Emerging risk factors for venous thromboembolism: The role of commonly prescribed drugs for cardiovascular disease and inflammatory disorders

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EMERGING RISK FACTORS FOR VENOUS THROMBOEMBOLISM

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Chapter 1

General introduction and outline of the thesis

GENERAL INTRODUCTION

Venous thromboembolism (VTE) is a common thrombotic condition that causes a major disease burden worldwide. VTE comprises deep vein thrombosis (DVT), pulmonary embolism (PE) or both. Although the disease presentation is acute, long-term sequelae may occur, such as post-thrombotic syndrome and chronic pulmonary hypertension, which can be associated with severe disability and reduced life expectancy [1]. Fatal PE also accounts for the disease-associated mortality [2].

The reported annual incidence rates of the disease ranges from 0.5 to 55.9 per 10000 individuals in the population, and increases exponentially with age in both men and women [1, 3] as illustrated in Figure 2. In addition, VTE mortality rates range from 0.94 to 3.23 per 10000 individuals per year [4] and the rate of disability-adjusted life years, which is the sum of the years of life lost and the years lived with disability, is estimated to be 0.12 per 10000 individuals per year [5]. Given the disease burden, the recognition of risk factors and the development of preventive measures and adequate treatment are warranted [1].

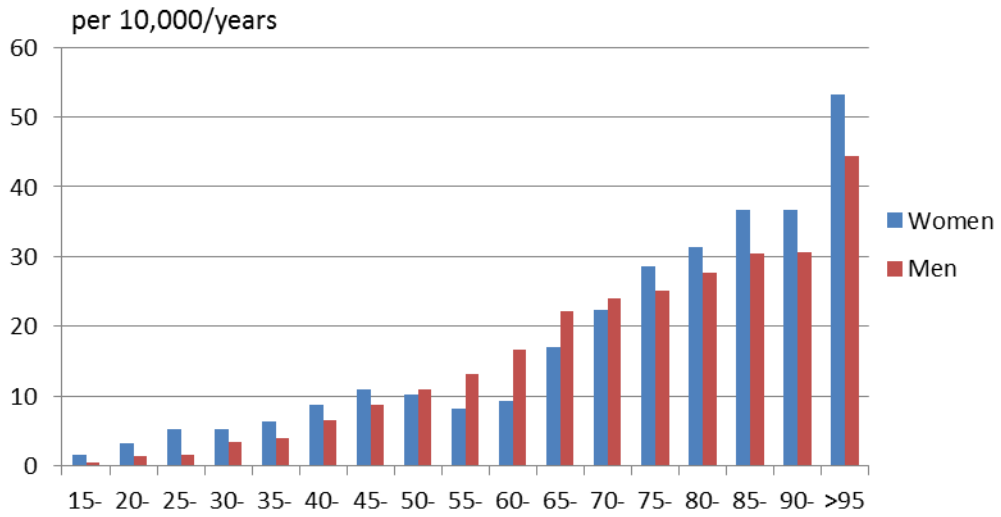

Figure 1. Incidence rates of first venous thromboembolism for men and women. Adapted from Kort et al. [3].

THE ROLE OF HEMOSTASIS IN THE PATHOGENESIS OF VTE

Hemostasis is a physiological dynamic process necessary to maintain the blood flow in a damaged vessel, which process is highly regulated to avoid either blood losses or thrombosis [6]. Hemostasis can be divided in three phases: primary hemostasis, coagulation and fibrinolysis. Primary hemostasis requires the participation of the vessel wall (mainly the media layer and the endothelium), platelet activation and von Willebrand factor (VWF), and is responsible for the formation of platelet thrombi [6].

Coagulation (or secondary hemostasis) is a cascade of enzymatic reactions that culminates in a burst of thrombin generation, and is responsible for thrombus consolidation. The coagulation pathways can be divided in initiation and amplification phases. The initiation phase starts when a damaged vascular endothelium allows plasma factor (F)VII to come into contact with tissue factor (TF)-bearing cells, the complex of TF and activated FVII activates FX and generates small amounts of thrombin [7]. In the amplification phase of coagulation, small amounts of thrombin activate FVIII and FV. Activated FVIII promotes the formation of the tenase complex (FIXa-FVIIIa) which activates FX. Activated FX moves to a complex with FVa (prothrombinase complex) that activates prothrombin (FII) and produces a large-scale thrombin burst, which is necessary for fibrinogen conversion to fibrin and thrombus consolidation [7].

Thrombin is also responsible for the activation of the anticoagulant system that, in turn, downregulates both initiation and amplification phases. Thrombin is responsible for the activation of tissue factor pathway inhibitor (TFPI), which is a protein that inhibits TF, FXa and FVIIa and downregulates the initiation phase of coagulation. Furthermore, thrombin binds to thrombomodulin (TMP) and to the endothelium protein C receptor (EPCR) to form a complex that promotes the protein C conversion to activated protein C. Activated protein C moves to a complex with protein S that inactivates factors Va and VIIIa [8].

Finally, fibrinolysis is responsible for the dissolution of the fibrin clot. The primary protein in fibrinolysis in plasmin, which is activated from plasminogen by either tissue plasminogen activator (t-PA), released by endothelial cells, and urokinase plasminogen activator (u-PA), released by monocytes. Both activators are inhibited by plasminogen activator inhibitor – 1 (PAI-1), which downregulates the fibrinolytic process [9].

The mechanisms that lead to thrombus formation was first described by Rudolf Virchow and comprise:1) reduction of blood flow, causing venous stasis; 2) vessel wall injury,

causing loss of vascular wall integrity; and 3) alteration of hemostasis in favor of a procoagulant state (hypercoagulability) [10]. Figure 2 illustrates the the Virchow triad. Interestingly, most of the risk factors for venous thrombosis are involved in pathological mechanisms that lead to hypercoagulability. Several genetic and acquired factors associated with imbalance in hemostasis have been consistently shown to increase the risk of VTE, such as increased levels of coagulation factors (FVIII, FIX, FXI, von Willebrand factor), deficiency of natural anticoagulants (protein S, protein C, antithrombin), gain of function mutations in factors V and II (FV Leiden and FII20210A), hypofibrinolysis, lupus anticoagulant, nephrotic syndrome, inflammatory diseases, estrogen use, pregnancy and malignancy [10-12]. That observation is consistent with the hypothesis that hypercoagulability is the most important component implicated in thrombus formation [6].

EMERGING RISK FACTORS FOR VTE

Despite the list of risk factors for VTE being extensive, only 50 to 60% of VTE episodes occur in the presence of major provoking factors [4]. Additionally, individuals with several risk factors may never develop VTE [11]. These observations underscore that the current knowledge on risk factors associated with VTE is not sufficient to identify all causes of the disease and potential risk factors are not being considered in the diagnostic workup [13].

Recently, inflammation has emerged as a mechanism potentially associated with thrombus formation via a process called immunothrombosis [14]. Thrombosis under certain circumstances is initiated by the innate immune system and has a major physiological role in immune defense, however when uncontrolled, immunothrombosis contributes to venous and arterial thrombosis [14]. In addition, inflammatory response leads to hypercoagulability, dyslipidemia, atherosclerosis and, in turn, increases the risk of thrombosis [15].

As hypercoagulability is the most important pathological determinant of VTE risk, theoretically any newly detected factor capable of causing impairment in the hemostatic process may cause or prevent VTE. However, determining a causal relationship between these emerging risk factors and VTE risk is challenging, as many other factors can influence or confound that association.

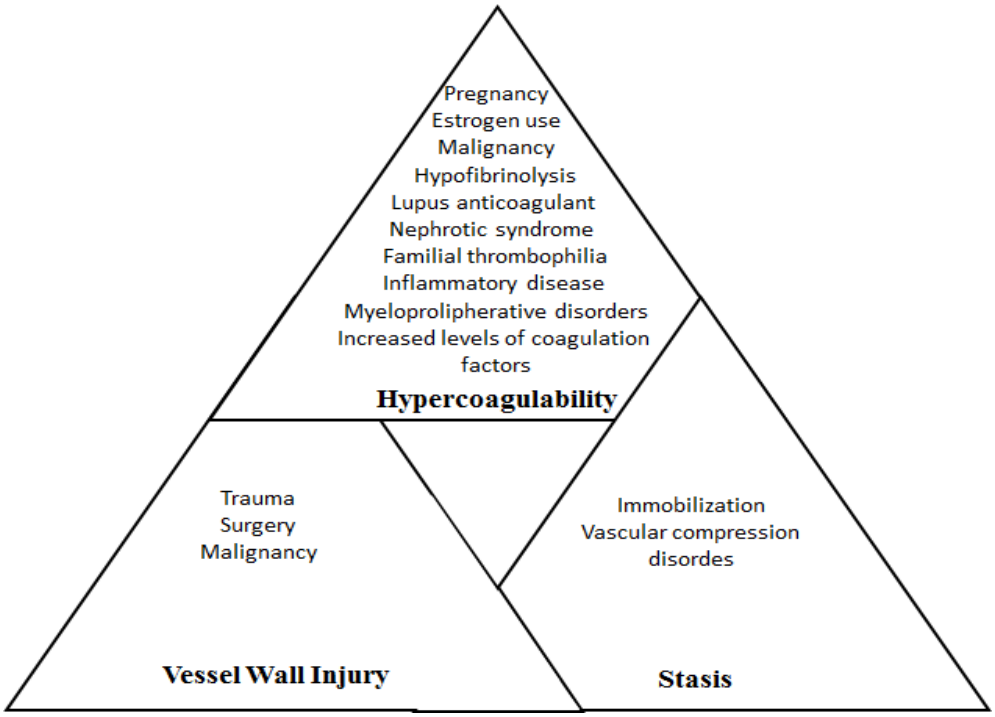

Figure 2. Virchow's triad and the pathological mechanisms by each factor affects the risk of thrombosis.

Therefore, the aim of this thesis is to identify emerging risk factors for VTE. To achieve this goal, we will describe the supposedly causal role of statin and glucocorticoid use (*i.e.* two drugs that can influence inflammation) with may lead to changes in hemostasis and VTE risk.

