issue have not been performed.

SPECIFIC ASPECTS RELATED TO THROMBOSIS

Risk situations for venous thrombosis that happened in the past but not related with a thrombotic event

Some patients with first venous thrombosis may have experienced many risk situations for thrombosis during their life time without actually developing it, while others may have experienced few of such risk situations and then developed venous thrombosis idiopathically or after a single provoked risk factor. In a previous study we hypothesized that those who had 'survived' many risk situations without developing venous thrombosis would, have a lower recurrence risk after a first event [41]. In this Brazilian cohort, risk situations for venous thrombosis that occurred in the past but did not result in venous thrombosis at that time were noted in 66% of patients. Although the high rate of positive responders to this question provided a large group to evaluate our hypothesis, the risk of patients with unprovoked venous thrombosis with or without past risk situations was still 3-fold higher than the risk of those who had a provoked risk factor present at time of first venous thrombosis. Hence, asking patients with venous thrombosis about risk situations for venous thrombosis that took place long before their first thrombotic event seems to be of little value for predicting if an individual patient has a low risk for thrombosis recurrence.

Infections

A recent prospective population-based cohort study showed that elevated C-reactive protein (CRP) levels were associated with an increased risk of venous thrombosis [42]. Interestingly, it was shown that this risk was highest when CRP levels were elevated during the period just before the onset of venous thrombosis. This latter finding supports the hypothesis that the coagulation system can be stimulated by transient infection [43], which has also been suggested by experimental studies [44]. Two retrospective cohort studies showed that patients with a transient infection such as urinary tract infection or pneumonia had a 2- to 3-fold increased risk of acute venous thrombosis [45,46]. Thus, in the presence of a transient infection, a clinician

could decide to withhold anticoagulant treatment after 3-6 months in a person with a first venous thrombosis at the time of such an episode of infection or inflammation. This approach is supported by the study by Baglin et al. [47] and is recommended by the ACCP Guidelines, based on a low risk of thrombotic recurrence when the provoking risk factor is known and transient [11]. However, because infections may also lead to transient periods of immobilization or hospitalization, this increased risk could also be explained by a concurrent period of immobilization leading to venous stasis that ultimately leads to thrombosis. Whether common transient or inflammatory diseases should therefore be considered as provoking risk factors for venous thrombosis is uncertain.

Thrombophilia

Thrombophilia can be identified in about half of all patients presenting venous thrombosis, and appears to provide at least a partial explanation for a previously poorly explained disease. Over the past decades, testing has increased tremendously for various indications, but whether the results of such tests aid the clinical management of patients has not been settled [48]. There is weak evidence that testing for thrombophilia could improve the risk prediction for venous thrombosis recurrence. Those with positive tests have at most a small increase in the risk of recurrence [7,9,49]. The association between natural anticoagulant deficiencies (protein C, protein S, antithrombin) and an increased risk of venous recurrence has been established [50,51], but the clinical relevance of this association is unknown. There is no proof that screening for thrombophilia helps patients with regard to treatment of the acute event or for prevention of recurrence [7,47,52].

Mode of first event presentation

The mode of presentation of venous thrombosis, as deep vein thrombosis or pulmonary embolism, could predict the likelihood and type of recurrence. In a recent meta-analysis on this issue, the risk for recurrence as pulmonary embolism was 3.1-fold greater in patients presenting with previous symptomatic pulmonary embolism than in patients with previous proximal deep vein thrombosis. Patients with proximal deep vein thrombosis had a 4.8-fold higher cumulative recurrence rate than those with distal deep vein thrombosis [53]. High risk of pulmonary embolism at recurrence in patients with an initial pulmonary embolism has been shown repeatedly [54-56]. It is not clear why patients with pulmonary embolism have a higher chance of

recurrence than patients with deep vein thrombosis [55-58]. However, given that the risk of fatal pulmonary embolism is two to four times higher in patients with symptomatic pulmonary embolism and the risk of chronic pulmonary embolism and pulmonary hypertension is 15 to 20 times more likely when the pulmonary embolism is recurrent [57,59,60], the mode of initial presentation appears to be an important factor in determining the duration of anticoagulant therapy in individual patients after a first episode of venous thrombosis. The current recommendation for anticoagulation duration is already shorter for patients with distal venous thrombosis [11]. However, more studies are needed in order to support different recommendations for the management of pulmonary embolism and proximal deep vein thrombosis.

Residual vein thrombosis

The presence of sequelae in the area affected by venous thrombosis is a possible risk factor for recurrence [61]. Two interventional studies used residual vein thrombosis to guide the duration of anticoagulation treatment after unprovoked proximal deep vein thrombosis [62,63]. Tailoring the duration of oral anticoagulant therapy on the basis of findings of repeated leg vein ultrasonography reduced the risk for recurrent venous thrombosis by 35% compared to the administration of conventional, fixed time treatment without an appreciable increase in hemorrhagic risk [62]. However, many patients without sequelae relapsed; therefore this assessment reduces, but does not exclude the possibility to misclassify patients as a being at low risk for recurrence [62]. Because there are currently no uniformly acknowledged criteria for the definition of vein decanalization, any clinical decision made on the basis of residual vein thrombosis assessment can only be premature [9,12,64].

D-dimer testing

D-dimer levels have been used as a predictive test for recurrence of venous thrombosis when remaining high after 30 days of discontinuation of oral anticoagulation [65]. An Italian multicenter interventional trial (PROLONG) evaluated adult patients after a first event of unprovoked venous thrombosis who were treated for at least three months with oral anticoagulants. The relative risk of events (recurrence or bleeding) was four-times higher in patients with abnormal D-dimer levels who had not resumed anticoagulation compared to those who restarted the anticoagulation [65]. The risk of

recurrence in patients with unprovoked venous thrombosis and normal D-dimer levels was similar to that of patients with a first provoked episode of venous thrombosis [66]. In the PROLONG II study, published in 2010, the Italian investigators reported that repeated testing of D-dimer concentrations after withdrawal of anticoagulation treatment following a first episode of unprovoked venous thrombosis could help establish the optimum duration of treatment [67]. Although these results are encouraging, it should be remembered that the positive predictive value of a high D-dimer concentration regarding recurrent venous thrombosis is low and therefore not very helpful for clinical decision making [68]. The measurement of D-dimer might be more useful in identifying patients at low risk of recurrence in whom the risk of bleeding during long-term anticoagulation might be higher than the risk of recurrence after stopping treatment.

RISK ASSESSMENT IN PATIENTS WITH UNPROVOKED FIRST VENOUS THROMBOSIS

A recently introduced approach for the assessment of risk for recurrent venous thrombosis is the combination of clinical characteristics of the patients (i.e., location of the thrombus, sex, or age) with laboratory or imaging tests. Rodger et al. [32] identified that a combination of 2 or more of the following risk factors in women: absence of symptoms suggestive of post-thrombotic syndrome, D-dimer concentration during anticoagulation treatment <250 ng/mL, body-mass index <30 kg/m², and age <65 years, could be predictive of a low recurrence risk. No combination of predictors identified a low-risk group of men. Following the same principles as those of the study by Rodger et al., Eichinger et al. [69] developed a nomogram that can be used to calculate risk scores and expected cumulative recurrence rates. The variables used were: sex, location of initial venous thrombosis and D-dimer levels in individual patients. Both prediction models need to be validated before they can be used in clinical care.

In summary, prevention of recurrent venous thrombosis will be more profitable if it becomes possible both to identify more precisely those persons who are at risk of recurrent venous thrombosis and to quantify the risk to which they are exposed. Consideration of the discussed new risk factors for recurrence may allow us a more optimal use of prophylactic strategies against recurrent venous thrombosis. More research on this topic is needed before conclusions can be drawn.

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Table 1. Risk factors for recurrent venous thrombosis.

Genetic	Acquired	Environmental	Mixed or not well established
Antithrombin deficiency	Malignancy	Surgery and major trauma	High factor VIII
Protein C deficiency	Antiphospholipid antibodies	Pregnancy and puerperium	High D-dimer
Protein S deficiency		Oral contraceptives	Factor V Leiden
		Hormone replacement therapy	Prothrombin G20210A
		Prolonged immobilization	Infection
			Sex
			Obesity
			Past risk situations
			Residual vein thrombosis
			Mode of first event type

Chapter 7

Past provoking venous thrombosis risk situations on the risk of a recurrent thrombotic event: a cohort study

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ABSTRACT

Objective: Strategies that can classify the risk for recurrent venous thrombosis are needed. Some patients may have experienced many risk situations during their life time without developing venous thrombosis (VT), while others may have experienced few of such risk factors and then develop VT idiopathically or after a single provoked risk factor. We hypothesized that those who had 'survived' many risk situations without developing VT would, after a first VT, have a low recurrence risk.

Methods: Brazilian tertiary hospital cohort was followed for an average of 30 months after anticoagulation withdrawal for a first VT. Patients with indication for indefinite anticoagulation were not included. The primary end point was objective recurrent VT.

Results: Recurrent VT was recorded in 7% of 378 eligible patients. Patients with a provoked first event and positive past risk situations for VT had an incidence rate of recurrence of 1.16 (95% confidence interval [CI], 0.47-2.39) per 100 patient-years. The incidence rate ratio (IRR) of this subgroup compared to patients with a provoked event without other past risk situations for VT was 1.1 (95% CI, 0.3-4.4). This IRR was 3.3 (95% CI, 1.3-8.7) in patients with an unprovoked event and positive past risk situations and 5.1 (95% CI, 1.6-16.1) in patients with an unprovoked event and no past risk situations.

Conclusions: Asking a patient about past exposure of venous thrombosis risk factors long before the occurrence of a first venous thrombosis occurred, does not provide information to classify patients at lower risk for recurrence of venous thrombosis.

INTRODUCTION

Venous thrombosis is a disease that occurs in approximately 1-2 per 1000 persons per year [1]. Despite adequate treatment, up to one quarter of patients with venous thrombosis will experience a recurrent event within the subsequent 5 years [2]. It has been recognized that the presence of a transient or reversible provoked risk factor at the time of venous thrombosis is associated with a decreased risk of recurrence after anticoagulant therapy is stopped [3]. Therefore, a relatively short period of anticoagulant treatment with vitamin K antagonists is advised for those with clear provoking risk factors for venous thrombosis [4]. However, because a substantial number of recurrent events occur without a clear provoking risk factor, many patients may need indefinite anticoagulant treatment after a first event occurred, that may lead to serious side effects such as major bleeding [5]. The challenge therefore is to identify other patient groups that may also have a low risk of recurrence and could receive anticoagulants for a fixed (short) time.

The thrombosis potential model takes into consideration that the thrombosis potential will drop sharply after the occurrence of a provoked event and that such an individual will not readily cross the thrombosis threshold again, consequently leading to a reduced risk for recurrence [6,7]. Considering this model we hypothesized that patients at risk for recurrent venous thrombosis, who had experienced transient provoked risk factors previously, yet not developed thrombosis at that time, would have a lower risk of recurrence in comparison with those whose first (provoked or unprovoked) event was not preceded by provoked risk factors. To study this, we analyzed a cohort of 378 patients who were questioned about past risk situations for venous thrombosis, yet not related to an episode of thrombosis at that time.

METHODS

Subjects

Subjects consisted of patients with one previous venous thrombotic event that were all seen by the same clinician (i.e. DDR) from April 2000 to September 2010 at the University Hospital of Federal University of Minas Gerais, Belo Horizonte, Brazil. Patients were referred from anywhere in the State of Minas Gerais by their doctors who looked after them during the first episode of venous thrombosis. There was no control over these referrals, they were included consecutively, but certainly they were not consecutive patients with thrombosis in the state. Throughout the study, patients' data were collected in a standardized method by using a form with questions related to first and recurrent venous thrombosis. A total of 786 patients who were diagnosed elsewhere with previous deep vein thrombosis (DVT) and/or pulmonary embolism (PE) were attended at the outpatients' clinic (Figure 1). We excluded 52 patients due to the absence of an objectively confirmatory test for first PE or DVT. Due to possible differences in the hemostatic system, patients younger than 16 years old were also excluded (N = 10). Other patients were excluded due to their high risk for recurrence: more than 1 previous episode of thrombosis (N = 35), presence of systemic lupus erythematosus or antiphospholipid syndrome (N = 75), sickle cell anemia (N = 1); diagnosis of cancer at the time of inclusion or within 6 months after the first venous thrombosis, homozygosity/double heterozygosity state for factor V Leiden or prothrombin G20210A, or antithrombin deficiency (N = 21). Therefore, these patients received indefinite treatment with oral vitamin K antagonists. Another 32 patients were also excluded because they had either another reason (atrial fibrillation, for example), or the wish to maintain use of anticoagulation indefinitely. Patients without an objectively confirmed recurrence were excluded (N = 138), and 2 patients died before enrollment. Despite efforts to follow-up all included patients, 42 patients (10%) were only seen at the time of enrollment and not afterwards. These patients were excluded from the analysis, which resulted from the study of 378 patients. Seventy five percent of the patients were seen after 2005, reflecting the growth curve of the outpatients' clinic. Patients were followed every 6 months either by phone call or by attending the clinic. Patients were not systematically screened with ultrasonography since we were only interested in symptomatic recurrent venous thrombosis. A standardized questionnaire was completed by a professional healthcare assistant. The study ended in November 2010. At that time we had full information of 343 patients (91%). Among the other 35 (9%) patients who were not re-interviewed after January 1st 2010, 14 were evaluated in 2009, and some of these patients had already completed the follow up period due to recurrence of venous thrombosis (N = 2). The definition of follow-up time is described in the "statistical analysis" heading. All patients were advised to seek the outpatients' clinic in case of suspected DVT or PE. All eligible patients for this study provided written informed consent, and the study was approved by the local ethics committees.