Statins to modulate hypercoagulability and thrombotic risk

The effect of statins on hemostasis and VTE risk confers particular therapeutic interest. Although anticoagulation therapy is indicated to prevent VTE episodes, it is not always prescribed because of the associated risk of bleeding [16]. The search for new medications capable of decreasing VTE risk without increasing the risk of bleeding is warranted. As statins do not increase the risk of bleeding, [17-19], the drug could become an alternative preventive treatment for patients who are at high risk of VTE and bleeding [20]. However, the effect of statins on reducing VTE risk is regarded with some skepticism, since the underlying mechanisms by which statins may control the hemostatic pathways are not clear.

Table 2 summarizes possible effects of statin treatment on hemostasis. Most of these mechanisms were reported “in vitro” and in animal models [20, 21] and controlled clinical

studies are scarce [21]. Furthermore, results from observational studies showing reduction on VTE risk by statins could be subject to several types of bias, while only one randomized trial was conducted to evaluate this issue.

For this reason, in **chapter 2** we performed a literature review to describe the epidemiology and potential hemostatic mechanisms behind the finding that statin use is related with a decreased risk of VTE. The review summarizes several etiological insights behind the supposed causal association, and includes recent trial evidence. It also combines and integrates different types of knowledge, *i.e.* from very fundamental to purely clinical, putting previous findings in a methodological context. The review summarizes recent data from randomized trials showing that statin therapy, in particular the high-potency statins atorvastatin and rosuvastatin, is associated with a decrease in levels of coagulation factors and D-dimer and an increase in levels of PAI-1 [22-25], even in patients with prior VTE [22]. It also highlights that, although increasing evidence indicates that statins, in particular rosuvastatin, may reduce the risk of first VTE, the estimated risk reduction varies from 11 to 54% [26, 27] and the strongest evidence is limited to a single randomized clinical trial (RCT) [28]. Clinical trials are still needed to determine if statins can play a role in recurrent VTE prophylaxis, although no randomized trial is being performed for that purpose.

In **chapter 3** we studied the effect of rosuvastatin on individual prothrombotic profiles. This was a secondary analysis of the STAtins Reduce Thrombophilia (START) trial, in which patients with prior VTE (n=247), who recently discontinued anticoagulant therapy, were randomized to rosuvastatin 20 mg/day for 4 weeks or no intervention. The primary analysis showed that rosuvastatin decreases plasma levels of FVIII, FXI and VWF [22]. In the current study, we evaluated whether rosuvastatin could affect thrombin generation potential, a coagulation marker that not only reflects the individual coagulation potential but also predicts the risk of incident and recurrent VTE. The primary outcome was the change in the endogenous thrombin potential and in the thrombin peak by rosuvastatin.

A possible explanation for the effect of statins on hemostasis is that lowering of lipids may affect hemostasis. Although this seems a reasonable pathophysiological hypothesis, no association between lipids and hypercoagulability or VTE risk has been confirmed so far [29-31]. However, only few lipids within established lipoprotein classes, such as low density lipoprotein (LDL-c), and very low-density lipoprotein (VLDL) and triglycerides, have been tested and other lipid species, in particular apolipoproteins (apos), have not been completely evaluated. Only recently, common apos A-I and B have been associated with levels of

hemostatic factors and VTE risk in population studies [30, 32]. Therefore, the effect of statins on hemostasis may be associated with the serum levels of apos. To test our hypothesis, we first evaluated whether levels of newly described apos C-II, C-III and E were associated with hemostatic factors and VTE risk. Since the development of high-sensitivity liquid chromatography–tandem mass spectrometry method for the absolute quantification of small molecules, progress has been made towards the quantification of these small apos. Since then, apos C-II, C-III and E have emerged as risk factors for arterial cardiovascular diseases. It was also demonstrated that apos C-III and E levels are reduced by statins [33]. Using this quantification method, in **chapter 4** we investigated whether these apos have prothrombotic properties and are associated with risk of VTE.

Role of glucocorticoids in thrombotic risk: players or viewers?

As with statins, glucocorticoids are frequently prescribed drugs with a potential effect on hemostasis and VTE risk. The potential effects of glucocorticoids on hemostasis are summarized in Table 3 [34-36]. However, evaluating the effect of glucocorticoids on VTE risk is challenging as the underlying condition that motivated the therapy may also be associated with thrombotic risk [37-39]. It has been shown that the risk of VTE increases two to three times in patients with chronic inflammatory diseases, such as chronic obstructive pulmonary disease [40], autoimmune diseases [41] and inflammatory bowel diseases [42, 43]. Whether the thrombotic risk associated with these diseases may also be affected by the use of glucocorticoids is not yet determined [39].

In **chapter 5** we evaluated the association of oral glucocorticoids use and the risk of incident and recurrent VTE. Confounding by indication is a major bias in any population study aiming to evaluate the effects of a drug. In **chapter 3** we overcame this potential bias by performing a randomized controlled trial (RCT). In **chapter 5**, as we aimed to evaluate an adverse effect of oral glucocorticoids, as performing a RCT is not ethically possible. Therefore, we determined the frequency of a first VTE event associated with the use of oral glucocorticoids employing the self-controlled case-series (SCCS) method. By using the SCCS method, we controlled for fixed confounding such as chronic disease and socioeconomic status [44, 45]. In order to distinguish the effects of the underlying disease from that of oral glucocorticoids on the risk of VTE, we separated the total period of exposure to oral glucocorticoids into five periods: one period immediately before the exposure, when we

expected to observe only the effect of the exacerbation of the disease on the risk of VTE and four periods with glucocorticoid treatment, which represented short and long-term glucocorticoid use [35, 46, 47]. In addition, we evaluated the effect of oral glucocorticoids on the risk of recurrent VTE in a cohort design. Studies on the association between oral glucocorticoids and recurrent VTE are unprecedented.

Finally, this thesis comprises studies that are aimed to improve the understanding of the pathophysiology of VTE, to further refine the assessment of VTE risk and to provide insight into new therapeutic approaches.

Table 2. Potential effects of statin therapy on hemostasis.

Phases of the process of hemostasis	
Primary hemostasis	
Von Willebrand levels	Decreased [48]
Platelet aggregation	Decreased [49]
Nitric oxide release	Increased [50]
P-selectin expression	Decreased [51]
Coagulation	
Tissue factor expression and activity	Decreased [52, 53]
TFPI levels	Decreased [54]
Factors Va, VII, VIII, XIII	Decreased [22, 48]
Thrombin	Decreased [53, 55]
Fibrinolysis	
Plasminogen activator inhibitor-1 (PAI-1)	Decreased [56, 57]
Tissue plasminogen activator (tPA)	Decreased [56, 57]

Table 3. Potential effects of glucocorticoid therapy on hemostasis [34, 58].

Phases of the process of hemostasis	**Absence of potentially influencing factors**	**Increased inflammatory activity setting**
Primary hemostasis		
Von Willebrand	Not changed	Decreased
Platelet aggregation	Not changed	Increased
P-selectin	Not changed	-x-
Coagulation		
Natural anticoagulants (proteins C, S and antithrombin)	-x-	Increased
Tissue factor	-x-	Increased
Prothrombin fragment 1+2	Decreased	Not changed
Antithrombin - Thrombin	Increased	Not changed
Fibrinolysis		
Plasminogen activator inhibitor-1 (PAI-1)	Increased	Increased

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Chapter 2

Statin therapy to revert hypercoagulability and prevent venous thromboembolism: a narrative review

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Summary

Venous thromboembolism (VTE) causes a major disease burden worldwide, so that effective preventive measures may be warranted. Although oral anticoagulation is effective in preventing VTE episodes, bleeding complications are a major concern that may lead to treatment avoidance. Statin therapy, which is widely used for prevention of arterial cardiovascular disease, is a promising alternative treatment for VTE prophylaxis, as the drug may affect hemostasis without increasing the risk of bleeding. In the past years, clinical studies have suggested that statins can interfere with blood coagulation and, in turn, reduce the risk of VTE. These effects, however, are still regarded with skepticism, as the underlying mechanisms by which statins may affect hemostasis in humans are not clear and data showing that statin therapy reduces VTE risk mostly came from observational studies, while only one randomized trial was conducted to evaluate this issue. In this review, we summarize the currently available evidence regarding the effect of statin therapy on coagulation and on VTE prevention. Recent randomized data show that statin therapy, in particular rosuvastatin, leads to decreased levels of coagulation factors in patients with prior VTE. This evidence provides a reasonable basis for interventional studies necessary to establish the efficacy of statins on reducing the risk of incident of recurrent VTE.

Keywords: blood coagulation, epidemiology, hydroxymethylglutaryl-CoA reductase inhibitors, prevention, venous thrombosis

INTRODUCTION

Venous thromboembolism (VTE) is a condition that comprises deep vein thrombosis (DVT), pulmonary embolism (PE) or both. The reported annual incidence of VTE ranges from 7.5 to 26.9 per 10,000 individuals in the population, with the highest rates being reported in the elderly [1]. In addition, VTE mortality rates range from 0.94 to 3.23 per 10,000 per year, varying across countries [2]. Due to the high incidence rates, associated disabilities and mortality, VTE causes a major disease burden worldwide [1], which underscores the necessity of effective preventive measures and adequate treatment.

Approximately 50-60% of VTE episodes are provoked by major risk factors, such as cancer, surgery, immobilization, estrogen therapy and pregnancy. Cases in which a transient or a persistent risk factor cannot be identified are categorized as unprovoked VTE [3, 4]. Because the risk of recurrent VTE is higher after an unprovoked event compared with a provoked one, categorizing VTE episodes is important to determine the duration of anticoagulation treatment [5]. After anticoagulation therapy is discontinued, the risk of VTE recurrence is up to 10% per year in patients with unprovoked VTE [6] and extended use of oral anticoagulants is associated with a 7-fold decrease in the risk of recurrent VTE [7, 8]. Prolonged oral anticoagulant therapy increases by 2-3 fold the risk of having a clinically significant bleeding episode compared with placebo [7, 8], which may result in treatment avoidance. Therefore, the search for new medications capable of decreasing VTE risk without increasing the risk of bleeding is warranted.