Definitions

The duration of anticoagulation for provoked venous thrombosis was 3-6 months and for unprovoked venous thrombosis 6-12 months. Some patients were treated with anticoagulants for longer or shorter time duration mainly because of individual patient preferences.

The first episode of venous thrombosis was considered established if proximal DVT was confirmed by compression ultrasound, and PE by ventilation/perfusion lung scanning or spiral computer tomography scanning. Recurrence was considered established if it was demonstrated by objective techniques at another site than the first event, or at the same site if previously repeated tests showed no residual venous thrombosis. If the recurrence of DVT was at the same site and we had not previously repeated tests to analyze if there was residual venous thrombosis, we only considered recurrence when the compressive ultrasound (CUS) showed new thrombus formation together with clinical symptoms that were absent previously. Only the objectively demonstrated PE was considered a recurrence. If these criteria were not fulfilled, anticoagulant treatment was withheld and the event was not classified as a recurrent venous thrombosis.

Venous thrombosis (either first or recurrent) was defined as provoked if it had occurred at or within 3 months after exposure to exogenous risk factors including surgery (with more than 30 minutes of duration), trauma leading to hospitalization, immobilization for more than 3 days (hospitalization for clinical reasons), limb immobilization in a plaster cast for more than 7 days, pregnancy, post-delivery period (2 months), the use of oral contraceptives or hormonal replacement therapy (at the time of thrombosis), presence of autoimmune diseases, or active malignancy. In the absence of these risk factors, venous thrombosis was classified as unprovoked.

During the medical visit, patients were asked about past risk situations for thrombosis at any time of life. The questions were related to use of oral contraceptives or hormonal replacement therapy, pregnancies, surgeries, immobilization with plaster cast in the lower limbs or any hospital admission for clinical reasons for more than 3 days. If they answered positive on any of these risk situations that occurred at least 3 months or more before the event occurred, they were considered as positive for past risk situations for venous thrombosis. Patients were submitted to CUS or ventilation/perfusion lung scanning or spiral computer tomography scanning around the end of anticoagulation use, and were separated in two groups: those with complete recanalization and those with incomplete or absent recanalization, according to the result of the image test. A family history was considered positive when the patient had one or more first degree relatives (parents, sibling and/or offspring) with venous thrombosis documented by image tests and/or with history of vitamin K antagonist use due to venous thrombosis.

Statistical analysis

Observation time started after vitamin K antagonists were withdrawn and ended at time of recurrence or at the last consultation. Incidence rates of recurrent thrombotic events were calculated as the number of events over the accumulated observation time. Incidences and 95% confidence intervals (95% CI) were calculated under the Poisson distribution assumption. Stratified analyses were used to assess the risk of thrombotic events recurrence by sex, idiopathic or provoked classification of first thrombotic event, status of image test around the end of anticoagulation (recanalization), presence or absence of family history of venous thrombosis and presence or absence of past risk situations for venous thrombosis. The presence of a triggering factor was evaluated together with past risk situations status and sex. The risk of recurrence in patients with unprovoked venous thrombosis was assessed by age at first event.

Including patient time before patients were enrolled could be problematic as this may create a survivor bias [8]. Therefore, we performed a sensitivity analysis in which observation time started at enrollment (and when vitamin K antagonists were withdrawn) and ended at time of recurrence or last consultation (model A). Another type of bias may arise when follow-up time is not ended on a specified date, because subjects may have a reason not to return to the clinic or answer a telephone call. Therefore, we performed a second sensitivity analysis in which follow-up time started when vitamin K antagonists were withdrawn and ended at time of recurrence or January 1st 2010, whichever occurred first (model B). Some patients did not use vitamin K antagonists at time of enrollment and model B allowed that observation time defined by “date of enrollment - date of withdrawing vitamin K antagonists” to be included. This is different from model C where the follow-up time started at time of enrollment (and when vitamin K antagonists were withdrawn) and ended at time of recurrence or January 1st 2010, whichever occurred first.

Further subgroup analyses such as distinction between major provoking risk factors (surgery.) and minor provoking risk factors (contraception, or type of contraception, pregnancy) or an interaction analysis on age and other covariates such as sex were not performed since our study was too small to yield meaningful results.

Statistical analyses were performed using SPSS software, version 16.0 (SPSS Inc., Chicago ILL).

RESULTS

The study cohort included 378 patients, clinical characteristics are shown in Table 1. Of patients, 291 (77%) were female, 253 (67%) had a provoked venous thrombosis, the median age at time of the first event was 36 years, 109 (29%) had a distal venous thrombosis, 175 (46%) had a proximal venous thrombosis, 71 (19%) had a PE and 22 (6%) had DVT and PE together (these patients were analyzed in the PE group). Oral contraceptives, hormone replacement therapy, pregnancy or postpartum period were the most prevalent provoked risk factors appearing 189 times (in 65% of the female population). Ultrasound/duplex was the diagnostic image method in 284 (75%) patients, ventilation/perfusion lung scan in 54 (14%) patients and spiral computer tomography scanning in 40 (11%) patients. A positive family history was present in 95 (25%) and positive past risk situations were present in 251 (66%) patients. The median time of anticoagulation, time between the end of anticoagulation and recurrence, interval between venous thrombosis, and of follow up were 8, 26, 42 months and 30 months, respectively. Recurrent venous thrombosis occurred in 25 (7%) patients. The median age at recurrence was 51 years. At a median of 7 months after their initial first event, patients were consulted by DDR.

Patients with a provoked first event and positive past risk situations (> 3months before the occurrence of a first venous thrombosis occurred) had an incidence rate of recurrence of 1.16 (95% CI, 0.47-2.39) per 100 patient-years (Table 2). The incidence rate ratio of patients with a provoked event without other past risk situations for venous thrombosis compared to patients with a provoked first event and positive past risk situations was 1.1 (95% CI 0.3-4.4). This incidence rate ratio was 3.3 (95% CI, 1.3-8.7) in patients with an unprovoked event and positive past risk situations and 5.1 (95% CI, 1.6-16.1) in patients with an unprovoked event and no past risk situations versus patients with a provoked first event and positive past risk situations. Sensitivity analyses taking period of follow up into account showed similar findings; i.e. previous risk situations mildly affected recurrent venous thrombosis risk.

In Table 3 it is shown that male sex was associated with an increased risk for recurrence (incidence rate ratio 3.8; 95% CI, 1.7-8.4), as was unprovoked first venous thrombosis (incidence rate ratio, 3.6; 95% CI, 1.6-7.9), and also residual vein thrombosis (incidence rate ratio, 3.1; 95% CI, 0.9-10.7). Patients with a positive family history had an incidence rate ratio for recurrence of 1.3 (95% CI, 0.5-3.0) compared to patients with a negative family history of venous thrombosis. Patients aged >40 with an unprovoked first event had an incidence rate ratio of 1.0 (95% CI, 0.3-2.9) for recurrence compared to patients aged ≤40 with an unprovoked first event.

Because the risk of recurrence in men compared to women may partly be explained by provoking hormonal factors [9], we performed another subgroup analysis in Table 4 wherein the first event type (unprovoked or provoked) was taken into account. Women experienced a provoked first event at a median age of 32 years; their incidence rate of recurrence was 0.97 (95% CI, 0.42-1.91) per 100 patient-years. Women with an unprovoked first event had a median age of 46 years; their risk of recurrence was 2.8 (95% CI, 0.9-8.5) fold increased compared to women with a first provoked event. Men with a provoked first event had a median age of 38 years and had a 3.4 (95% CI, 0.7-15.9) fold increased risk compared to women with provoked first venous thrombosis, while men with an unprovoked first event had a median age of 42 years and a 5.5 (95% CI, 2.1-13.4) fold increased risk of recurrence. Sensitivity analyses, in which differences in follow-up time were taken into account, did not materially affect outcomes.

DISCUSSION

This cohort study showed, in agreement with other studies, that patients with first provoked venous thrombosis have a 2-3 fold lower risk for a recurrence than those with unprovoked events [10,11]. Risk situations for venous thrombosis that occurred in the past yet did not result in venous thrombosis at that time were noted in 66% of patients. Although the high rate of positive responders to this question provided a large group that, according to our hypothesis, could also be at lower risk for recurrence compared to those who experienced their first event without ever having had a venous thrombotic risk situation, their risk was still 3-fold higher than those who had a provoked risk factor present at time of first venous thrombosis. Hence, asking patients with venous thrombosis about risk situations for venous thrombosis that took place long before their first thrombotic event occurred seems to be of little value for predicting if an individual patient is at low risk for recurrence.

The young age of patients enrolled in our study (median age 36 years) clearly shows that the cohort does not represent a normal distribution of patients with first venous thrombosis, as the median age at onset of a first venous thrombotic event in the community is 62 years [12]. To test whether selection bias influenced our relative risk estimates, we created subgroups that made it able to compare whether our results are similar to cohorts that included consecutive patients from normal populations [2,3]. It turned out that these risk estimates were similar to normal populations. We did find, as other studies, that the risk of recurrent venous thrombosis is approximately 3-4 times higher in men compared to women [13,14]. Some studies suggested that the lower risk of recurrent venous thrombosis in women can be explained by hormonal risk factors [9,15]. This also appeared partly to be the case in our study where women with a first provoked event had a very low absolute risk for recurrence. Nevertheless, when we compared women who had an unprovoked first event to men, men still had an increased risk for recurrence, as has also been observed in other studies [13]. Whether residual vein thrombosis increases the risk of recurrence has not been studied extensively, but seems to be a risk factor for recurrence [16]. This finding was confirmed in our study although it had broad confidence intervals (incidence rate ratio 3.1; 95% CI, 0.9-10.7). Therefore, it should be treated with caution. Nevertheless, the point estimate is quite similar to a much larger study (N= 538) on this issue that was performed in a more generalizable cohort than ours [16,17]. Furthermore, age an important risk factor for a first episode of venous thrombosis, seemed to play a little role in the risk of recurrence, as was also observed in other studies [13,18]. Although consistent with other reports, this finding should be interpreted with caution as our cohort was composed of young patients who may have a

different risk for recurrence than older patients with venous thrombosis. Not many studies have reported on positive family history as a risk factor for recurrence, but the few that did report no influence on recurrence risk [19]. This is also in agreement with our findings. Because the subgroup analyses performed in this study revealed similar findings to normal population studies, this added strength that the main outcome in the present study (i.e. that past venous thrombosis risk situations only slightly influences recurrent venous thrombosis risk) also accounts to normal populations.

Some other methodological aspects of our study warrant further comments. First, although the absolute risk for recurrence was high in our study (3 per 100 person years), it is similar to absolute risks for recurrence found in the community [11,13]. Second, our total event rate for recurrence was low (N = 25), making it difficult to adjust our data with Cox proportional hazard models. However, stratified analysis demonstrated results that were in agreement with the literature. Third, many patients were excluded from the study because they did not have an image test confirming the first event or the possible recurrence. Indeed, studies have shown that a diagnosis of either first or recurrent venous thrombosis based only on clinical approach is not considered the state of the art practice, given the large number of false positive diagnosed venous events [20,21]. Fourth, an interesting question that our study could not analyze due to small numbers is whether prolonged periods with vitamin K antagonists result in an overall decreased risk of recurrent venous thrombosis after anticoagulation is withdrawn. However, a randomized study on this issue previously showed that although patients on vitamin K antagonists have a low risk for recurrence, the risk reappears as soon as anticoagulation is stopped [22]. Finally, patients with distal vein thrombosis were included although they seem to have a lower probability for recurrence [23,24]. Small numbers did not enable us to exclude these patients.