In this context, statins could be an alternative medication for VTE prophylaxis because they may have antithrombotic effect without causing bleeding complications [9]. Although results from the SPARCL trial [10] indicated that atorvastatin at 80 mg per day was associated with a 66% increase in the risk of hemorrhagic stroke compared to placebo (hazard ratio, 1.66; 95% confidence interval [CI] 1.08 to 2.55), these observations were not confirmed in other trials.[11-14] This suggests that results from SPARCL were due to a type I error. In the Heart Protection Study [11] no difference in hemorrhagic stroke was observed between simvastatin at 40 mg per day and placebo (incidence rate ratio, 0.95; 95% CI: 0.65 to 1.40). The JUPITER trial found no difference between rosuvastatin at 20 mg per day and placebo in the rates of intracranial hemorrhage [12] or other bleeding episodes [13]. A meta-analysis of randomized trials and observational studies [14] demonstrated that statins were not associated with an increased risk of intracerebral hemorrhage. The pooled risk ratio for bleeding on statin in randomized trials was 1.10 (95% CI: 0.86 to 1.41) and was 0.94 (95% CI: 0.81 to 1.10) in

cohort studies. Finally, the American Heart Association/American Stroke Association guidelines[15] state that there is insufficient data to recommend restrictions on use of statin agents for the management of intracerebral hemorrhage. In addition, long term use of statins, which is required for prophylactic treatments, is well tolerated and side effects are manageable [16].

Statins are widely used in patients with cardiovascular risk factors for prophylaxis of cardiovascular disease [17]. Since cardiovascular disease and VTE have some common risk factors, such as older age, male sex, smoking history, sedentary lifestyle, obesity and hypercoagulability [18-21], statins are frequently recommended anyway to patients requiring VTE prophylaxis. Thus, the possibility of using one single drug to prevent arterial cardiovascular diseases and VTE would diminish the pill burden associated with the use of several classes of drugs. Therefore, statin therapy could become an alternative treatment for VTE prophylaxis if statins are proven to downregulate hemostasis and prevent VTE episodes.

In this article, evidence of association between statins, hemostasis and VTE risk will be reviewed. For this purpose, we searched MEDLINE electronic databases to select the manuscripts. The searches combined the MESH terms related to the intervention (i.e., hydroxymethylglutaryl-CoA reductase inhibitors) and outcomes (i.e., hemostasis, blood coagulation, venous thromboembolism, embolism and thrombosis). Basic science studies and clinical studies, particularly meta-analysis and systemic reviews, were selected for this review.

THE EFFECT OF STATINS ON HEMOSTASIS

Statins are a class of drugs that decrease the serum levels of cholesterol through the inhibition of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase. By inhibiting this enzyme, statins reduce the hepatocyte cholesterol content, thus promoting enhanced expression of low-density lipoprotein (LDL) receptors on the cell membrane. The increased expression of this receptor leads to increased receptor-mediated endocytosis of LDL and, in turn, to decreased serum levels of LDL [22].

Statin use decreases the risk of CVD not only because of the lipid lowering effect but also because of an anti-inflammatory effect.[23] Results from the JUPITER trial demonstrated that the risk of CV events decreased by 79% in participants who achieved the targets of hsCRP less than 1 mg/L and LDL cholesterol less than 1.8 mmol/L (hazard ratio 0.21, 95% CI: 0.09

to 0.51). While the CV risk reduction was 51% (hazard ratio 0.49, 95% CI: 0.37 to 0.66) in those who achieved only the target of LDL cholesterol [23].

Besides the lipid lowering and the anti-inflammatory effects, statins are alleged to have pleiotropic effect on the process of hemostasis. Basic research and clinical studies have investigated whether statins can interfere with platelet activity, coagulation, thrombin generation and fibrinolysis. These studies will be reviewed in this topic.

Effects of statins on platelet activity

Studies *in vitro* and in animal models have demonstrated that statins can decrease platelet activation by several mechanisms. Rosuvastatin was shown to inhibit platelet degranulation in rat models of myocardial infarction [24], and atorvastatin to decrease platelet activation and upregulate the synthesis of *nitric oxide* (NO) by platelets and endothelial cells in mice models of cerebral ischemia [25-27]. Pravastatin was shown to suppress platelet-dependent procoagulant activity *in vitro* by decreasing the expression of P-selectin and the deposition of tissue factor (TF), thrombin and fibrin on adherent platelets [28, 29].

The first clinical study that evaluated the effects of statins on platelet activity enrolled patients with hypercholesterolemia, and showed that pravastatin could affect platelet aggregation and platelet-dependent thrombin generation in association with reduced serum cholesterol levels [30]. Atorvastatin, simvastatin, fluvastatin and cerivastatin were also shown to reduce P-selectin levels [31] and platelet aggregation with different agonists, such as arachidonic acid [32, 33], ADP, collagen, and epinephrine [34, 35]. Conversely, the discontinuation of statins led to increased platelet activity [33]. In patients with hypercholesterolemia, the effect of statins on platelet aggregation seemed to occur in association with increased platelet-derived NO release, in a dose-dependent manner and independently of cholesterol-lowering effect [34]. In healthy individuals, use of simvastatin for 7 days led to a reduction in platelet aggregation triggered by arachidonic acid [36]. Although these studies point to an effect of statins on platelet activity, most of the data came from non-randomized studies, and therefore these results may be biased by confounding or due to regression to the mean.

In the STAtins Reduce Thrombophilia (START) trial, in which individuals with prior VTE were randomized to receive either rosuvastatin at 20 mg daily for 28 days or no intervention, rosuvastatin use had no effect on platelet activation mediated by thromboxane A_2

(TxA_2) [37]. Although the observations from this randomized controlled trial (RCT) do not confirm that rosuvastatin decreases platelet activation mediated by TxA_2, it is not possible to exclude potential other antiplatelet effects of rosuvastatin since other platelet function assays were not performed.

Effect of statins on coagulation

Evidence with regard to the effect of statins on coagulation was first demonstrated in the late 1990s and early 2000s. The first studies, performed *in vitro*, demonstrated that both simvastatin and fluvastatin were capable of reducing TF gene expression in human monocytes, endothelial cells and smooth muscle cells, in a dose dependent manner [38, 39]. Not only TF was suppressed but also thrombin formation could be inhibited by simvastatin *in vitro* [40]. Lovastatin, simvastatin and mevastatin were shown, *in vitro*, to enhance the activated protein C-mediated suppression of thrombin generation, in part via increased levels of thrombomodulin, in a concentration-dependent manner, that is dependent on inhibition of the Rho/Rho-kinase pathway [41]. Later on, the inhibitory effect of statins on TF expression was replicated *in vivo*. Studies in hyperlipidemic mice models, showed that statins diminished the expression of TF in atherosclerotic lesions and monocytes [42] independently of the reduction in plasma lipids [43, 44]. The mechanisms of statin-induced TF inhibition involved a direct inhibitory effect on Rho/Rho-kinase pathway and, in turn, TF expression [39, 45]. This lipid-independent mechanism was further confirmed in a study with a atherosclerotic rabbit model showing that fluvastatin directly interfered with the transcriptional activation of *TF* gene, by downregulating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway in endothelial cells [46]. Therefore, basic research studies consistently demonstrated that statins can downregulate TF expression [38, 39, 42] through a direct pathway, independent of the lipid-lowering mechanism [45, 46].

Clinical trials have also demonstrated that statin therapy, either with simvastatin [47], atorvastatin [48-50] or cerivastatin [35, 51] affects coagulation factors and thrombin generation, although the mechanisms behind these effects are less clear. The first study, published in 1997 [52] showed that simvastatin treatment decreased plasma levels of prothrombin fragment F1+2 (F1+2) by 35% and monocyte TF antigen and activity by 68% and 61% , respectively, in hypercholesterolemic patients. The effect of simvastatin and cerivastatin in reducing tissue factor pathway inhibitor (TFPI) and factor (F) VII was also demonstrated in

crossover studies [35, 51] and in a small randomized trial [53]. In an RCT aimed to compare atorvastatin with placebo in patients eligible for two-step carotid endarterectomy (CEA), atorvastatin reduced TF and TFPI antigen levels in blood (29% and 18% reduction respectively), and TF activity (56% reduction) in plaques removed at the second CEA, as compared with placebo [48]. Further clinical studies demonstrated that 12 weeks of atorvastatin therapy could lead to decreased FVII levels and activity [49, 50], coinciding with a decrease in the serum levels of LDL-cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL-C) and triglycerides [32]. The effect of statin on TF was also evaluated in individuals without dyslipidemia or cardiovascular disease. In a study of experimental endotoxemia, 20 healthy men were randomized to receive either simvastatin (80 mg/d) or placebo for 4 days before intravenous administration of l*ipopolysaccharide*s (LPS). Simvastatin premedication attenuated the increase in monocyte TF expression and reduced the formation of F1+2 in response to LPS without affecting platelet aggregation [54].

The effect of statin therapies on FV [55-57], von Willebrand factor (VWF) [55, 58, 59], and natural anticoagulants [59] has also been reported in several studies [55-59]. Two small clinical studies performed in patients with cardiovascular disease [56, 57] showed that early simvastatin therapy decreased FVa generation and increased FVa inactivation by protein C in blood samples collected from sites of microvascular injury (bleeding-time wounds). Simvastatin and pravastatin reduced VWF levels in patients with hypercholesterolemia after 3 months of therapy. In a clinical trial, 45 patients with unstable angina were randomized to receive either atorvastatin or placebo for 6 weeks. Levels of coagulation markers, such as plasminogen activator levels (tPA), protein C, protein S, FV, FVII and VWF, were measured after 1 week and 6 weeks with treatment and compared with baseline [55]. In the placebo arm, all coagulation markers, except for protein S, increased from baseline to the end of the first week with treatment. At six weeks, protein C and antithrombin remained elevated in patients receiving placebo, when compared to baseline. These parameters did not change substantially in patients who received atorvastatin, suggesting that atorvastatin could regulate the levels of liver-derived coagulation markers [55]. Furthermore, in a randomized trial conducted with 60 patients with acute coronary syndrome, both atorvastatin low (10 mg) and high (80 mg) dose prevented elevation of VWF from baseline to the end of the first week with treatment, when compared to placebo [59]. After two weeks with treatment, levels of VWF were similar between both treatment arms and placebo [59]. Besides the reduction of some coagulation factors, simvastatin [47, 51, 56, 60], pravastatin [58] and atorvastatin [61, 62] therapies were

also shown to reduce the thrombin generation potential and the level of D-dimer [63], both markers of hypercoagulability. In the LIPID study, 9014 patients were randomized to pravastatin 40 mg or placebo for 3 to 36 months after an acute coronary syndrome. After one year of treatment, D-dimer levels in placebo group did not change from baseline (mean change of 1 ng/mL) while D-dimer levels in patients receiving pravastatin decreased significantly (mean change -12ng/mL). The difference between groups in absolute change of D-dimer levels from baseline to the end of the first year of the study was statistically different (mean levels at year 1 were 172ng/mL in placebo arm and 166ng/mL in patients receiving pravastatin) [64].