In conclusion, in this study the risk for recurrence was related with the nature of a first provoked or unprovoked event and only slightly with the presence of other past risk situations for venous thrombosis. Asking a patient about past exposure of venous thrombosis risk factors long before the occurrence of a first venous thrombosis occurred, does not provide information to classify patients at lower risk for recurrence of venous thrombosis.

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Table 1: Demographics characteristics

Patients		N (%)		
Sex	Female	Male	Total	
	291 (77)	87 (23)	378 (100)	
Risk Factors	Yes	No	Missing	
	253 (67)	122 (32)	3 (1)	
Number of risk factors at the time of VT	One	Two	Three	Total
	253 (83)	46 (15)	7 (2)	306 (100)
Type of risk factors (all patients):				
Surgery	48 (16)			
Imobilization	39 (13)			
Oral contraceptive	139 (45)			
Hormone replacement therapy	14 (5)			
Pregnancy or postpartum period	36 (12)			
Autoimmune diseases	9 (3)			
Neoplasia or chemotherapy	13 (4)			
Others	8 (3)			
VT Place	Proximal DVT	Distal DVT	PE/DVT	Missing
	175 (46)	109 (29)	93 (24.7)	1 (0.3)
Diagnostic method	Ultrasound/duplex	V/P Lung Scan	Angio CT	
	284 (75)	54 (14)	40 (11)	
Age first event	Mean (Years)	Median (Years)		
	40	36		
Age at recurrence	51	51		
Anticoagulation Period	Mean (Months)	Median (Months)		
	13	8		
Time between the end of VKA and recurrence	29	26		
Time between VTs	40	42		
Follow up	40	30		
Family History of VT	Yes	No	Unknown	Missing
	95 (25)	275 (73)	5 (1.2)	3 (0.8)
Recurrence	25 (7)	353 (93)		
Risk factors for recurrence	5 (20)	20 (80)		
Recurrence place	Proximal DVT	Distal DVT	PE	Both
	10 (40)	10 (40)	4 (16)	1 (4)
Recanalization	Absent	Incomplete	Complete	Missing
	4 (1)	187 (49)	131 (35)	56 (15)
Past history of risk situations	Yes	No	Missing	
	251 (66)	105 (28)	22 (6)	

VKA denotes, vitamin K antagonist; VT, venous thrombosis; DVT, deep venous thrombosis;

PE, pulmonary embolism; CT, computer tomography; V/P, ventilation/perfusion.

Table 2: Risk of recurrent venous thrombosis

First event Type	Past risk factor situation	Observational years	Median age at first event	Number of events	IR per 100 PY (95% CI)	IRRatio (95% CI)	IRRatio † (95% CI)	IRRatio * (95% CI)	IRRatio # (95% CI)
Provoked	Present (N=168)	601	35	7	1.16 (0.47-2.39)	Reference	Reference	Reference	Reference
Provoked	Absent (N=71)	224	29	3	1.33 (0.28-3.91)	1.1 (0.3-4.4)	1.1 (0.3-4.3)	1.1 (0.3-4.2)	1.1 (0.3-4.1)
Unprovoked	Present (N=83)	260	46	10	3.85 (1.84-7.07)	3.3 (1.3-8.7)	2.9 (1.1-7.6)	3.1 (1.2-8.1)	2.9 (1.1-7.6)
Unprovoked	Absent (N=33)	84	39	5	5.95 (1.93-13.89)	5.1 (1.6-16.1)	4.2 (1.3-13.3)	2.9 (1.1-7.6)	4.2 (1.3-13.2)

† Model A

* Model B

Model C

IR denotes, incidence rate; PY, patient years; CI, confidence interval.

Table 3: Venous thrombosis in different subgroups

	Observational years	Median age at first event	Number of events	IR per 100 PY (95% CI)	IRRatio (95% CI)	IRRatio † (95% CI)	IRRatio * (95% CI)	IRRatio # (95% CI)
Sex								
Female (N=291)	1026	34	13	1.27 (0.67-2.17)	Reference	Reference	Reference	Reference
Male (N=87)	248	41	12	4.83 (2.50-8.45)	3.8 (1.7-8.4)	3.8 (1.7-8.4)	3.6 (1.7-8.0)	3.8 (1.8-8.4)
Risk factors								
Provoked (N=253)	888	32	10	1.13 (0.54-2.07)	Reference	Reference	Reference	Reference
Unprovoked (N=122)	373	45	15	4.02 (2.25-6.63)	3.6 (1.6-7.9)	3.2 (1.4-7.1)	3.5 (1.6-7.7)	3.2 (1.4-7.2)
Recanalization ‡								
Complete (N=131)	387	36	3	0.78 (0.16-2.27)	Reference	Reference	Reference	Reference
Incomplete (N=191)	660	34	16	2.42 (1.38-3.93)	3.1 (0.9-10.7)	3.0 (0.9-10.4)	2.9 (0.8-10.0)	2.9 (0.8-9.8)
Family history ‡								
Absent (N=275)	897	35	16	1.78 (1.02-2.90)	Reference	Reference	Reference	Reference
Present (N=95)	352	38	8	2.27 (0.98-4.48)	1.3 (0.5-3.0)	1.3 (0.6-3.0)	1.2 (0.5-2.8)	1.2 (0.5-2.8)
Age (years) unprovoked events								
< 40 (N=43)	125	32	5	4.00 (1.30-9.33)	Reference	Reference	Reference	Reference
> 40 (N=79)	248	51	10	4.03 (1.93-7.41)	1.0 (0.3-2.9)	0.9 (0.3-2.8)	1.0 (0.3-2.8)	1.0 (0.3-2.8)

† Model A

* Model B

Model C

‡ Data were missing for some patients in these subgroups.

IR denotes, incidence rate; PY, patient years; CI, confidence interval.

Table 4: Risk of recurrence venous thrombosis stratified by firsts event type and sex.

First event type	Sex	Observational years	Median age at first event	Number of events	IR per 100 PY (95% CI)	IRRatio (95% CI)	IRRatio † (95% CI)	IRRatio * (95% CI)	IRRatio # (95% CI)
Provoked	Female (N=228)	826	31.7	8	0.97 (0.42-1.91)	Reference	Reference	Reference	Reference
Unprovoked	Female (N=60)	186	45.8	5	2.69 (0.87-6.27)	2.8 (0.9-8.5)	2.3 (0.7-7.0)	2.7 (0.9-8.3)	2.3 (0.8-7.1)
Provoked	Male (N=25)	61	38.3	2	3.28 (0.40-11.84)	3.4 (0.7-15.9)	3.3 (0.7-15.5)	3.2 (0.7-14.8)	3.2 (0.7-15.0)
Unprovoked	Male (N=62)	186	42.4	10	5.38 (2.58-9.88)	5.5 (2.2-14.1)	5.3 (2.1-13.4)	5.3 (2.1-13.4)	5.4 (2.1-13.7)

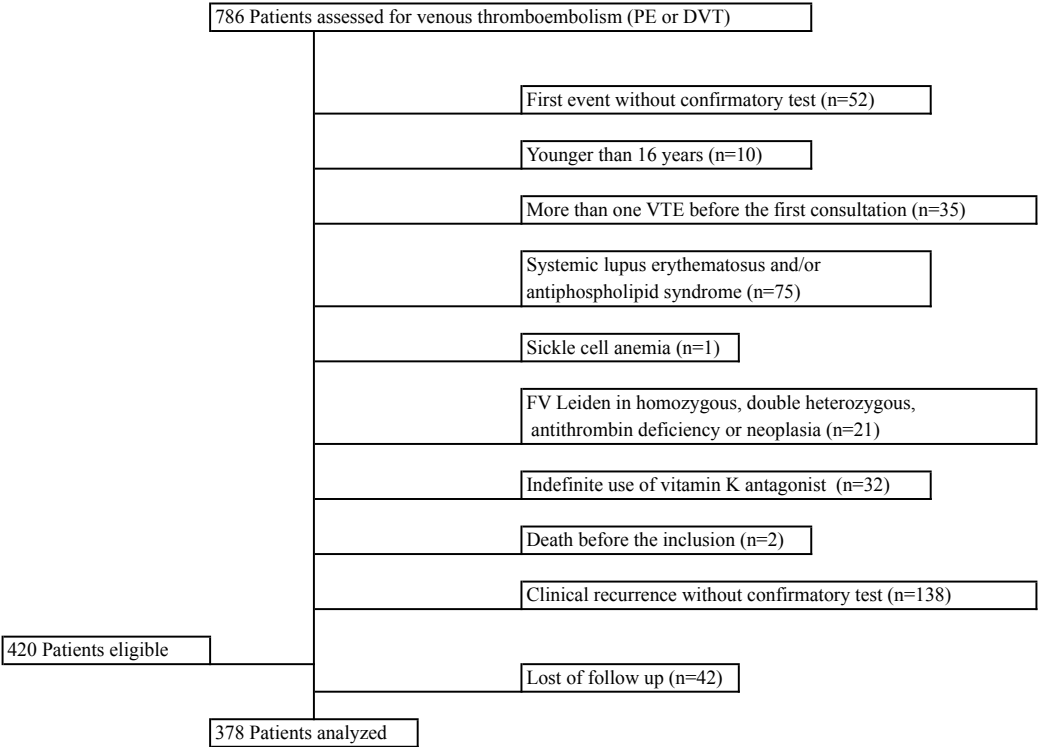
† Model A

* Model B

Model C

IR denotes, incidence rate; PY, patient years; CI, confidence interval.

Figure 1: Flow diagram of the study cohort.



Chapter 8

Risk of recurrent venous thrombosis related to past provoking risk situations: follow-up of a cohort study

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ABSTRACT

Objective: Strategies targeting at classifying the risk for recurrent venous thrombosis (VT) are needed. We previously hypothesized, by studying a cohort of patients, that those who had ‘survived’ risk situations for VT without developing it would, after a first VT, have a low recurrence risk. Therefore, we re-evaluated the same cohort, now with a longer follow-up.

Methods: Patients, after a first confirmed VT event, were followed for an average of 43 months after suspension of anticoagulation. Patients with indication for indefinite anticoagulation were not included. The primary end point was objective recurrent VT.

Results: Recurrent VT was recorded in 9% of 378 eligible patients. Patients with a provoked first event and positive past risk situations for VT had an incidence rate of recurrence of 1.26 (95% CI, 0.60-2.31) per 100 patient-years. The incidence rate ratio (IRR) of this subgroup compared to patients with a provoked event without other past risk situations for VT was 0.8 (95% CI 0.2-2.9). This IRR was 2.8 (95% CI, 1.2-6.5) in patients with an unprovoked event and positive past risk situations and 7.1 (95% CI, 3.0-17.1) in patients with an unprovoked event and no past risk situations. When only idiopathic first events were evaluated the IRR was 2.5 (95% CI, 1.1-5.9) for patients without past risk situation compared with those with these history.

Conclusions: In this study asking a patient about past exposure to risk factors for VT long before the occurrence of a first VT occurred, could be used to classify patients with a first unprovoked VT at higher risk for recurrence of VT.

INTRODUCTION

Venous thrombosis (VT) occurs in 1-2 per 1000 persons per year [1]. Approximately 30% of patients with VT will experience a recurrent event within the subsequent 5 years [2]. It has been recognized that the presence of a transient or reversible risk factor at the time of VT is associated with a decreased risk of recurrence after anticoagulant therapy is stopped [3]. Therefore, a relatively short period of anticoagulant treatment with vitamin K antagonists is advised for those patients [4]. However, because about 50% of events occur without a clear provoking risk factor, many patients may need long-term or even indefinite anticoagulation after the occurrence of a first event. This treatment may lead to serious side effects such as major bleeding [5]. Therefore, the challenge is to identify new subgroups of patients with unprovoked VT who have a low rather than high risk of recurrence. This would allow shortening the duration of the anticoagulant treatment in these patients.

The thrombosis potential model takes into consideration that the thrombosis potential will drop sharply after the occurrence of a provoked event and that such an individual will not readily cross the thrombosis threshold again, consequently leading to a reduced risk for recurrence [6,7]. Considering this model, we previously hypothesized that those who had ‘survived’ many risk situations without developing VT would, after a first VT, have a low recurrence risk [8]. We observed that the risk for recurrence seemed related with the presence of past risk situations for VT, but as our total event rate for recurrence was low (N=25) and our follow up was relatively short, we could not rule out a possible chance finding. For this reason, we re-evaluated the same cohort of 378 patients, now with a median follow-up of 13 more months.