It is noteworthy that most of these data came from non-randomized studies in which confounding and regression to the mean cannot be ruled out. Stronger evidence that statins can affect coagulation came from two meta-analyses of randomized trials [65, 66]. These meta-analyses demonstrated a significant decrease in the plasma levels of VWF (standardized mean difference [SMD] -0.54, 95 %CI: -0.87 to -0.21) [65] and D-dimer (SMD -0.988 µg/ml, 95% CI: -1.590 to -0.385) [66] following statin therapy. In both studies, the effects on VWF and D-dimer levels were more evident after atorvastatin or simvastatin therapy was commenced, when compared to no statin use. Limitations of the two meta-analyses are the inclusion of heterogeneous patient populations and treatment assignments and, according to funnel plot analyses, the possibility of publication bias.

Evidence of the effect of statin therapy on coagulation factors recently came from the START trial, in which patients with a prior VTE were randomized to rosuvastatin treatment or non-statin [67]. The mean FVIII:C levels decreased 7.2 IU/dL (95% CI: 2.9 to 11.5) in rosuvastatin users from baseline to the end of treatment, while among non-users, no change in FVIII:C was observed (mean difference -0.1; 95% CI: -3.0 to 2.9). Rosuvastatin therapy also decreased the levels of FVII:C (mean change -3.6 IU/dL; 95% CI: -0.2 to -7.1) and FXI:C (mean change -5.9 IU/dL; 95% CI: - 2.7 to -9.0) from baseline to the end of the study. Subgroup analyses revealed that the effect of rosuvastatin on decreasing the levels of coagulation factors was more pronounced in participants with unprovoked VTE or with cardiovascular risk factors [67]. The results are illustrated in Figure 1.

Effect of statins on fibrinolysis

Clinical studies that evaluated the effect of statins on fibrinolysis have presented controversial results. In a study with 46 patients with coronary artery disease randomized to receive either

placebo or fluvastatin, patients receiving fluvastatin had a decrease in tPA and an increase in plasminogen activator inhibitor-1 (PAI-1) activity [68]. Pravastatin decreased tPA [69] and PAI-1 [69, 70] levels by 10-20% among hyperlipidemic patients, accompanied by a decrease in LDL-C levels. Atorvastatin also increased the levels of plasmin-antiplasmin complex (PAP) by 50%, decreased PAI-1 activity by 34% and decreased platelet-dependent thrombin generation by 48% in hyperlipidemic patients [71]. A shift to a pro-fibrinolytic profile related to statin therapy was also observed in sets of patients with coronary disease undergoing coronary artery bypass grafting [72] in hyperlipidemic women [73] in patients with essential hypertension [74] and in patients with chronic obstructive pulmonary disease (COPD) [75]. In healthy individuals, a large cohort study showed that participants using statins had lower levels of D-dimer and FVIII and higher levels of fibrinogen and PAI-1, in comparison with non-statin users [76]. These results, however, seem contradictory since lower D-dimer and FVIII suggest a decrease in hemostatic activity, while higher fibrinogen and PAI-1 levels suggest hypercoagulability.

The strongest evidence for the effect of statin therapy on fibrinolysis came from a recent meta-analysis of RCTs, where the pooled analysis revealed that statin therapy reduced plasma levels of PAI-1compared with no statin therapy [77]. Although these findings suggest that statins stimulate fibrinolysis, result could also be plagued by publication bias. No additional evidence from RCTs of the effect of statin therapy on fibrinolysis has been published recently.

The importance of the lipid-lowering effect on hemostasis

Basic research studies have consistently shown that the effect of statins on hemostasis is independent of the drug-related lipid-lowering effect. Studies *in vitro* have confirmed that statins can directly inhibit the Rho/Rho-kinase pathway and consequently NF-kB activity and TF expression in monocytes and endothelial cells [39, 40, 45, 46]. Such mechanisms of action are different from that involved in the lipid-lowering process [22].

Additionally, studies in hyperlipidemic mice and rabbits have shown that statins can decrease the expression of TF in atherosclerotic lesions and monocytes independently of the decrease in plasma lipids [43, 44]. The effect of statins on increasing platelet-derived NO release and, in turn, decreasing platelet activity was also shown to be independent of the lipid-lowering effect [26, 34].

In the clinical data, the distinction between a potential direct effect of statin and the lipid-lowering effect on hemostasis is less clear. Most of the studies were conducted in patients with dyslipidemia, and although studies in individuals without dyslipidemia have also demonstrated an effect of statin therapy on hemostasis [54, 67, 76], it is not possible to rule out that the observed effect was due to decreased serum levels of lipids, since many lipoproteins are related to coagulation activity [29].

THE EFFECT OF STATINS ON THE RISK OF VENOUS THROMBOEMBOLISM

Evidences from basic research studies

Data on the effect of statins on *in vivo* venous thrombosis are limited to few studies. In a mouse model of venous thrombosis, mice treated with high dose atorvastatin or rosuvastatin displayed 25% reduction in venous thrombus mass and accelerated thrombus resolution, compared to control animals treated with saline [78]. The reduction in thrombus formation observed in statin treated mice was accompanied by several changes in platelet activity, fibrinolysis and coagulation factors [78]. More recently, a study in a murine model of hyperlipidemic *APOE* knock-out mice showed that mice treated with high doses of rosuvastatin had a 12% decrease in venous thrombus formation compared to controls [79]. The effect of statin on reducing thrombus formation occurred independently of the effect on lipid levels [79]. Results from these animal studies confirm a biological effect of statins on venous thrombus formation. However, supratherapeutic doses of statins were used in the studies, and doses currently used in the clinical scenario were not tested.

Statins to prevent first venous thromboembolism

The first study to suggest that statin therapy was associated with a reduced risk of VTE was conducted in a selected population of postmenopausal women [80]. Since then, several population-based studies have aimed to evaluate the effectiveness of statin therapy on VTE prevention. Most of them are observational studies and were performed in selected populations of patients with cardiovascular disease [81].

The only RCT supporting the effect of statins on decreasing VTE risk was published in 2009. In the JUPITER trial [13] 17,802 apparently healthy individuals, with normal cholesterol levels, were randomized to receive rosuvastatin at 20 mg per day or placebo and were followed

for a median period of 1.9 years. Rosuvastatin reduced the incidence of VTE by 40% (hazard ratio: 0.57; 95% CI: 0.37 to 0.86) compared to placebo.

Since the results of the JUPITER trial were published, additional evidence of the association between statin therapy and reduced VTE risk came from at least four meta-analyses of observational studies and clinical trials. A meta-analysis of RCTs, published in 2012, compared statin use with no statin (20 trials), and also high dose versus standard dose statin (7 trials) [82]. Results from the pooled analysis did not confirm a risk reduction of VTE by statin treatment. Albeit the confidence intervals were wide, the point estimates suggest that rosuvastatin, which is the statin that is most related with halting/regression of atherosclerosis, dyslipidemia, and inflammation [23, 83, 84] also provides the largest risk reductions for the occurrence of VTE. The results are illustrated in Figure 2.

A meta-analysis of 36 studies (13 cohort studies and 23 RCTs) of statin treatment versus no-statin (placebo or control) was published in 2017 [85]. When evaluating RCTs, the meta-analysis demonstrated a pooled relative risk for VTE of 0.85 (95% CI: 0.73 to 0.99) when statin was compared with a non-statin treatment. The lowest relative risk for VTE was observed in individuals who were randomized to rosuvastatin (relative risk, 0.57; 95% CI: 0.42 to 0.75). Among observational studies, the pooled relative risk for VTE was 0.75 (95% CI: 0.65 to 0.87). A stronger effect, i.e. a 54% risk reduction, was observed in populations with pre-existing disease or at high VTE risk, in contrast with a 14% VTE risk reduction in studies that recruited participants from the general population. These results are in line with those from a previous meta-analyses of 7 observational studies that demonstrated that statin therapy was associated with a 40% decrease in VTE risk, as compared to no statin use [86].

Although the results from the JUPITER trial and the meta-analyses of observational studies and RCTs might encourage the use of statins for VTE prevention, these data must be interpreted with caution for several reasons. First, meta-analyses of observational studies have limitations that are related to potential biases in the studies. Some examples: 1) the underlying disease severity in patients selected for statin therapy may be different, even milder than that in patients for whom statin was not indicated (healthy user effect) [87], 2) the inclusion of prevalent statin users results in missing the events that occurred in statin users before the inclusion, early after starting treatment (survivor bias), and 3) observational studies are not able to control for those patients who adhere or not to statin treatment (adherence bias) [88]. Second, the meta-analyses of RCTs included non-published data and were influenced by the results of the JUPITER trial. Finally, the results on VTE from the JUPITER trial came from the analysis

of a secondary endpoint in the trial, in which the primary outcome was the occurrence of a first major cardiovascular event. These results may have run into the statistical problem of small numbers, since in the statin treatment arm only 34 participants developed a venous thrombotic event, and randomness, or a type I error, may have influenced this result. Replication of the results from the JUPITER trial is important, therefore, to confirm that statin therapy reduces the risk of VTE.