METHODS

The subjects of the cohort were described previously [8]. The study started in April 2000. Patients were seen in our outpatients' clinic (Hematology Unit, University Hospital, Universidade Federal de Minas Gerais, Belo Horizonte) or contacted by phone at least once a year. All but 62 patients were contacted by phone or had a clinical evaluation at July 1st 2011. Patients were not systematically screened with ultrasonography since we were only interested in symptomatic recurrent VT. All patients were advised to seek the outpatients' clinic in case of suspected DVT or PE. A standardized questionnaire with study questions was completed by a professional healthcare assistant in each patient visit. All eligible patients for this study provided written informed consent, and the study was approved by the local ethics committees.

Definitions

The duration of anticoagulation for provoked VT was 3-6 months and for unprovoked VT 6-12 months. Some patients were treated with anticoagulants for longer or shorter time duration mainly because of individual patient preferences. The first episode of VT was considered established if proximal DVT was confirmed by compression ultrasound, and PE by ventilation/perfusion lung scanning or spiral computer tomography scanning. Recurrence was considered established if it was demonstrated by objective techniques at another site than the first event, or at the same site if previously repeated tests showed no residual VT. If the recurrence of DVT was at the same site and we had not previously repeated tests to analyze if there was residual VT, we only considered recurrence when the compressive ultrasound (CUS) showed new thrombus formation in combination with clinical symptoms that were absent previously. Only the objectively demonstrated PE was considered a recurrence. If these criteria were not fulfilled, anticoagulant treatment was withheld and the event was not classified as a recurrent VT. VT (either first or recurrent) was defined as provoked if it had occurred at or within 3 months after exposure to exogenous risk factors including surgery (with more than 30 minutes of duration), trauma leading to hospitalization, immobilization for more than 3 days (hospitalization for clinical reasons), limb immobilization in a plaster cast for more than 7 days, pregnancy, puerperium (until 2 months after delivery), use of oral contraceptives or hormonal replacement therapy (at the time of thrombosis), presence of autoimmune diseases, or active malignancy. In the absence of these risk factors, VT was classified as unprovoked.

During the medical visit, patients were asked about past risk situations for VT at any time of life. The questions were related to use of oral contraceptives or hormonal replacement therapy, pregnancies, surgeries, immobilization with plaster cast in the lower limbs or any hospital admission for clinical reasons for more than 3 days. If they answered positive on any of these risk situations that occurred at least 3 months or more before the event occurred, they were considered as positive for past risk situations for VT. Patients were submitted to CUS or ventilation/perfusion lung scanning or spiral computer tomography scanning around the end of anticoagulation use, and were separated in two groups: those with complete recanalization and those with incomplete or absent recanalization, according to the result of the image test. A family history was considered positive when the patient had one or more first degree relatives (parents, sibling and/or offspring) with VT documented by image tests and/or with history of vitamin K antagonist use due to VT. Follow up period started after vitamin K antagonist were withdrawn and finished at 01.07.2011 or last consultation or death, whichever occurred first.

Statistical analysis

Observation time started after vitamin K antagonists were withdrawn and ended at the time of the recurrence or at the last consultation. Incidence rates of recurrent thrombotic events were calculated as the number of events over the accumulated observation time. Incidences and 95% confidence intervals (95% CI) were calculated under the Poisson distribution assumption. Stratified analyses were used to assess the risk of thrombotic events recurrence by age, sex, idiopathic or provoked first thrombotic event, status of image test around the end of anticoagulation (recanalization), family history of VT and presence or absence of past risk situations for VT. Including patient time before patients were enrolled (i.e. patients who stopped anticoagulant treatment before the first appointment with the researcher) could be problematic as this may create a survivor bias [9]. Therefore, we performed 3 sensitivity analyses to take observation time into close account (Figure 1). Statistical analyses were performed using SPSS software, version 17.0 (SPSS Inc., Chicago ILL).

RESULTS

The study cohort comprised 378 patients: 291 (77%) were female, 253 (67%) had a provoked VT, the median age at time of the first event was 36 years, 109 (29%) had a distal VT, 175 (46%) had a proximal VT, 71 (19%) had a PE and 22 (6%) had DVT and PE together (these patients were analyzed in the PE group). Oral contraceptives, hormone replacement therapy, pregnancy or puerperium were the most prevalent provoked risk factors appearing 189 times (in 65% of the female population). Ultrasound/duplex was the diagnostic image method in 284 (75%) patients, ventilation/perfusion lung scan in 54 (14%) patients and spiral computer tomography scanning in 40 (11%) patients. Family history for VT was present in 95 patients (25%) and positive past risk situations (patients who experience a well-known risk situation for VT in the past and did not develop thrombosis at that moment) were present in 251 patients (66%). Recurrent VT occurred in 35 patients (9%), trigger factors were present in 6 (17%) patients and the thrombotic event was unprovoked in 29 (83%). The median age at recurrence was 49 years. At a median of 7 months after their initial first event, patients were consulted by the researcher. Only two patients died during the follow up and the causes were unknown. The median follow-up time was 43 months.

Patients with a provoked first event and positive past risk situations had an incidence rate of recurrence of 1.26 (95% CI, 0.60-2.31) per 100 patient-years (Table 1). The incidence rate ratio of patients with a provoked event without other past risk situations for VT compared to patients with a provoked first event and positive past risk situations was 0.8 (95% CI 0.2-2.9). This incidence rate ratio was 2.8 (95% CI, 1.2-6.5) in patients with an unprovoked event and positive past risk situations and 7.1 (95% CI, 3.0-17.1) in patients with an unprovoked event and no past risk situations versus patients with a provoked first event and positive past risk situations. The incidence rate ratio was 2.5 (95% CI, 1.1-5.9) for patients without past risk situation compared with those with these history when only idiopathic first events were evaluated. Sensitivity analyses taking period of follow up into account showed similar findings; i.e. previous risk situations affected recurrent VT risk only in patients with unprovoked first event.

Male sex was associated with an increased risk for recurrence (incidence rate ratio 5.5; 95% CI, 2.8-10.8), as was unprovoked first VT (incidence rate ratio, 4.1; 95% CI, 2.1-8.1). Residual vein thrombosis was a risk factor for recurrence (incidence rate ratio, 2.2; 95% CI, 0.9-5.4), although presented broad confidence intervals (Table 2). Patients with a positive family history had an incidence rate ratio for recurrence of 1.3 (95% CI, 0.6-2.6) compared with patients with a negative family history of VT. Patients older than 40 years with an unprovoked first event had an incidence rate ratio of 0.9 (95% CI, 0.4-2.1) for recurrence compared with patients younger than 40 with an unprovoked first event.

DISCUSSION

We have re-evaluated a cohort of 378 patients with a first confirmed VT event for recurrent VT and concluded that previous risk situations for VT affected recurrence risk only in patients with an unprovoked first event. To our knowledge, this is the first approach to explore past risk situations as a risk factor for VT.

This incidence rate ratio for recurrence was 2.8 in patients with an unprovoked event and positive past risk situations and 7.1 in patients with an unprovoked event and no past risk situations when compared with patients with a provoked first event and positive past risk situations. Thus, collecting information about risk situations for VT that took place long before the occurrence of a first thrombotic event seems to add information about the risk for recurrence when the event was unprovoked.

Results from this cohort study are in agreement with other studies, in which patients with first provoked VT have a lower risk of recurrence than those with unprovoked events [10,11]. Risk situations for VT that occurred in the past yet did not result in VT at that time were noted in 66% of patients. The high rate of positive responders to this question provided a large group that, according to our hypothesis, could also be at lower risk of recurrence compared to those who experienced their first event without ever having had a venous thrombotic risk situation. However, we could only observe this when the first event was unprovoked.

The young age of patients enrolled in our study (median age 36 years) clearly shows that the cohort does not represent a normal distribution of patients with first VT, as the median age at onset of a first venous thrombotic event in the community is 62 years [12]. To test whether selection bias influenced our relative risk estimates, we created subgroups to compare whether our results are similar to cohorts that included consecutive patients from normal populations [2,3]. It turned out that these risk estimates were similar to those of general populations.

In our study, we also found that the risk of recurrent VT was higher in men than in women, as has been shown by others [13,14]. Furthermore, age an important risk factor for a first episode of VT, was not a risk factor for recurrence in this study. This has also been reported by other studies [13,15]. Residual vein thrombosis seems to be a risk factor for recurrence [16]. This finding was confirmed in our study although it had broad confidence interval (incidence rate ratio 2.2; 95% CI, 0.9-5.4). Nevertheless, the point estimate is similar to a larger study (n= 538) that also explored this question [16,17]. Even though, the difficulty in the standardization of residual thrombosis is always a problem in these studies and our study.

Few studies have reported on positive family history as a risk factor for recurrence, but the few that did report no influence on recurrence risk [18]. This is also in agreement with our findings.

Some methodological aspects of our study warrant further comments. First, although the absolute risk of VT recurrence was high in our study (2.09 per 100 person years), it is similar to absolute risks of recurrence found in the community [11,13]. Second, as the total number of recurrences was low (n=35), it was difficult to carry out a multivariate analysis. However, stratified analysis demonstrated results that were in agreement with the literature. Third, patients with distal vein thrombosis were included in this study although they seem to have a lower probability of recurrence [19,20]. Finally, because the subgroup analyses performed in this study revealed similar findings to general population studies, we believe that the main results of the present study (i.e. that past VT risk situations decrease recurrent VT risk) are generalizable to other cohorts. Nevertheless, at least one other cohort study should confirm our findings before implications from a clinical level can be inferred from our findings, and such study should also include patients more representative for a general population.

In conclusion, in this study asking a patient about past exposure to risk factors for VT long before the occurrence of a first VT occurred, could be used to classify patients with a first unprovoked VT at higher risk for recurrence of VT.

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Table 1: Risk of recurrent venous thrombosis

First event Type	Past risk factor situation	Observational years	Median age at first event	Number of events	IR per 100 PY (95% CI)	IRRatio (95% CI)	IRRatio † (95% CI)	IRRatio * (95% CI)	IRRatio # (95% CI)
Provoked	Present (n=168)	796	35	10	1.26 (0.60-2.31)	Reference	Reference	Reference	Reference
Provoked	Absent (n=72)	303	29	3	0.99 (0.20-2.89)	0.8 (0.2-2.9)	0.7 (0.2-2.8)	0.8 (0.2-2.7)	0.7 (2.0-2.7)
Unprovoked	Present (n=83)	342	46	12	3.51 (1.81-6.13)	2.8 (1.2-6.5)	2.6 (1.1-5.9)	2.7 (1.2-6.2)	2.5 (1.1-5.8)
Unprovoked	Absent (n=34)	112	39	10	8.93 (4.28-16.42)	7.1 (3.0-17.1)	6.3 (2.6-15.1)	7.2 (3.0-17.4)	6.1 (2.5-14.7)

† Model A

* Model B

Model C

IR denotes, incidence rate; PY, patient years; CI, confidence interval.