Statins to prevent recurrent venous thromboembolism

Over the last years, clinical studies have aimed to determine the role of statins in preventing VTE recurrence, with heterogeneous results. Cohort studies that observed a protective effect of statin therapy on the risk of recurrent VTE have reported a risk reduction that varies from 26 to 38% [89-91]. A recently published meta-analysis of observational cohort studies demonstrated that the pooled relative risk for VTE recurrence was 0.73 (95% CI: 0.68-0.79) in statin users in comparison with no use [92]. Statin therapy reduced the risk for recurrent PE by 25% and the risk of recurrent DVT by 34%, in comparison with no statin [92].

A more detailed analysis of the cohort studies, however, reveals that the results may have been affected by the inclusion of long-term (or prevalent) statin users. Long-term statin users may be healthier than non-users and less susceptible to VTE recurrence, which results in survivor bias and healthy user effect [93]. As an example, in a large cohort study, Schmidt et al [90] reported a 29% decrease (hazard ratio, 95% CI: 0.58–0.87) in the risk of recurrent VTE in long-term statin users as compared with non-users, while among new users the relative risk reduction dropped to a non-significant 17% (0.45–1.52). Therefore, a biased association between statin use and prevention of VTE recurrence cannot be ruled out.

SUMMARY AND CONCLUSIONS

Several studies have consistently demonstrated that statins affect hemostasis, particularly by downregulating TF expression on endothelium cells and monocytes through a direct, lipid-lowering independent effect on transcriptional activation of *TF* gene. This drug effect is, thus far, the best described mechanism by which statins may reduce thrombus formation. Additionally, statins were also shown to impair platelet activation, decrease multiple coagulation factors and increase fibrinolysis. A summary with documented biological influence of statins on the different phases of hemostasis is provided in Figure 3.

Recent data from randomized trials pointed to a potential effect of statins on decreasing the levels of coagulation factors. Although these studies have provided stronger evidence that statins may affect coagulation in humans, the exact mechanisms by which this happens are not elucidated. Furthermore, the question whether the observed effect of statin therapy on coagulation is sufficient to prevent VTE is still open.

From the clinical perspective, recent meta-analyses of randomized trials have demonstrated that rosuvastatin therapy may reduce the risk of incident VTE, reinforcing the results reported by the JUPITER trial. However, a direct relation between statin therapy and recurrent VTE risk cannot be assumed from the current clinical data, since the results still need to be replicated. The lack of randomized data also provides uncertainty about the effect of statin therapy on reducing the risk of recurrent VTE.

Clinical trials are currently underway for assessing whether rosuvastatin (NCT01524653) or simvastatim (NCT02285738) therapy would reduce the risk of incident VTE in patients with cancer. The two studies have surrogate biomarkers of hypercoagulability as a primary endpoints, D-dimer levels and soluble P-selectin levels respectively. A third study aims to evaluate whether rosuvastatin can reduce post-thrombotic syndrome in VTE patients (NCT02679664). A fourth trial (NCT02331095) aims to determine whether atorvastatin plus anticoagulation can reduce the thrombin generation potential in patients with acute VTE compared to anticoagulation alone. The results of these trials will contribute to the body of evidence on the association between statin therapy and hemostasis. However, the question whether statin therapy is effective to prevent incident or recurrent VTE will probably remain unanswered for the following years, since no studies on this issue have been started yet.

In conclusion, findings from clinical studies point to a potential effect of statins on decreasing the levels of coagulation factors and reducing the risk for VTE. These effects were mainly attributed to rosuvastatin, which is the most potent LDL-C and atherosclerosis reducing statin. However, stronger evidence on efficacy of statins in preventing VTE, in particular the recurrent events, is still lacking. Recent data showing the effect of rosuvastatin on levels of coagulation factors in patients with prior VTE provides a solid basis for interventional studies necessary to establish the efficacy of statins on reducing the risk of incident of recurrent VTE.

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Legends to Figures

Figure 1 Effects of rosuvastatin on coagulation factors VIII, XI, VII, von Willebrand Factor and D-dimer according to (A) VTE classification (unprovoked and provoked) and (B) the presence/absence of cardiovascular risk factors. Results from the START (STAtins Reduce Thrombophilia) trial.[63]

CI confidence interval. CV cardiovascular

Figure 2 Effect of rosuvastatin, pravastatin, atorvastatin and simvastatin on VTE [77,78].

[a] excluding results from the JUPITER (The Justification for the Use of *Statins* in Prevention: an Intervention *Trial* Evaluating Rosuvastatin) trial. CI confidence interval.

Figure 3 Biologically documented effects of statins on distinct phases of the process of hemostasis.

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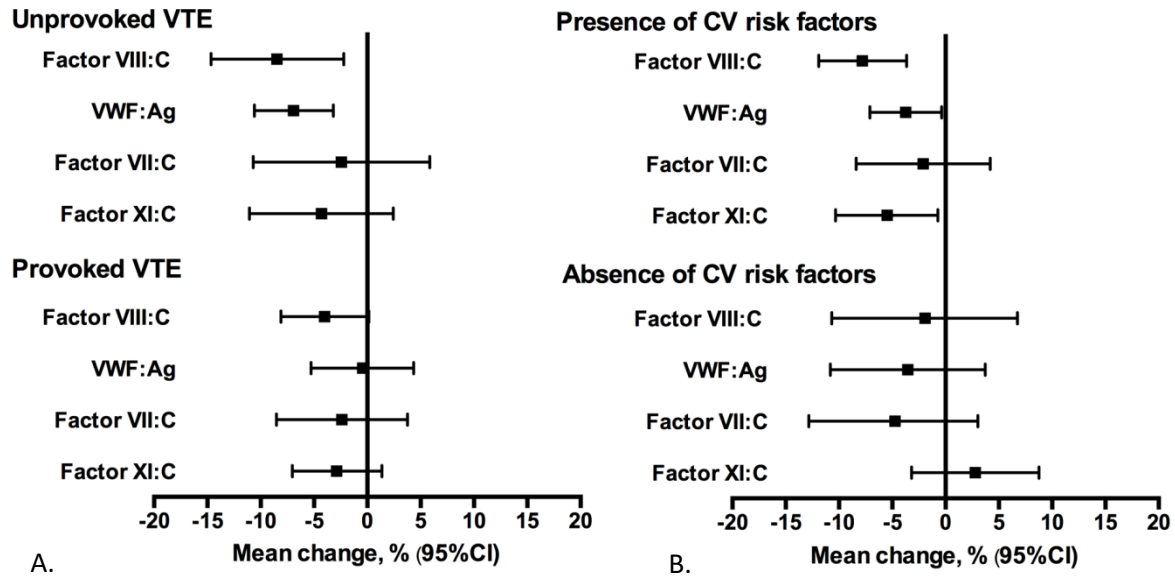

Figure 1 Effects of rosuvastatin on coagulation factors VIII, XI, VII, von Willebrand Factor and D-dimer according to (A) VTE classification (unprovoked and provoked) and (B) the presence/absence of cardiovascular risk factors. Results from the START (STAtins Reduce Thrombophilia) trial.[63]

CI confidence interval. CV cardiovascular

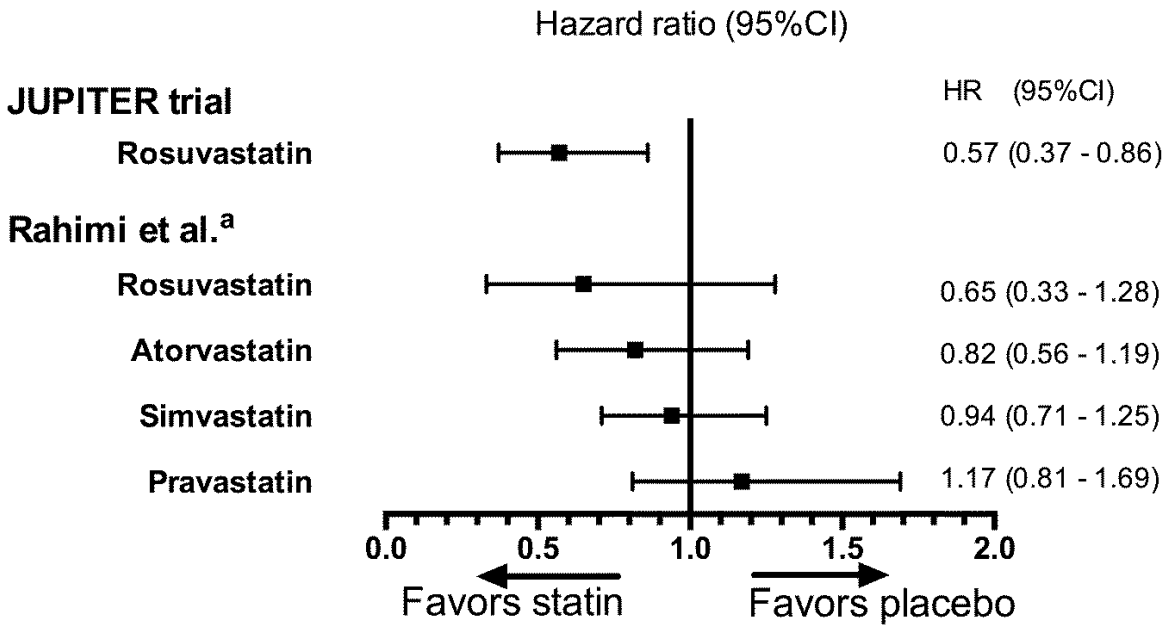

Figure 2 Effect of rosuvastatin, pravastatin, atorvastatin and simvastatin on VTE risk [77,78]. CI confidence interval. [a] excluding results from the JUPITER (The Justification for the Use of *Statins* in Prevention: an Intervention *Trial* Evaluating Rosuvastatin) trial.

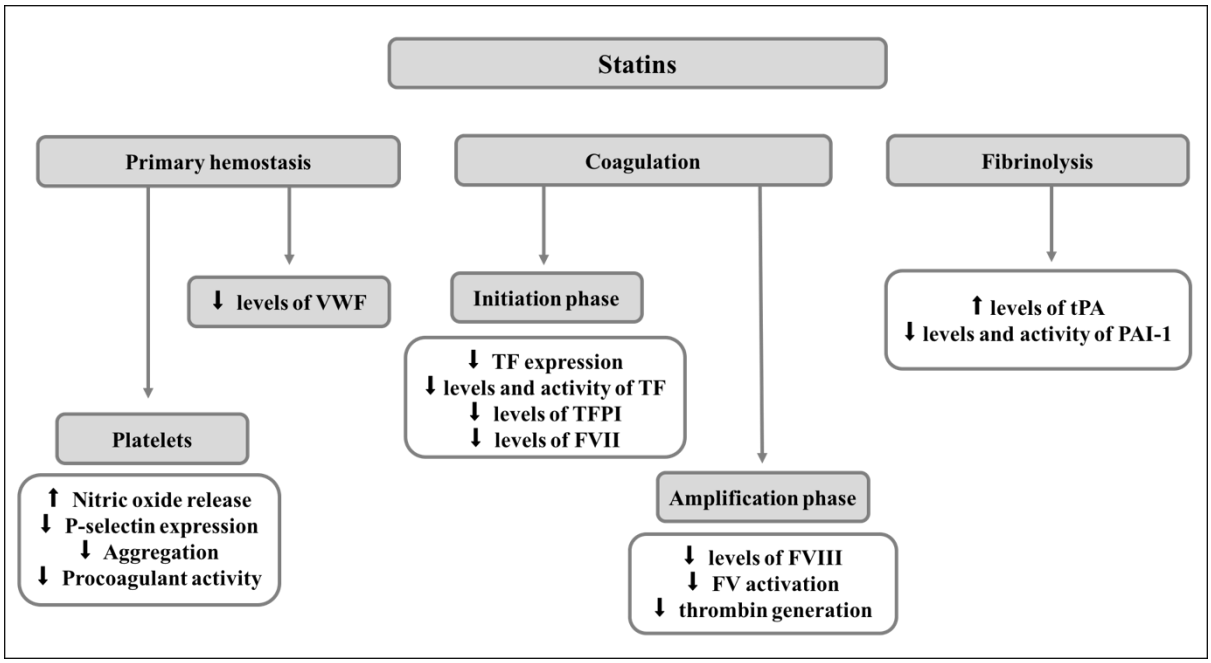

Figure 3 Biologically documented effects of statins on distinct phases of the process of hemostasis. Data from basic research and clinical studies have suggested that statins can decrease platelet activity, downregulate both initiation and amplification phases of coagulation and stimulate fibrinolysis. So far, the best known mechanism by which statins may impair hemostasis is the direct downregulation of TF expression on endothelial cells and monocytes through a direct inhibitory effect on transcriptional activation of *TF* gene. VWF von Willebrand factor, TF tissue factor, TFPI tissue factor pathway inhibitor, FVII factor VII, FVIII factor VIII, FV factor V, tPA tissue plasminogen activator levels, PAI-1 plasminogen activator inhibitor-1

Chapter 3

Rosuvastatin use reduces thrombin generation potential in patients with venous thromboembolism: a randomized controlled trial

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Summary

Background: Statin therapy could form an alternative prophylactic treatment for venous thromboembolism (VTE) if statins are proven to downregulate hemostasis and prevent recurrent VTE, without increasing bleeding risk. **Objectives**: The STAtins Reduce Thrombophilia (START) trial investigated whether statin affects coagulation in patients with prior VTE. **Patients/Methods:** After anticoagulation withdrawal, patients were randomized to rosuvastatin 20mg/day for 4 weeks or no intervention. Plasma samples taken at baseline and at the end of the study were analyzed employing thrombin generation assay. **Results and Conclusions:** The study comprised 126 rosuvastatin users and 119 non-users. Mean age was 58 years, 61% were men, 49% had unprovoked VTE and 75% had cardiovascular (CV) risk factors. Endogenous thrombin potential (ETP) increased from baseline to end of study in non-statin users (mean 97.22nM*min; 95%CI 40.92 to 153.53) and decreased in rosuvastatin users (mean -24.94nM*min; 95%CI -71.81 to 21.93). The mean difference in ETP change between treatments was -120.24nM*min (95%CI -192.97 to -47.51), yielding a 10.4% ETP reduction by rosuvastatin. Thrombin peak increased in both non-statin (mean 20.69nM; 95%CI 9.80 to 31.58) and rosuvastatin users (mean 8.41nM; 95%CI -0.86 to 17.69). The mean difference in peak change between treatments was -11.88nM (95%CI -26.11 to 2.35), yielding a 5% peak reduction by rosuvastatin. Other thrombin generation parameters did not change substantially. The reduction in ETP and peak by rosuvastatin was more pronounced in the subgroups of participants with CV risk factors and with unprovoked VTE. We conclude that rosuvastatin reduces thrombin generation potential in patients who had VTE.

INTRODUCTION

Venous thromboembolism (VTE) contributes significantly to the global disease burden, and therefore, preventive measures and adequate treatment are warranted [1]. While anticoagulation is the treatment of choice for preventing VTE episodes [2], bleeding complications are a major concern and may lead to treatment avoidance in many cases [3]. The latter underscores the need for alternative treatment options for VTE prophylaxis. Statins may provide a promising alternative treatment for thromboprophylaxis because these drugs are alleged to have pleiotropic effects on hemostasis and may reduce VTE risk, although strong clinical evidence supporting these effects is still scarce [4].

Previous studies have reported that statins reduce the risk of first VTE by 14 to 54% [5-9] and the risk of recurrent VTE by 27% [10]. However, healthy user effects, survivor bias and adherence bias could have influenced these results [11]. Moreover, the strongest evidence on the effect of rosuvastatin on first VTE still comes from one randomized clinical trial [8], while no randomized trials have investigated the impact of statin therapy on the risk of recurrent VTE. Despite the need for additional randomized trials, the lack of knowledge on the mechanisms that are at the basis of the supposed causal association between statin use and a reduced risk of VTE may discourage the conduction of such interventional studies.

Recently, we have shown in the STAtins Reduce Thrombophilia (START) trial that one month of treatment with rosuvastatin at 20 mg/day led to an improved coagulation profile as compared with non-statin users in patients with prior VTE, most notably by reducing factor VIII plasma levels [12]. These observations from the START trial were the first randomized evidence indicating that rosuvastatin reduces coagulation factor levels in patients with prior VTE and confirmed similar findings previously observed for other statins [13-15]. To better understand the effect of rosuvastatin on individual prothrombotic profiles, we evaluated here whether rosuvastatin could interfere with thrombin generation, a global coagulation test that reflects not only the coagulation potential [16-18] of an individual but also predicts the risk of a first and recurrent VTE [19-21].

METHODS

Trial design

The START trial is a randomized, open label, controlled, clinical trial conducted in the Netherlands that investigated whether the coagulation profile in persons with a history of VTE

and not taking anticoagulants is improved when using rosuvastatin. Details of the study design are described elsewhere [12]. The study was undertaken in accordance with the Declaration of Helsinki and International Conference on Harmonization guidelines for Good Clinical Practice. All participants gave written informed consent prior to participation. START was approved by the Medical Ethics Committee of the Leiden University Medical Center, Leiden, the Netherlands, and is registered at www.clinicaltrials.gov as NCT01613794.

Participants

Participants were recruited at three anticoagulation clinics in the Netherlands (Leiden, Hoofddorp and Rotterdam) between June 2012 and January 2017. Individuals aged 18 years or older, with confirmed symptomatic proximal deep vein thrombosis or pulmonary embolism, were eligible for inclusion in the study if their physicians approved the cessation of oral anticoagulant treatment. Exclusion criteria were: current use of statins or lipid lowering drugs, or any contraindications for rosuvastatin at 20 mg/day use as provided by the instruction leaflet of the manufacturer.

Intervention

Informed consent was obtained at the study baseline visit. The study baseline visit was defined at the time of the last regular visit of the patient to the anticoagulation clinic. After informed consent, participants were screened on acquired risk factors for thrombosis through a questionnaire and tested for liver and kidney functions. At randomization, participants were allocated to receive either rosuvastatin at 20 mg/day or no study medication. The random allocation sequence was implemented by central telephone and the sequence was concealed until interventions were assigned.

The duration of the study was 28 days, based on the consideration that some small, non-randomized, studies showed beneficial effects of statins on the coagulation system as early as after a three-day statin administration [12].

Measurements

Patients stopped using their vitamin K antagonist for one month (to allow the anticoagulant drugs to wear off) after which a blood sample was drawn at randomization visit and at the end

of the study period (*i.e.* 28 days later). All blood drawings were performed between 08:00 and 15:00. Blood was collected in tubes containing sodium citrate (3.2%) and centrifuged within 3 hours of venepuncture at 2500 g for 15 minutes at 18°C, after which plasma was immediately stored at -80°C. Laboratory technicians, who were unaware of which participants were rosuvastatin-users, performed the assays after all participants had completed the study.

The thrombin generation potential was assessed by means of the thrombin generation assay (TGA), which is a global coagulation test that reproduces the kinetics of thrombin formation [22, 23], using the Calibrated Automated Thrombogram® (Diagnostica Stago, France) according to the manufacturer's specifications [24]. Briefly, plasma samples were mixed with the assay reagents (tissue factor and phospholipids) and tested in duplicate. As internal control, normal pooled plasma, derived from citrated plasma from 64 healthy men and women not taking oral contraceptives, was tested in each assay and a thrombin calibrator was used for each plasma duplicate. The fluorescent signal representing generated thrombin was monitored in a Fluoroskan Ascent fluorometer (Thermo Scientific, USA) and the parameters were calculated with the Thrombinoscope software (Thrombinoscope BV). The TGA parameters determined were: endogenous thrombin potential (ETP), thrombin peak, time to peak, lag time and velocity index. ETP, or area under curve, represents the total amount of thrombin generated over time. The thrombin peak represents the maximum amount of thrombin that can be generated. Time to peak indicates the time required to reach the maximum amount of thrombin formed. The lag time measures the length of time between the start of the assay (addition of triggers) and the initiation of thrombin generation. Velocity index is defined as [peak height/(time to peak – lag time)] and represents the rate of thrombin generation [20].