Table 2: Venous thrombosis in different subgroups

	Observational years	Median age at first event	Number of events	IR per 100 PY (95% CI)	IRRatio (95% CI)	IRRatio † (95% CI)	IRRatio * (95% CI)	IRRatio # (95% CI)
Sex								
Female (n=291)	1352	34	15	1.11 (0.62-1.83)	Reference	Reference	Reference	Reference
Male (n=87)	327	41	20	6.11 (3.73-9.45)	5.5 (2.8-10.8)	5.5 (2.8-10.8)	5.4 (2.8-10.6)	5.4 (2.8-10.6)
Risk factors								
Provoked (n=253)	1175	32	13	1.11 (0.59-1.89)	Reference	Reference	Reference	Reference
Unprovoked (n=122)	485	45	22	4.54 (2.84-6.87)	4.1 (2.1-8.1)	3.8 (1.9-7.5)	4.1 (2.1-8.1)	3.8 (1.9-7.6)
Recanalization ‡								
Complete (n=131)	541	36	6	1.11 (0.41-2.41)	Reference	Reference	Reference	Reference
Incomplete (n=192)	868	34	21	2.42 (1.38-3.93)	2.2 (0.9-5.4)	2.2 (0.9-5.4)	2.2 (0.9-5.5)	2.2 (0.9-5.5)
Family history ‡								
Absent (n=275)	1195	35	23	1.92 (1.22-2.89)	Reference	Reference	Reference	Reference
Present (n=95)	451	38	11	2.44 (1.22-4.36)	1.3 (0.6-2.6)	1.3 (0.6-2.7)	1.2 (0.6-2.4)	1.2 (0.6-2.5)
Age (years) unprovoked events								
< 40 (n=43)	164	32	8	4.88 (2.10-9.61)	Reference	Reference	Reference	Reference
>40 (n=79)	322	51	14	4.35 (2.37-7.30)	0.9 (0.4-2.1)	0.9 (0.4-2.1)	0.9 (0.4-2.0)	0.9 (0.4-2.1)

† Model A

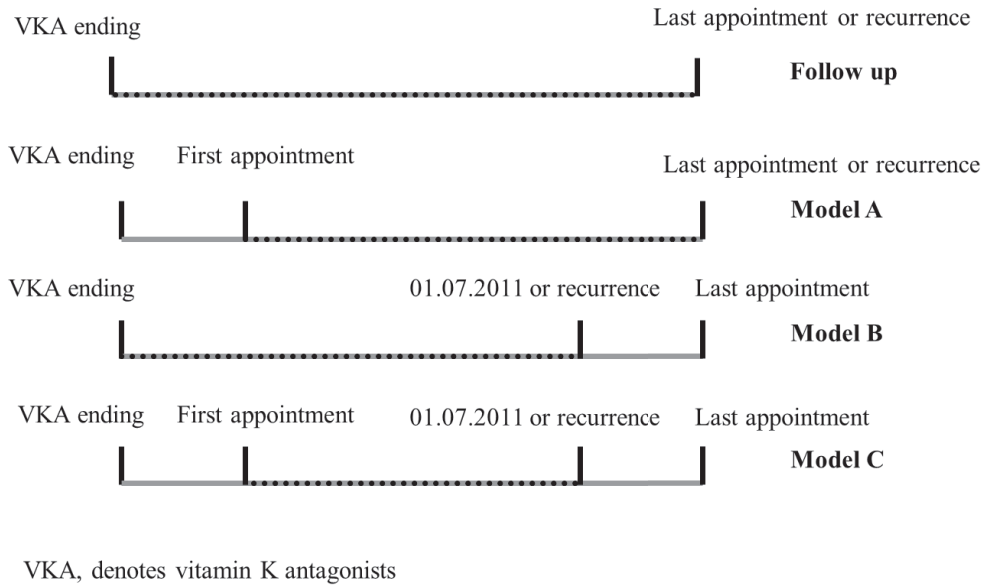
* Model B

Model C

‡ Data were missing for some patients in these subgroups.

IR denotes, incidence rate; PY, patient years; CI, confidence interval.

Figure 1: Schematic descriptions of sensitive analyses performed



The sensitivity analysis was aimed to take observation time into account. In model A, observation time started at enrollment (and when vitamin K antagonists were withdrawn) and ended at time of recurrence or last appointment. In model B, the follow-up time started when vitamin K antagonists were withdrawn and ended at the time of recurrence or July 1st 2011, whichever occurred first. In model C the follow-up time started at the time of enrollment (and when vitamin K antagonists were withdrawn) and ended at the time of recurrence or July 1st 2011, whichever occurred first.

Chapter 9

The influence of prothrombotic laboratory abnormalities on the risk of recurrent venous thrombosis

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Venous thrombosis (VT) is a disease that occurs in approximately 1-2 per 1000 persons per year [1]. Despite an appropriate treatment, up to one quarter of patients with VT will experience a recurrent event within the subsequent 5 years [2]. It has been recognized that the presence of a transient or reversible provoked risk factor at the time of VT is associated with a decreased risk of recurrence after stopping anticoagulant therapy [3]. Therefore, a relatively short period of anticoagulant treatment with vitamin K antagonists is advised for those with clear provoking risk factors for VT, such as oral contraceptive use, hospitalization, or surgery [4]. However, approximately 30%-50% of events occur without association with a provoking risk factor. Since the risk for recurrent VT is higher when the first event is unprovoked, such patients may need indefinite anticoagulant treatment. This approach could, however, lead to major side effects such as severe bleeding [5]. The challenge, therefore, is to identify patient groups who suffered an unprovoked event and still have a low risk of recurrence. These patients might benefit of anticoagulants for a fixed (shorter) time.

Since prothrombotic alterations can be demonstrated in at least 50% of patients with VT [6], testing patients with a first VT has gained great interest. Potential advances of testing patients might be the chance to elucidate the cause of the thrombosis and to track unaffected family members. However, there are also potential disadvantages. Although the presence of prothrombotic alterations is currently considered a weak predictor of recurrence in patients with a first episode of VT, [3,7,8] these results were mostly obtained in patients with either provoked and unprovoked first VT. Whether prothrombotic alterations have a predictive value for recurrence in patients who had a first unprovoked event is less well studied. Furthermore, most studies that were published on this issue [7,9], measured prothrombotic laboratory abnormalities (such as levels of factor VIII or homocysteine) only once, increasing the chance of a false positive (high) value and dilution of risk estimates.

We performed a prospective cohort study to assess the risk of recurrence in patients with provoked and unprovoked first VT, related to the presence or absence of prothrombotic alterations. In addition, these abnormalities were only considered present when they were confirmed in at least two consecutive measurements.

The subjects of the cohort and definitions of the study were described previously [10]. Briefly, subjects were patients with one previous venous thrombotic event followed from April 2000 to July 2011 at the University Hospital of Universidade Federal de Minas Gerais and at Hematológica, a specialized medical center in Hematology, Belo Horizonte, Brazil. All patients were attended by the same clinician (i.e. DDR) Patients were referred from anywhere in the State of Minas Gerais by their doctors who

looked after them during the first episode of VT. There was no control over these referrals. Patients were included consecutively, but certainly they were not consecutive patients with thrombosis in the State. Throughout the study, patients' data were collected using a standardized form with questions related to first and recurrent VT. Venous thrombosis (either first or recurrent) was defined as provoked if it had occurred at or within 3 months after exposure to exogenous risk factors including surgery (with more than 30 minutes of duration), trauma leading to hospitalization, immobilization for more than 3 days (hospitalization for clinical reasons), limb immobilization in a plaster cast for more than 7 days, pregnancy, post-delivery period (2 months), the use of oral contraceptives or hormonal replacement therapy (at the time of thrombosis), presence of autoimmune diseases, or active malignancy. In the absence of these risk factors, venous thrombosis was classified as unprovoked. Despite efforts to follow-up all included patients, 42 patients (10%) were only seen at the time of enrolment. These patients were excluded from the analysis, which resulted in 378 eligible patients. All but 62 patients who had incomplete follow-up (16%) were contacted by phone or had a clinical evaluation at July 1st 2011. The last consultation of the 62 patients with incomplete follow-up was between 09.03.2005 and 30.03.2011 (2 in 2011, 41 in 2010, 7 in 2009, 5 in 2008, 5 in 2007 and 1 in 2005). At that moment they were questioned about recurrent VT and included in the analysis up to this date. Recurrence was considered established if it was demonstrated by objective techniques at another site than the first event, or at the same site if previously repeated tests showed no residual venous thrombosis. If the recurrence of DVT was at the same site and we had not previously repeated tests to analyze if there was residual venous thrombosis, we only considered recurrence when the compressive ultrasound showed new thrombus formation together with clinical symptoms that were absent previously. Only the objectively demonstrated PE was considered a recurrence. If these criteria were not fulfilled, anticoagulant treatment was withheld and the event was not classified as a recurrent venous thrombosis. We had access to all discharge letters. Laboratory test information was available for factor VIII activity, homocysteine, factor V Leiden, (rs6025), prothrombin G20210A (rs1799963) and blood group. We analysed these risk factors individually and grouped. We considered high factor VIII as levels > 150 IU/dL in two occasions after at least three months apart. Similarly a high level of homocysteine (> 20 μ mol/L) was only considered when confirmed in a subsequent testing. All eligible patients for this study provided written informed consent, and the study was approved by the local ethical committee. Observation time started after vitamin K antagonists were withdrawn and ended at time of recurrence or at the last consultation. Incidence rates of recurrent thrombotic events were calculated as the number of events over the accumulated observation time in different

groups of patients with specific prothrombotic alterations. The incidence rate ratio was calculated and patients without prothrombotic alterations were considered as the reference group. Incidences and 95% confidence intervals (95% CI) were calculated under the Poisson distribution assumption.

Statistical analyses were performed using SPSS software, version 17.0 (SPSS Inc., Chicago ILL).

The study cohort included 378 patients; 291 (77%) were women, 253 (67%) had a provoked VT, the median age at time of the first event was 36 years, 109 (29%) had a distal VT, 175 (46%) had a proximal VT, 71 (19%) had a PE and 22 (6%) had DVT and PE (these patients were analyzed in the PE group). The total follow-up was 1573 person-years, for a median follow-up of 43 months. Recurrent VT occurred in 35 (9%) patients, for an incidence of 22 per 1000 person-years. The median age at recurrence was 49 years. At a median of 7 months after their initial first event, patients were consulted by one physician (DDR). Only two patients died during the follow up and the causes were not related to VT. In patients with elevated factor VIII, 5 (11%) out of 47 had a second event of VT. Recurrence was also identified in 1 (8%) out of 13 with high levels of homocysteine. The presence of factor V Leiden or prothrombin 20210A mutation were associated with VT recurrence in 2 (5%) out of 37 and 3 (15%) out of 20, respectively. Among the 190 patients with blood group non O 19 (10%) had a second event of VT (Table 1). The broad confidence interval of some groups reflects the small numbers. Despite this, the relative risk estimates are approximately 1 for all comparisons showing no association between VT recurrence risk and prothrombotic laboratory abnormalities (Table 1). A stratified analysis showed no increase in the risk for VT recurrence by sex and for patients with a provoked first event. However, in patients with an unprovoked first event there was a 4-fold increase in the recurrence risk in those with abnormal prothrombotic tests, albeit again with wide confidence intervals.

It has been suggested that the identification of prothrombotic abnormalities could lead to reduced VT recurrence due to changes in clinical management, such as prolongation of initial anticoagulant treatment or intensified prophylaxis during high-risk situations. However, no different recurrence risk was found when tested patients were compared with patients who were not tested [11]. Despite a slightly different study question, the presence of prothrombotic abnormalities did not increase the risk for recurrence in our study, corroborating the findings of Coppens *et al* and others [11]. However, these latter studies did not stratify on type of first event (if provoked or unprovoked). In the present study, we observed that patients with a first unprovoked event and prothrombotic abnormalities had a 4.4-fold increased risk of recurrence. Although numbers were small, and therefore a chance result cannot be ruled out, this finding can be supported by taking the

thrombosis potential model into account [12]. Unprovoked events are those events of which we do not know the underlying cause. However, there may be several reasons why patients with unprovoked thrombosis had their event. If these reasons were in some part transient, such as an infection [13], or a flare of inflammatory bowel disease [14], and in some part chronic (e.g., prothrombotic abnormalities), one would expect the risk of recurrence to be increased in patients with prothrombotic abnormalities. In this case, the thrombosis potential in the latter (chronic causes) is not altered after the first event, but the thrombosis potential in the former group (transient causes) significantly drops after the first event. These patients will have a lower risk for recurrence than those with lasting prothrombotic abnormalities [12].

Our study has limitations including small sample size and the selection of referred patients, as our Hospital is a tertiary care center. Patients were on average nearly 30 years younger than the average age of first VT in the community [15]. In addition, only 0.5% of 378 patients died during follow-up, but as 16% of patients were lost to follow up, it is likely that at least some of these patients died, possibly due to thrombosis. However, because thrombophilia is not associated with a reduced life expectancy in previous studies [16,17], it is not likely that mortality of patients who were lost to follow-up biased our results.

We conclude from our study that thrombophilia testing in all patients with a first VT is neither clinically feasible nor useful. Future studies are required to further elucidate if patients with a first unprovoked event and a negative result for prothrombotic abnormalities are at reduced risk for VT recurrence and could, therefore, benefit from a shorter period of anticoagulant treatment.