Outcomes

Because the ETP and thrombin peak have been consistently associated with VTE risk [25-31], the primary endpoints were defined as the difference in change in ETP and thrombin peak from baseline to the end of the study between rosuvastain users and non-users. The differences in the change in lag time, time to peak or velocity index were considered secondary endpoints. The study was originally powered on factor VIII [12]. Nevertheless, we observed in the non-statin users that the mean ETP was 1245 mM*min (SD 322) at randomization. Therefore, we a-priori expected to find a powered mean difference of at least 76 nM*min or 6% decrease between participants at end of study with a 2-sided alpha of 0.05 and 80% power.

Statistical analysis

Final analyses were done by modified intention-to-treat since there were post-randomization exclusions. The mean levels and 95% confidence intervals (95% CI) of all pre-specified thrombin generation assay parameters were calculated at the time of randomization (baseline), at the end of the study period and for the change between these two time periods within each treatment group. We also calculated the percentage of change within groups by subtracting the baseline value from the end of the study value, dividing it by the baseline value, and multiplying the result by 100%.

To determine the between-groups difference in thrombin generation parameters, the mean difference in change and 95% CI between treatment groups (rosuvastatin users vs. non-users) was calculated by means of linear regression methods. We performed both unadjusted and age and sex adjusted analysis, because more men were randomized to non-rosuvastatin use and non-rosuvastatin users were slightly older than those who were randomized to rosuvastatin. In a predefined sensitivity analysis, we excluded all participants who reported signs or symptoms of an infection during the study, as infections may affect thrombin generation [32, 33].

Next, we plotted the end of study-expected and the end of study-observed thrombin generation among rosuvastatin users. To do so, we assumed that if patients assigned to rosuvastatin had not received the drug, they would have had the same change in thrombin generation as those assigned to non-statin treatment. Thus, the expected end of study-thrombin generation among rosuvastatin users was estimated by adding the mean change in thrombin values (at each time point of the thrombin generation curve) within non-statin users to the corresponding baseline-thrombin value in rosuvastatin users.

Additionally, we performed a subgroup analysis according to the following potential or established prognostic determinants of recurrent venous thrombosis: male/female sex, unprovoked/provoked first event, deep vein thrombosis or pulmonary embolism, and presence or absence of self-reported cardiovascular risk factors.

A post-hoc analysis was performed to investigate whether the coagulation factors VIII, VII, XI and D-dimer was associated with the effect of rosuvastatin on ETP. For this purpose, we performed linear regression with those coagulation factors entering as independent

variables, along with the randomization groups and sex and age, and ETP entering as dependent variables. All analyses were performed with SPSS version 23.0 (SPSS Inc, Chicago, Ill).

RESULTS

Study population

A total of 255 patients were randomized between December 2012 and December 2016, 131 were assigned to receive rosuvastatin and 124 were allocated to non-statin treatment. Figure 1 shows the trial profile. Two participants allocated to rosuvastatin treatment did not start treatment and another six randomized, three in each study arm, did not complete the study. The thrombin generation assay could not be performed in two patients because of technical issues, who both had been assigned to non-statin treatment. Table 1 presents baseline characteristics in the 245 participants who completed the study; 126 assigned to rosuvastatin and 119 assigned to non-rosuvastatin treatment.

Non-rosuvastatin users were slightly older than rosuvastatin users, mean ages were 58.4 years (range 21 to 80) and 56.8 years (range 19 to 82), respectively. More men were assigned to non-statin treatment, the proportion of men was 54% among rosuvastatin users and 69% among non-users. Other reported exposures, such as body mass index (BMI), type and classification of venous thromboembolism, and presence of cardiovascular risk factors, were balanced at baseline. (Table 1)

Outcomes

Results of all measured thrombin generation parameters are shown in Table 2. ETP increased 7.8% from baseline to end of study in non-statin users (mean change, or intraindividual variability, within non-users 97.22 nM*min; 95%CI 40.92 to 153.53) and decreased 1.9% from baseline to end of study in rosuvastatin users (mean change in rosuvastatin users: -24.94 nM*min; 95%CI -71.81 to 21.93). The mean difference between treatments, after adjustment for age and sex, was -120.24 nM*min (95%CI -192.97 to -47.51). After the exclusion of patients who reported an infection at the end of study, as pre-specified by the study protocol, the age and sex- adjusted mean difference in ETP between treatments was -129.39 nM*min (95%CI -202.29 to -56.49). The mean difference between treatments yielded a treatment effect

of 10.4% (95%CI 4.5 to 16.2%) reduction in ETP by rosuvastatin, when compared with non-statin treatment (Figure 2).

While the thrombin peak increased in both rosuvastatin and non-statin users from baseline to the end of the study, the percentage change was higher for non-users (7.6%) relative to the rosuvastatin users (2.9%). The mean change in thrombin peak was 20.69 nM (95%CI -9.80 to 31.58) for the non-users and 8.41 nM (95%CI -0.86 to 17.69) for the rosuvastatin users, which resulted in a mean difference in change between both treatments, adjusted for age and sex, of -11.88 nM (95%CI -26.11 to 2.35). The mean difference between the treatments yielded a treatment effect of 5.0% (95%CI -0.2 to 10.2%) reduction in thrombin peak by rosuvastatin, when compared with non-statin treatment (Figure 2).

The time to peak decreased 6.4% from baseline to the end of the study in rosuvastatin users (mean change -0.28 min; 95%CI -0.35 to -0.21), and 1.5% in non-statin users (mean change -0.07 min; 95%CI -0.23 to 0.09). The mean difference in these changes between treatments was -0.21 min (95%CI -0.38 to -0.03), which was equivalent to a treatment effect of 4.8% (95%CI 0.9 to 8.5) reduction in time to peak by rosuvastatin, when compared with non-statin treatment (Figure 2). The results were not materially affected by excluding the 8 participants who reported an infection. Changes in lag time and velocity index were not different between treatments (Figure 2).

Figure 3 illustrates the difference between expected and observed thrombin generation in rosuvastatin users by the end of the study.

Supplementary tables 1 to 5 show all measures of thrombin generation parameters in the subgroups of sex, unprovoked or provoked first VTE, deep vein thrombosis or pulmonary embolism, and presence or absence of self-reported cardiovascular risk factors. These subgroup analyses revealed that the decrease in ETP and thrombin peak by rosuvastatin was more pronounced in patients with unprovoked venous thrombosis, pulmonary embolism or cardiovascular risk factors, than in those with provoked venous thrombosis, deep vein thrombosis or without cardiovascular risk factors, respectively (Figure 4). A relative decrease in ETP following rosuvastatin use was also more pronounced in men than in women, while the effects of rosuvastatin on thrombin peak were similar between sexes. Subgroup analysis of the effect of rosuvastatin on other thrombin generation parameters revealed similar results as in the main analysis.

As we have recently reported that treatment with rosuvastatin led to a decrease in the levels of D-dimer and coagulation factors VIII, VII and XI as compared with non-statin in START, we performed a post-hoc analysis to evaluate whether the observed effect of rosuvastatin on thrombin generation could be explained by the levels of these factors at the end of the study. As shown in table 3, the effect of rosuvastatin on thrombin generation was reduced by 33% with factor VII, but not by the other coagulation factors/ D-dimer.

DISCUSSION

In this randomized clinical trial (START), we have shown that treatment with rosuvastatin leads to a relative reduction in thrombin generation potential, decreasing the ETP by 10.4% (adjusted mean difference between treatments -129.39 nM*min) and decreasing the thrombin peak by 5% (adjusted mean difference between treatments -13.69 nM), in comparison with non-statin treatment. Our results confirm previous clinical studies that also demonstrated that statin therapy, either with rosuvastatin [30], simvastatin [34], atorvastatin [35, 36] or cerivastatin [37], affects coagulation factors and thrombin generation.

Additionally, these findings are consistent with previous results from the START trial, in which rosuvastatin treatment was shown to decrease the plasma factor VIII levels by 6% (adjusted mean difference in change between treatments -8.2 IU/dL; 95%CI -13.6 to -2.9), those of FXI by 4% (adjusted mean difference in change between treatments -4.9 IU/dL; 95%CI 9.9 to -0.1), coinciding with a decrease in D-dimer by 3% and factor VII levels by 4% [12]. The results from the START trial point to the same direction of an effect of rosuvastatin on the individual coagulation profile, but the observed decrease in thrombin generation potential was only partially mediated by factor VII and by D-dimer, factor VIII or XI. Since thrombin generation is a product of a synergic combination of multiple coagulation factors [38], [18], it is possible that the mechanism behind the effect of rosuvastatin on decreasing thrombin generation potential relies on the reduction of several coagulation factors, some of them not measured in the START trial. Whether this effect of rosuvastatin on changing the coagulation profile has clinical significance in terms of reducing VTE risk deserves to be addressed in clinical trials aimed to evaluate this question. However, it is possible to speculate on a potential clinical impact of statins on VTE risk if the current findings are evaluated in the light of previous studies. Studies on thrombin generation and VTE risk have demonstrated that both the ETP and thrombin peak are associated with a first VTE [16, 28, 29, 31] and can predict the risk of recurrent VTE [25-27, 30]. A cohort study of 188 patients with VTE [28] described that the

risk of recurrent VTE increased by 25% per 100 nM*min increase in ETP (hazard ratio 1.25 per 100 nM*min increase, 95% CI 1.01 to 1.55). The Austrian Study on Recurrent Venous Thromboembolism (AUREC), which is a cohort study with patients with an unprovoked first episode of VTE, showed that the risk of recurrent VTE increased by 1.4% for each 1% increase in ETP (hazard ratio 1.014 per 1% increase in ETP, 95% CI 1.0 to 1.03; P 0.06) [25]. Another study derived from the AUREC cohort showed that the relative risk of recurrent VTE increased by 4% (relative risk [RR] 1.04; 95% CI, 1.02 to 1.06) for each 10nM increase in thrombin peak [27]. The Vienna Cancer and Thrombosis Study (CATS), a prospective cohort study of patients with cancer, demonstrated that patients who developed VTE had 10% higher thrombin peak at baseline than those without VTE events (peak values 556nM; 95% CI 432 to 677 and 499nM; 95%CI 360 to 603, respectively) [39]. Considering ETP and thrombin peak as surrogate markers of recurrent VTE risk, as described in the aforementioned trials, our results suggest that rosuvastatin has the potential to decrease the risk of recurrent VTE by 14 to 25%. Interestingly, a meta-analysis of observational studies reported that statins reduced the overall risk of recurrent VTE by 27% (RR 0.73; 95% CI, 0.68 to 0.79) [10]. Therefore, our finding that statins are capable of modulating the pro-thrombotic profile in patients after a first VTE episode could be interpreted as statins having the potential to decrease the risk of recurrence.