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Table 1: Risk for venous thrombosis recurrence in subgroups based on laboratory findings

	Number (%)	Patients with VT recurrence (%)	IR per 100 PY (95% CI)	IRRatio (95% CI)
Thrombophilia				
Factor VIIIc				
Normal	298(86)	27(8)	1.98 (1.31-2.88)	Reference
Elevated (>150%)	47(14)	5(1)	2.82 (0.92-6.59)	1.4 (0.6-3.7)
Homocysteine				
Normal	340(96)	31(9)	2.03 (1.38-2.88)	Reference
Elevated (>20 mMol/L)	13(4)	1(0.3)	2.13 (0.05-11.85)	1.1 (0.1-7.7)
Factor V Leiden				
Wild type	329(90)	31(9)	2.20 (1.50-3.13)	Reference
Heterozygote	37(10)	2(0.5)	1.04 (0.13-3.76)	0.5 (0.1-2.0)
Protrombin mutation				
Wild type	337(94)	29(8)	1.92 (1.29-2.76)	Reference
Heterozygote	20(6)	3(0.8)	4.41 (0.91-12.89)	2.3 (0.7-7.5)
Blood group				
O	71(27)	5(2)	1.62 (0.53-3.79)	Reference
Non-O	190(73)	19(8)	2.27 (1.37-3.55)	1.4 (0.5-3.7)
All thrombophilia				
No	52(19)	3(6)	1.31 (0.28-4.00)	Reference
Any ‡	224(81)	22(10)	2.27 (1.41-3.42)	1.6 (0.5-5.5)

‡ Presence of at least one of the following: high factor VIII or homocysteine, presence of factor V Leiden or prothrombin mutation and blood group non-O

IR denotes, incidence rate; PY, patient years; CI, confidence interval; VT, venous thrombosis

Table 2. Risk for venous thrombosis recurrence for laboratory thrombophilias in subgroups

	IRRatio		IRRatio		IRRatio		IRRatio	
	95% (CI)	Unprovoked	95% (CI)	Provoked	95% (CI)	Male	95% (CI)	Female
Thrombophilia								
No	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Any ‡	1.6 (0.5-5.5)	4.4 (0.6-33.3)	0.7 (0.1-3.2)	1.4 (0.3-6.3)	1.8 (0.3-14.7)			

‡ Presence of at least one of the following: high levels of factor VIII, elevated homocysteine, presence of factor V Leiden, presence of prothrombin mutation or blood group non-O
 IR denotes, incidence rate; CI, confidence interval

Chapter 10

Summary and Discussion

SUMMARY

Chapter 1 describes the outline of this thesis and the rationale to subdivide this thesis into two parts: risk factors for first venous thrombosis, and risk factors for recurrent venous thrombosis.

Part I: Risk factors for first venous thrombosis

Chapter 2 describes the association between pneumonia and risk of venous thrombosis in a large population-based case-control study (MEGA study), and whether this risk was influenced by immobility or unhealthy lifestyle factors. Gene-environment interactions were also assessed (i.e., the combined effect of pneumonia and prothrombotic genes), as this association could be clinically relevant for targeted thromboprophylaxis. Odds ratios were calculated as estimates of the relative risk, and were adjusted for age and sex by logistic regression methods to take the matching into account. Subgroup analysis involved stratification of patients with deep vein thrombosis of the leg only, or with pulmonary embolism with or without diagnosed deep vein thrombosis. We adjusted our findings for classical venous thrombosis risk factors, for unhealthy life style and for immobilization to determine whether the associations could be explained by confounding through these factors. The combined effect of prothrombotic genes and pneumonia was evaluated through a stratification analysis.

Pneumonia was associated with an increase in risk for venous thrombosis (5 times more likely to have venous thrombosis, odds ratio 5.0; 95% CI, 3.9-6.3). This risk was higher when pulmonary embolism was considered (odds ratio 7.9; 95% CI, 6.1-10.3, for pulmonary embolism with or without deep vein thrombosis) than when deep vein thrombosis only was considered (odds ratio 3.0; 95% CI, 2.2-4.0). None of the adjustments substantially changed the results. A joint positive effect was observed for factor V Leiden /prothrombin G20210A and pneumonia to venous thrombosis risk (odds ratios > 17).

This study shows that pneumonia is a moderate to strong risk factor for venous thrombosis. This study also provides evidence that the association between infectious or inflammatory disease and venous thrombosis cannot be explained by concurrently unhealthy life style.

In **Chapter 3**, we had the opportunity to study circannual variability of venous thrombosis in three regions (i.e. Milan/ Italy, Leiden/the Netherlands and Tromsø/Norway). These three regions have a similar climate type (Köppen classification Cfa, Cfb, Cfb/Dfc, respectively), and are similarly situated around sea level, but differ in the annual temperature, which is highest in Milan, lower in Leiden and lowest in Tromsø. The regions are therefore matched on demographic and climate variables, but differ in terms of temperature, sunlight and air pollution. Three different data set were used in the analysis, the Multiple Environmental and Genetic Assessment (MEGA) study (Leiden), the Tromsø study (Tromsø) and from the Lombardy region (Milan). We evaluated the absolute risk differences between seasons of prevalent venous thrombosis.

Approximately half of the patients were men, and the mean age at time of thrombosis was 49 years (interquartile range 39-61). In all three studies, the prevalence of venous thrombosis was lowest in Spring as compared with the other seasons (absolute risk difference - 2.3%, 95% CI, -4.0 to -0.7). The absolute risk difference (taking Spring as a reference group) was -2.6% (95% CI, -4.3 to -1.0) to Winter, -2.2% (95% CI -3.8 to -0.6) to Summer and -2.2% (95% CI, -3.8 to -0.5) to Autumn, respectively. When unprovoked events, or incident pulmonary embolism, or men only were analyzed separately, a similar pattern was observed, that is: a lower prevalence of thrombosis in Spring than in the other seasons.

We observed a difference in the distribution of venous thrombosis over the seasons in all three regions, wherein the prevalence was lowest in Spring. Though the absolute difference of venous thrombosis between Spring and the other seasons was statistically significant, the clinical relevance is negligible. The causal relation between weather and venous thrombosis incidence could be real, although a causal interpretation of this finding is hard to provide. To proof causality, one would like to know an intervention strategy, and the intervention “Spring” seems too ambiguous to use as an intervention tool. Nevertheless, we inferred some things from this study. First, our findings cannot be explained by a sole linear increase of temperature. Second, transient infections are also not very likely to explain our results. Third, the results are also probably not explained by sunlight exposure. Fourth, air pollution, seemed also not to explain the difference in risk over the seasons. Finally, a referral bias seemed also unlikely. To conclude, in our study the risk of venous thrombosis was different between the seasons. We were unable to provide a reason for the difference in venous thrombosis risk between the seasons.

In **Chapter 4** we investigated whether the presence of factor (F)V Leiden with blood group non-O modifies the risk for venous thrombosis in individuals with different body mass index strata in a case-control study (N=11253). We observed that a progressive increase in body mass index was associated with an ever increasing risk for venous thrombosis, odds ratio 1.9 (95%CI, 1.6-2.3) for those in the upper tertile, as compared with individuals in the first body mass index tertile, blood group O, and no FV Leiden (the reference group). The addition of FV Leiden and blood group non-O in the model increased the risk in all body mass index tertiles; odds ratios were 3.8 (95%CI, 3.2-4.6) in the third body mass index tertile of individuals with blood group non-O, and 5.4 (95% CI, 3.5-8.5) in the third body mass index tertile of individuals with FV Leiden, respectively. When both FV Leiden and blood group non-O were present, odds ratios were 9.1 (95% CI, 5.9-14.0) in the first body mass index tertile, 9.4 (95% CI, 6.6-13.5) in the second body mass index tertile, and 12.5 (95% CI, 8.9-17.6) in the third body mass index tertile. In conclusion, individuals with a high body mass index, blood group non-O or factor V Leiden are at higher thrombosis risk than expected when these prothrombotic factors are analyzed separately. The high risks of venous thrombosis in some subgroups may justify targeted screening and thromboprophylaxis decisions.

In **Chapter 5** we aimed to determine whether protein S deficiency is associated with venous thrombosis in a population-based case-control study (n=5317) as it is in thrombophilic family studies. Subjects were regarded protein S deficient when protein S levels were <2.5th percentile of the controls. Free- and total protein S deficiency was not associated with venous thrombosis: free protein S <53 U/dL, odds ratio [OR] 0.82 (95%CI, 0.56-1.21) and total protein S <68 U/dL, OR 0.90 (95%CI, 0.62-1.31). Restriction, stratifications and adjustments for possible confounders did not change findings. When lower cut-off values were applied, it appeared that individuals at risk of venous thrombosis could be identified at levels <0.10th percentile of free protein S (<33 U/dL, OR 5.4; 95% CI, 0.61-48.8). In contrast, even extremely low total protein S levels were not associated with venous thrombosis. *PROS1* was sequenced in 48 subjects with free protein S level <1st percentile (<46 U/dL), and copy number variations were investigated in 2718 subjects, including all subjects with protein S (free or total) <2.5th percentile. Mutations in *PROS1* were detected in five patients and five controls reinforcing the observation that inherited protein S deficiency is rare in the general population. Our results show that protein S testing and subsequent testing on *PROS1* mutations should not be considered in unselected patients with venous thrombosis.

Part II: Risk factors for recurrent venous thrombosis

Chapter 6 is a review which outlines what is currently known about the epidemiology of recurrent venous thrombosis, and focuses in more detail on potential new risk factors for venous recurrence. The general implications of these findings in patient management are discussed. Venous thrombosis, including deep vein thrombosis and pulmonary embolism, is a common disease that frequently recurs. Recurrence can be prevented by anticoagulants, but this comes at the risk of bleeding. Therefore, prevention of recurrent venous thrombosis will be more profitable if it becomes possible both to identify more precisely those individuals who are at risk of recurrent venous thrombosis and to quantify the risk to which they are exposed. Consideration of the discussed new risk factors for recurrence may allow us a more optimal use of prophylactic strategies against recurrent venous thrombosis.

In **Chapter 7**, we evaluated the possibility of new strategies to classify the risk for recurrent venous thrombosis. Some patients may have experienced many venous thrombosis risk situations during their life time without developing venous thrombosis, while others may have experienced few of such risk factors and then develop venous thrombosis idiopathically or after a single provoked risk factor. We hypothesized that those who had 'survived' many risk situations without developing an event would, after a first venous thrombosis, have a low recurrence risk. Patients who were included in a Brazilian tertiary hospital were followed after anticoagulation withdrawal for a first venous thrombosis. Patients with indication for indefinite anticoagulation were not included. The primary end point was objective recurrent venous thrombosis.

Recurrent venous thrombosis was recorded in 7% of 378 eligible patients. Patients with a provoked first event and positive past risk situations for venous thrombosis had an incidence rate of recurrence of 1.16 (95% confidence interval [CI], 0.47-2.39) per 100 patient-years. The incidence rate ratio of this subgroup compared with patients with a provoked event without other past risk situations for venous thrombosis was 1.1 (95% CI, 0.3-4.4). This incidence rate ratio was 3.3 (95% CI, 1.3-8.7) in patients with an unprovoked event and positive past risk situations and 5.1 (95% CI, 1.6-16.1) in patients with an unprovoked event and no past risk situations.

In this study the risk for recurrence was related with the nature of a first provoked or unprovoked event and only slightly with the presence of other past risk situations for venous thrombosis. Asking a patient about past exposure of venous thrombosis risk factors long before the occurrence of a first venous thrombosis occurred, does not provide information to classify patients at lower risk for recurrence of venous thrombosis.

In **Chapter 8**, we reevaluated the same cohort described in chapter 7, but now with a longer follow up. The objective was the same, i.e., to try and find a variable that could help us in the decision about to keep or not the anticoagulation treatment.

Patients, after a first confirmed venous thrombosis event, were followed for an average of 43 months after suspension of anticoagulation and the primary end point was objective recurrent venous thrombosis. Patients with a provoked first event and positive past risk situations had an incidence rate of recurrence of 1.26 (95% CI, 0.60-2.31) per 100 patient-years and this was our “reference group”. The incidence rate ratio of patients with a provoked event without other past risk situations for venous thrombosis compared with the reference group was 0.8 (95% CI 0.2-2.9). This incidence rate ratio was 2.8 (95% CI, 1.2-6.5) in patients with an unprovoked event and positive past risk situations and 7.1 (95% CI, 3.0-17.1) in patients with an unprovoked event and no past risk situations versus our control group. The incidence rate ratio was 2.5 (95% CI, 1.1-5.9) for patients without past risk situation compared with those with these history when only idiopathic first events were evaluated.

We conclude that previous risk situations for venous thrombosis affected recurrence risk only in patients with an unprovoked first event.

In **Chapter 9**, we performed a prospective cohort study to assess the risk of recurrence in patients with provoked and unprovoked first venous thrombosis, related to the presence or absence of prothrombotic alterations. Since prothrombotic alterations can be demonstrated in at least 50% of patients with venous thrombosis, testing patients with a first venous thrombosis has gained great interest. Subjects were patients with one previous venous thrombotic event followed from April 2000 to July 2011 at the University Hospital of

Universidade Federal de Minas Gerais. The study cohort included 378 patients and the total follow-up was 1573 person-years, with a median follow-up of 43 months.

Laboratory test information was available for factor VIII activity, homocysteine, factor V Leiden, (rs6025), prothrombin G20210A (rs1799963) and blood group. We analysed these risk factors individually and grouped. We considered high factor VIII as levels > 150 IU/dL in two occasions after at least three months apart. Similarly a high level of homocysteine (> 20 μ mol/L) was only considered when confirmed in a subsequent testing.

Recurrent venous thrombosis occurred in 35 (9%) patients, for an incidence of 22 per 1000 person-years. The relative risk estimates were approximately 1 for all comparisons showing no association between venous thrombosis recurrence risk and prothrombotic laboratory abnormalities. In those with abnormal prothrombotic tests and an unprovoked first event there was a 4-fold increase in the recurrence risk. From our results, we concluded that thrombophilia testing in all patients with a first venous thrombosis is not useful. Future studies are required to further elucidate whether patients with a first unprovoked event and a negative result for prothrombotic abnormalities are at reduced risk for venous thrombosis recurrence.

DISCUSSION

Venous thrombosis is a common disorder that can affect apparently healthy individuals as well as hospitalized patients. One estimate is that there are worldwide about 10 million people suffering from venous thrombosis per year with admission to hospital, with an equal incidence in high-income and low-income countries, but with more deaths in the latter (ISTH Steering Committee for World Thrombosis Day; *J Thromb Haemost.* 2014;12:1580–1590). Venous thrombosis is probably best understood as a multicausal disease in which more than one genetic or environmental condition coincides to produce clinically apparent thrombosis.

Venous thrombosis can easily be prevented by anticoagulants, but at the risk of side-effects, most notably hemorrhage. Anticoagulant-associated bleeding is the number one cause of iatrogenic admission to hospital. Hence, prevention should be tailored to those individuals who have the highest risk to develop venous thrombosis.

Searching for risk factors or a combination of concurrently present risk factors can improve our capacity of identification the risk situations in specific individuals, therefore reaching a personalized relation between risk and benefits for thromboprophylaxis applications. The aim of this thesis was to understand better and to search for such risk situations.

In this thesis, it was shown that pneumonia increases the risk for venous thrombosis. The positive interaction between pneumonia and factor V Leiden or prothrombin G20210A besides assets our findings, and should be taken into consideration for thromboprophylaxis decisions that should be studied in clinical trials. In our seasons study (**chapter 3**) we observed a difference in the distribution of venous thrombosis over the year, wherein the prevalence was lowest in Spring, and somewhat similar over the other seasons. The difference of venous thrombosis between spring and the other seasons was (2%). Although this difference may seem relevant, we concluded from this study that the clinical applicability is negligible, as we also explained in a rebuttal (see *J Thromb Haemost.* 2013;11:570-2) to a comment (in *J Thromb Haemost.* 2013;11:568-70). First, we explained that when we considered spring as our reference season (23.2% of all events occurred in spring), and compared this finding with winter (25.9% of all events occurred in winter), we would have found a $25.9/23.2 \times 100 = 12\%$ higher rate of venous thrombosis in winter than in spring. This relative increase was in the same range as the authors in the rebuttal found for risk of venous thrombosis in winter as compared with spring (19% higher rate). Next, we explained that a relative increase of a first venous thrombosis of even 19% on a bad winter day as compared with a good summer day can ever be clinically useful. As the absolute risk of venous thrombosis is 1 in

1000 persons per year or $1/365.25$ in 1000 persons per day, a relative increase of 19% of a bad winter day as compared with a good summer day would mean that about 5000 persons ($1000/0.19$) would need to be treated with a good summer day annually to prevent 1 venous thrombotic event which is about 1.8 million per day to prevent one venous thrombosis event. If we could give one person a summer season instead of a winter season, we would need to give this to 400 000 people to prevent one thrombotic event, and likewise for other interventions. Even if we were able to completely replace 3 months of Dutch winter by summer, for 3 months, we would need to maintain this for hundreds of years to prevent one case of thrombosis. Furthermore, the intervention ‘good summer day’, however desirable, seems a difficult tool to introduce into clinical practice. Though we tried, we were unable to provide a reason for the difference in venous thrombosis risk between the seasons. The latter is somewhat disappointing, as according to the principle of sufficient reason everything must have a reason or a cause. Others may be more successful than we were here. By courtesy of professor le Cessie, another issue about the calculated “2%” should be mentioned here, that is that spring was not prespecified as being the season in which venous thrombosis would have occurred less as compared with the other seasons and that for this reason it would have been better to compare the four observed percentages in the four different seasons to the expected percentages (25; 25; 25; 25), yielding a p-value of 0.03.

That venous thrombosis is a multicausal disease was (again) shown in **chapter 4**, where it was shown that individuals with a high body mass index, blood group non-O or factor V Leiden are at a 12.5 fold higher venous thrombosis risk than when these prothrombotic factors are absent. When other transient risk situations such as hormone use, immobilization and recent travel were analyzed in subgroups, the same pattern was observed. As the daily life situations described above are frequent, even a small increase in relative risks can be clinically relevant.

Changing paradigms, these two words help to discuss **Chapter 5**. Although individuals with very low levels of free protein S appeared to be at increased risk of unprovoked venous thrombosis (odds ratio 2.31, 95%CI, 1.06-5.05), the prevalence of venous thrombosis patients with such levels was very low (0.4%), making it unpractical to use free protein S levels to help identify patients at risk in a clinical setting. Total protein S levels were not able to identify subjects at risk of venous thrombosis even when these levels are very low. Therefore, a relevant question, about the utility of searching for protein S deficiency in any patient with venous thrombosis is answered with “No”. Maybe only patients with venous thrombosis who originate from thrombophilic families should be screened for protein S deficiency.

The optimal duration of anticoagulant therapy in patients with a first episode of venous thrombosis, occurring in the absence of antecedent major risk factors, is unknown. Based on a 5-year cumulative risk of disease recurrence of 25 to 30% in these patients, current American College of Chest Physicians guidelines recommend at least 3 months of treatment with a vitamin K antagonist, and an option for life-long anticoagulation in patients at low risk of bleeding. While preventing most recurrences, this approach has some drawbacks. Whereas the risk of recurrence appears to diminish with time, the risk of anticoagulant related hemorrhage increases with ongoing anticoagulation and advancing age. Hence, the net clinical benefit of indefinite anticoagulation may vary considerably over the long term. The risk of recurrent venous thrombosis may be lower in certain patients. Therefore, at least in some patient subgroups, the risk of recurrence approximates the annual risk of anticoagulant-related major hemorrhage. Another challenge that was faced in this thesis was to identify venous thrombosis patient groups that may have a low risk of recurrence and could for this reason receive anticoagulants for only a short time.

We hypothesized (**in chapters 6-8**) that patients at risk for recurrent venous thrombosis, who had experienced transient provoked risk factors previously, yet not developed thrombosis at that time, would have a lower risk of recurrence than those whose first (provoked or unprovoked) event was not preceded by provoked risk factors. This hypothesis was based on the thrombosis potential model, first described by Frits Rosendaal in 1999, where all individuals have a thrombosis threshold and a thrombosis potential; when this limit is crossed venous thrombosis occurs. Therefore, if someone has already been pushed through a risk situation for venous thrombosis in the past and an event did not happen, it suggests that this person has a high thrombosis threshold (or a low thrombosis potential) and, therefore, probably a lower risk for venous thrombosis recurrence. From **chapters 7 and 8** we concluded that the absence of past exposure to risk factors for venous thrombosis long before the occurrence of a first venous thrombosis occurred, may be used to classify patients with a first unprovoked venous thrombosis at higher risk for recurrence of venous thrombosis. Although the cohort in which this result originated from was validated through stratified analyses using risk factors for recurrence already described, the finding needs to be validated by other groups and in different populations before it can be incorporated in clinical practice.

We finally assed whether identification of prothrombotic abnormalities could lead to reduced venous thrombosis recurrence due to changes in clinical management, such as prolongation of initial anticoagulant treatment or intensified prophylaxis during high-risk situations. However, as also shown by previous studies, no different recurrence risk was found. Although

we observed that patients with a first unprovoked event and prothrombotic abnormalities had an increased risk of recurrence, numbers were small and the confidence interval was wide. Another point is that we evaluated thrombophilias as one group, “present or not”, and that we could not simultaneously test for all concurrent known laboratory abnormalities related with an increased risk for first venous thrombosis. It has recently been shown in the MEGA follow-up study that such a strategy, may, after all, be useful in the prediction of recurrent thrombosis. Therefore, the finding in **chapter 9** must be observed with restrictions.

In conclusion, in this thesis, the thrombosis potential model was successfully applied to several known risk factors for venous thrombosis to better understand why first and recurrent venous thrombosis can develop in an individual patient. Thanks to the *old concept* of the thrombosis potential model, which supposes that patients with past provoking risk factors should have a lower risk of recurrent venous thrombosis than those whose first (provoked or unprovoked) event was not preceded by provoked risk factors, has led to the *new insight* for clinicians that these past provoking risk factors might be of use to stratify patients at low or high risk of recurrent venous thrombosis to which they can adapt thrombosis prevention strategies.

Future directions:

The most important risk factor for recurrence is the presence of any triggering factor during or close to the venous thrombotic event. Therefore, the discovery of unknown/new risk factors in patients with unprovoked venous thrombosis will add to our ability to predict recurrence. An easy, costless, anamnestic test for physicians is to ask patients with venous thrombosis about preceding triggering factors, yet not developed thrombosis at that time. These patients had a lower risk of recurrence than those whose first (provoked or unprovoked) event was not preceded by provoked risk factors in this thesis and may, for this reason, be candidates who do not need to be treated with anticoagulants for a prolonged (indefinite) time. Nevertheless, one cannot be confident that this finding, as well as all other findings that are reported in this thesis, will prove true in the long run. For this reason one needs replication studies, and it is to be hoped that the results from this thesis will lead to such replication studies. That replication studies are vital to science and progress is not only true for the studies that are reported in this thesis: it is true for all studies, including clinical trials from pharmaceutical companies. The reason to specifically mention these clinical trials here, is because we are now entering an era in which the direct oral anticoagulants (DOACS) are introduced as anticoagulant treatment to patients with venous thrombosis. These trials

showed (for the specific DOAC classes) that treatment with DOAC was non-inferior to vitamin K antagonist treatment. They are now given to patients with venous thrombosis on a global scale, but none of these studies have been replicated thus far, for which the reason is unknown. It could be due to costs. Trials are expensive and are very time consuming enterprises. It could also be because patients, researchers, clinicians and/or politicians think that replication studies are unnecessary: a waste of time, effort and money. If the latter is the reason to not replicate trials (and other studies), then we cannot be sure that medical science is built on a solid foundation. Indeed, researchers showed in *Nature* (Begley et al, *Nature* 2012) that in hemato-oncology 47 out of 53 highly promising results from pharmaceutical trials could not be replicated. And the epidemiologist Ioannidis has argued that failure or non-willingness to replicate study results in medical science is a reason why most published research findings are false (Ioannidis, *PLoS Med* 2005). It is therefore not with modesty, but with urgency to draw the attention to the necessity to replicate research findings from all theses, including this thesis. Following such a strategy will lead to new insights from old concepts, as this thesis has shown.

Addendum

Nederlandse Samenvatting

List of publications

Curriculum Vitae

Acknowledgments

NEDERLANDSE SAMENVATTING

Veneuze trombose (waaronder diepe veneuze trombose en longembolie wordt verstaan) is een vaak voorkomende aandoening in zowel de algemene bevolking als bij patiënten die zijn opgenomen in het ziekenhuis. Wereldwijd krijgen ongeveer 10 miljoen mensen per jaar veneuze trombose waarvoor zij tijdelijk dienen opgenomen te worden in een ziekenhuis. Trombose komt even vaak voor in landen met een hoog gemiddeld inkomen als in ontwikkelingslanden, ofschoon het aantal doden ten gevolge van trombose hoger is in ontwikkelingslanden (ISTH Steering Committee for World Thrombosis Day; J Thromb Haemost. 2014;12:1580–1590). Het ontstaan van veneuze trombose kan het best worden begrepen als een aaneenschakeling van genetische en omgevingsfactoren, waar een bepaalde omstandigheid de spreekwoordelijke druppel de emmer doet overlopen (multicausaal model).

Met antistollingsmiddelen kan veneuze trombose relatief gemakkelijk voorkomen worden, maar dergelijke middelen hebben bijwerkingen, waarbij de meest gevreesde bijwerking ernstige (orgaan)bloedingen zijn. Bloedingen ten gevolge van antistollingsmiddelen zijn de meest voorkomende oorzaak van ziekenhuisopnamen ten gevolge van bijwerkingen van medicijngebruik. Daarom worden deze middelen liefst alleen voorgeschreven aan mensen die een intrinsiek hoog risico hebben op het krijgen van veneuze trombose. Het identificeren van nieuwe risicofactoren van trombose of het beoordelen van interactie tussen bekende risicofactoren voor trombose kunnen ons begrip waarom iemand veneuze trombose krijgt, vergroten. Hierdoor kan vervolgens een individuele inschatting worden gemaakt wie er wel en wie niet een verhoogd risico heeft op het krijgen van veneuze trombose. Daarna kan empirisch afgewogen worden wie er antistollingsmiddelen voor een bepaalde duur moeten krijgen vanwege het hoge risico op trombose. Het doel van dit proefschrift is om dergelijke hoog-risico situaties te identificeren en beter te begrijpen.

Dit proefschrift heeft aangetoond dat longontsteking (pneumonie) het risico op het krijgen van veneuze trombose verhoogt. Mensen met longontsteking die een onderliggende genetische variant dragen die het risico op veneuze trombose verder verhoogt (zoals factor V Leiden of protrombine G20210A variant) hebben veruit het hoogste risico op het krijgen van veneuze trombose (**hoofdstuk 2**). Dit resultaat bevestigt dat veneuze trombose een multicausale ziekte is, en dat vooral mensen met een genetische variant als factor V Leiden of protrombine G20210A mogelijk baat hebben bij het krijgen van antistolling ten tijde van een longontsteking. In **hoofdstuk 3** wordt een verschil aangetoond in de distributie van het ontstaan van veneuze trombose over de seizoenen: in de lente komt veneuze trombose minder vaak

voor dan in de andere drie seizoenen. Het verschil in prevalentie is 2% ten faveure van de lente ten opzichte van de andere seizoenen. Waarom dit verschil aanwezig is, kon niet met deze studieopzet aangetoond worden. Wel kon de ‘klinische relevantie’ van dit verschil worden toegelicht aan de hand van een getallenvoorbeeld. Dat hebben wij gedaan nadat anderen zich sterk hadden gemaakt dat een verschil van 2% over de seizoenen klinische consequenties zou kunnen hebben (J Thromb Haemost. 2013;11:568-72). Ten eerste legden wij uit dat een prevalentie van ziekte niet gelijk is aan het optreden van ziekte. Een prevalentie van 2% meer trombose komt overeen met een relatieve toename van 10-20%. Gezien het absolute risico op het krijgen van veneuze trombose 1 per 1000 per jaar is, is het risico op veneuze trombose 1/365.25 per 1000 per dag is (aangezien een jaar gemiddeld 365.25 dagen telt). Met deze getallen rekenden wij uit dat wat betreft het wisselen van een druilerige koude winterse dag met een mooie zonnige dag er ongeveer 5000 mensen een jaar lang met een mooie zonnige dag dienen te worden ‘behandeld’ om één trombose te voorkomen of 1.8 miljoen mensen één dag moeten worden ‘behandeld’ met een mooie zonnige dag om één veneuze trombose te voorkomen. Dit getallenvoorbeeld toont aan dat de klinische relevantie van deze bevinding afwezig is, ofschoon uiteraard een mooie warme Braziliaanse lentedag te prefereren is boven een koude, druilerige Nederlandse winterdag.

Dat veneuze trombose het best omschreven kan worden als een multicausale ziekte werd (wederom) aangetoond in **hoofdstuk 4**, waar mensen met een hoog BMI, bloedgroep niet-0 en factor V Leiden een 12.5-voudig verhoogd risico hebben op het krijgen van veneuze trombose in vergelijking met degenen die deze risicofactoren niet bezitten. In subgroepanalyses toonden wij aan dat een zelfde verhoogd risico op trombose aanwezig is bij vrouwen die de anticonceptiepil slikken, recentelijk langdurig gereisd hebben of die tijdelijk geïmmobiliseerd zijn. Aangezien deze factoren vaak aanwezig zijn binnen de bevolking, kunnen deze hoge relatieve risico's klinisch relevant zijn.

Veranderend paradigma, deze twee woorden helpen in het samenvatten van **hoofdstuk 5**. Een familiale afwijking in het hebben van een te lage hoeveelheid in het bloed van anticoagulant eiwit “proteïne S” levert een hoog risico op het krijgen van veneuze trombose. Dit vonden wij ook terug, zoals beschreven in hoofdstuk 5, waarbij mensen uit de algemene bevolking met erg lage bloedwaarden van vrij proteïne S een verhoogd risico bleken te hebben op het krijgen van veneuze trombose (odds ratio 2.31, 95%BI, 1.06-5.05). Echter, de prevalentie van deze afwijking in patiënten met onverklaarde veneuze trombose is laag (0.4%). Een dergelijke lage prevalentie maakt het onwaarschijnlijk dat het testen op te laag vrij proteïne S in alle patiënten met veneuze trombose zinvol kan zijn: de vooraf-trefkans op het vinden van een dergelijke afwijking is te laag. Het meten van totaal

proteïne S antigeen leverde geen enkel aanknopingspunt op dat mensen uit de algemene bevolking een verhoogd risico hebben op het krijgen van veneuze trombose. Deze bevindingen suggereren dat alleen mensen met veneuze trombose waarbij deze ziekte vaak voorkomt in de familie getest zouden kunnen worden op proteïne S.

Het is onbekend wat de optimale behandeling met antistollingsmiddelen is bij patiënten die een eerste veneuze trombose hebben doorgemaakt zonder daarbij eerder blootgesteld te zijn geweest aan tromboserisicofactoren. Hoewel de internationale richtlijn van de ‘American College of Chest Physicians’ adviseert om iedereen met trombose minimaal 3 maanden te behandelen en mogelijk levenslang voor degenen die een laag basisrisico hebben om een bloeding te krijgen onder antistollingstherapie, is dit advies niet zonder risico’s. Enerzijds neemt het jaarlijkse risico op het krijgen van een nieuwe trombose met de tijd af, maar anderzijds neemt het jaarlijkse bloedingsrisico ten gevolge van antistollingstherapie met de leeftijd toe. Daarom is het onzeker of mensen met een eerste veneuze trombose wel levenslang antistollingstherapie moeten blijven gebruiken. Dit geldt zeker voor patiëntengroepen bij wie het basisrisico op een nieuwe veneuze trombose laag is. In **hoofdstuk 6-8** wordt getracht om van patiënten met veneuze trombose met anamnestic verkrege informatie het recidief risico in te schatten. Degenen bij wie dit risico als laag wordt ingeschat, zouden na 3-6 maanden veilig kunnen stoppen met antistollingstherapie. In deze hoofdstukken wordt gebruik gemaakt van het ‘trombose potentiaal model’ dat in 1999 voor het eerst is beschreven door Frits Rosendaal. De hypothese was dat patiënten met veneuze trombose die eerder geen trombose hadden ontwikkeld terwijl ze toch eerder een ‘hoogrisico’ situatie hadden doorgemaakt, een lager risico zouden hebben op een recidieftrombose dan degenen die niet eerder blootgesteld waren aan een dergelijke hoogrisico situatie. Uit **hoofdstuk 7 en 8** blijkt dat patiënten met dergelijke ‘hoogrisicofactoren’ in de voorgeschiedenis, inderdaad een lagere kans hebben op een recidieftrombose. In beide hoofdstukken wordt geadviseerd om deze studies eerst te repliceren in andere studies alvorens er klinische consequenties aan verbonden kunnen worden.

In het laatste hoofdstuk van dit proefschrift (**hoofdstuk 9**) werd beoordeeld of protrombotische bloedafwijkingen het risico op recidief veneuze trombose verhogen. Evenals eerdere studies naar dit onderwerp werd geen effect gevonden op het optreden van recidieftrombose bij patiënten met protrombotische bloedafwijkingen. Aangezien de aantallen in deze studie klein waren kon er geen onderscheid worden gemaakt tussen het simultaan bestaan van meerdere protrombotische afwijkingen en het optreden van een recidief, maar slechts kon worden beoordeeld of het al dan niet hebben van een of meerdere protrombotische afwijkingen het recidief tromboserisico

verhoogt. Een grotere studie in de MEGA follow-up heeft aangetoond dat de strategie waarbij meerdere protrombotische factoren bij elkaar worden opgeteld om het recidief risico te bepalen, wel succesvol kan zijn in het voorspellen van wie wel en wie niet een recidieftrombose krijgt. De interpretatie van hoofdstuk 9 moet daarom voorzichtig zijn.

In conclusie: in dit proefschrift is het multicausale trombosepotentiaalmodel verscheidene malen succesvol toegepast om te begrijpen of en waarom eerste en recidief veneuze trombose kan ontstaan in een individuele patiënt. Met dank aan het *oude concept* van het trombosepotentiaalmodel, dat veronderstelt dat patiënten met veneuze trombose die eerder een hoog risico situatie hebben doorgemaakt, en toch geen trombose kregen, kon aangetoond worden dat deze theorie de praktijk volgt. Dit kan leiden tot het *nieuwe inzicht* bij clinici dat het uitvragen van deze eerdere hoog risicofactoren van invloed kan zijn in het bepalen welke patiënt met veneuze trombose al dan niet langdurig antistollingstherapie behoort te krijgen.

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CURRICULUM VITAE

Daniel Dias Ribeiro was born to Brazilian parents on the 6th of October 1971 in Belo Horizonte, Brazil. He started in the Brazilian private education system in 1974. In 1989 he graduated from the Sistema Educacional de Ensino Pitágoras, Belo Horizonte. In 1990, Daniel commenced his medical studies at the Universidade Federal de Minas Gerais and graduated in 1995. From January 1996 to December 1998 he attended a Medical Residency in Clinical Pathology at the Hospital das Clínicas, Universidade Federal de Minas Gerais. From January 1999 to December 2001 he attended a Medical Residency in Hematology and Hemotherapy at the Hospital das Clínicas, Universidade Federal de Minas Gerais. A deeper contact with research started in 2004 during his post-graduation as a Master of Science (MSc) in Hematology - Universidade Federal de Minas Gerais, entitled, "Association between etiology of cirrhosis, Child's classification, presence of ascites and conventional coagulogram, and the immediate preoperative thromboelastographic profiles in patients submitted to liver transplantation" Under the supervision of Professor Teresa Cristina de Abreu Ferrari. The knowledge in researching was improved during his PhD, under the supervision of Professor Suely Meireles Resende, the thesis was entitled "Incidence of Recurrence of deep vein thrombosis in patients following in hemostasis clinics". This PhD program started in 2008 and finished in 2012. From August 2010 to August 2011 he lived in Leiden, The Netherlands, a program sponsored by CAPES (Brazil) and NUFFIC (The Netherlands), to improve his knowledge in epidemiology. During his stay in Leiden he was invited by Professor Doctor Frits Rosendaal to start a Dutch PhD program under his supervision, having as copromotors Dr Willem Lijfering, Dr Suzanne Cannegieter. In Brazil, he has occupations in a public and private institutions. Hospital das Clínicas from Universidade Federal de Minas Gerais, a public institution, he supervises Hematologists during their Medical Residency and is the Head of hemostasis clinic since 2001. His activities in the private health system are in a Laboratory of clinical pathology – Laboratório São Paulo – 1996-currently. He has activities related with coagulations disorders, and a Clinical of Hematology – Clínica de Hematologia Ltda. HEMATOLÓGICA – 2001-currently. Assisting patients with coagulations disorders and he is the Head of hemostasis section.

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Risk factors for first and recurrent venous thrombosis

New insights from old concepts

Daniel Dias Ribeiro



The idea behind “the thrombosis potential model” is that an individual is at risk for venous thrombosis throughout life, which is reflected in the 'thrombosis potential' and that each risk factor contributes to increase the potential. Only when the combination of thrombosis risk factors reach a certain potential, venous thrombosis will occur (crossing of the thrombosis threshold).

In this thesis, the thrombosis potential model will be applied to several known risk factors for venous thrombosis to better understand why first and recurrent venous thrombosis can develop in an individual patient.