We also observed that the relative treatment effect of rosuvastatin on ETP was mainly driven by the absence of an increase in this parameter among rosuvastatin users, in contrast to a significant increase in ETP in patients not using statins. This is consistent with a previous observation from this trial demonstrating that the difference in D-dimer levels between both treatment groups was driven by the absence of an increase in D-dimer following rosuvastatin use [12]. As both thrombin generation and D-dimer are markers of hypercoagulability [25, 26], the current results provide further evidence that rosuvastatin may prevent a rebound phenomenon; *i.e.,* a shift to a more procoagulant profile along with increased risk of a recurrence of VTE after the sudden withdrawal of anticoagulant treatment [40, 41]. Preventing such a rebound hypercoagulability may be further beneficial to patients with previous VTE in whom anticoagulation is withdrawn.

It is worth noting that the decrease in ETP and thrombin peak appeared strongest in participants with unprovoked VTE and in those with cardiovascular risk factors. This potential benefit for patients who had unprovoked VTE is interesting because these patients are at high risk of recurrent VTE [2], and anticoagulants may not be prescribed if a patient is considered to be at high risk of anticoagulation-related bleeding [42]. Secondary prevention with statin

therapy may be a convenient alternative treatment, as statins do not increase the risk of bleeding complications [43]. In addition, a benefit among patients with cardiovascular risk factors is noteworthy because most of these patients are already likely to receive statins [44]. Therefore, the possibility of using one single drug to prevent both cardiovascular diseases and VTE could diminish the medication burden associated with the use of several classes of drugs and decrease the risk of adverse effects, thus increasing the changes of treatment efficacy [45].

Although our results point to a decrease in thrombin generation potential by rosuvastatin, not all thrombin generation parameters were modified after the treatment. The lag time and velocity index did not change substantially, while the time to peak decreased in rosuvastatin users, in comparison with non-statin users. Despite the fact that a reduced time to peak may indicate a hypercoagulable state [20], the real significance of this parameter is not known, since it is not associated with the risk of VTE. Conversely, as time to peak is calculated based on the thrombin values, a shortened time to peak may be explained by a relative reduction in ETP and thrombin peak [46]; a similar phenomenon was reported in a previous study, wherein a protraction of the thrombin generation curve lengthened the time to peak [47].

There are some aspects in this study that need to be highlighted. First, the trial was not blind to participants and physicians involved; however, it was considered unlikely that knowledge on the treatment could affect a laboratory surrogate outcome. Second, we previously noticed that the distribution of sex and age after randomization was different between the groups, for which we *a-priori* decided to adjust the analysis for these potential confounding factors [12]. These adjustments did not influence our results. Third, we decided *a-priori* to perform a sensitivity analysis excluding participants who developed an infection during the follow-up due to the possibility of an acute phase reaction affecting the thrombin generation potential, which did not materially change the results. Fourth, although the results from our subgroup analyses suggest that statins may have the strongest potential to decrease thrombin potential in individuals with CV risk factors or unprovoked VTE, these subgroup analyses must be handled with caution as the study was not designed or powered to analyze differences in subgroups [48]. Finally, the assessment of thrombin generation potential is dependent on the assay conditions, which vary according to different laboratory protocols and may affect the clinical interpretation of the results [49]. Besides the potential limitations, the START trial evaluated the effect of rosuvastatin on six coagulation parameters related with the risk of VTE: VWF, factors VIII, VII, XI, D-dimer, ETP and thrombin peak. The values of all parameters were consistently pointing towards a decreased level with rosuvastatin treatment,

as compared with no statin. Altogether, these results confirm that rosuvastatin is capable of affecting several components of coagulation and modifying the coagulation profile of patients with a prior VTE.

We conclude that rosuvastatin use of 20mg/day improves the coagulation profile in patients with VTE by reducing the thrombin generation potential after anticoagulation withdrawal. These results of the START trial suggest that statin therapy might be beneficial in patients at risk of recurrent VTE and provide a clinical rationale for the conduction of a randomized controlled trial to evaluate the effectiveness of rosuvastatin in decreasing the risk of recurrent VTE.

Addendum

F.A.Orsi performed the statistical analyses and drafted the manuscript; J.S. Biedermann performed the statistical analyses and revised the manuscript; M.J.H.A. Kruip, F.J. van der Meer, F.R. Rosendaal, A. van Hylckama Vlieg and F.W.G. Leebeek revised the manuscript; M.H.A. Bos was responsible for the laboratory analyses and revised the manuscript; S.C. Cannegieter designed the analyses and revised the manuscript and W.M. Lijfering was responsible for the START study concept, designed the analyses and revised the manuscript.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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Legends to Figures and Tables

Figure 1. Trial profile. study enrolment, randomization, follow-up and reasons for withdrawal (*one participant admitted to hospital with a diagnosis of acute asthma exacerbation)

Table 1. Baseline characteristics of participants

Table 2. Effects of rosuvastatin on measures of thrombin generation parameters

Table 3. Mean difference in change in thrombin generation parameters between roasuvastatin users and non-users (reference) adjusted for coagulation factors

Figure 2. Relative effect of rosuvastatin treatment on thrombin generation. This figure illustrates the changes in endogenous thrombin potential, from baseline to the end of treatment, compared between rosuvastatin users versus non-statin users.

Figure 3. Thrombin generation curves (A) mean values of thrombin generation over time in non-statin users at baseline and at the end of study. (B) mean values of thrombin generation over time in rosuvastatin users at baseline and at the end of study. (C) expected mean thrombin generation values (if rosuvastatin would not have a treatment effect on thrombin generation) and observed mean thrombin generation values by the end of study in patients receiving rosuvastatin.

Figure 4. Relative effect of rosuvastatin treatment on thrombin generation potential by subgroups. The relative effect of rosuvastatin treatment on endogenous thrombin potential (A) and on thrombin peak (B) in pre-specified subgroups: sex (female/male), type of VTE (DVT/PE), VTE classification (provoked/unprovoked) and presence of cardiovascular (CV) risk factors (no CV risk/CV risk) compared with non-statin treatment.

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Table 1. Baseline characteristics of participants

	Rosuvastatin users (n=126)		Non-users (n=119*)	
General				
Age (years)	56.8	(19-82)	58.4	(21-80)
Male	68	(54)	82	(69)
Body mass index (kg/m2)	27.4	(19.2-43.5)	27.7	(17.2-43.3)
Aspirin use	5	(4)	5	(4)
Venous thrombosis characteristics				
Type of venous thromboembolism				
Deep vein thrombosis	72	(57)	64	(54)
Pulmonary embolism	54	(43)	55	(46)
Unprovoked	57	(45)	62	(52)
Provoked, by	69	(55)	57	(48)
Surgery/ Trauma/ Immobilization	32	(25)	30	(25)
Travel > 4 hrs	22	(18)	14	(12)
Estrogen use (% in women)	24	(41)	14	(38)
Pregnancy/ puerperium (% in women)	0	(0)	2	(5)
Malignancy	2	(2)	8	(7)
Recurrent venous thrombosis	10	(8)	8	(7)
Cardiovascular risk factors				
Cardiovascular risk	89	(71)	94	(78)
Current smoking	18	(14)	16	(13)
Hypertension	24	(19)	20	(17)
Diabetes	3	(2)	0	(0)
Overweight[#]	54	(43)	51	(43)
Obesity[##]	29	(23)	34	(28)

Continous variables denoted as mean (range), categorical variables as number (%)
*technical issued in 2 non-users
[#] Overweight was defined as body mass index (BMI) between 25 and 30kg/m2
[##] Obesity was defined as BMI above 30kg/m2

Table 2. Effects of rosuvastatin on measures of thrombin generation parameters

	Mean levels (SD)		Mean*	Mean difference‡	Mean^	Mean difference#
	Baseline	End of study	change (95% CI)	in change (95% CI)	change (95% CI)	in change (95% CI)
THROMBIN GENERATION						
ETP (nM*min)						
Non users	1245.01 (321.47)	1343.85 (290.17)	97.22 (40.92, 153.53)	Reference	94.62 (37.78, 151.46)	Reference
Rosuvastatin users	1284.04 (263.97)	1259.10 (205.37)	-24.94 (-71.81, 21.93)	-120.24 (-192.97, -47.51)	-38.49 (-85.19, 8.21)	-129.39 (-202.29, -56.49)
Thrombin Peak (nM)						
Non users	273.33 (62.09)	294.47 (52.32)	20.69 (9.80, 31.58)	Reference	20.39 (9.42, 31.37)	Reference
Rosuvastatin users	288.86 (62.68)	297.27 (52.25)	8.41 (-0.86, 17.69)	-11.88 (-26.11, 2.35)	5.99 (-3.31, 15.29)	-13.69 (-27.98, 0.60)
Lag Time (min)						
Non users	2.23 (0.49)	2.19 (0.72)	-0.04 (-0.16, 0.08)	Reference	-0.04 (-0.17, 0.08)	Reference
Rosuvastatin users	2.16 (0.43)	2.05 (0.38)	-0.11 (-0.15, -0.07)	-0.07 (-0.20, 0.05)	-0.12 (-0.16, -0.08)	-0.08 (-0.21, 0.05)
Time to peak (min)						
Non users	4.55 (0.89)	4.48 (1.06)	-0.07 (-0.23, 0.09)	Reference	-0.06 (-0.22, 0.10)	Reference
Rosuvastatin users	4.37 (0.77)	4.09 (0.71)	-0.28 (-0.35, -0.21)	-0.21 (-0.38, -0.03)	-0.28 (-0.35, -0.21)	-0.22 (-0.39, -0.04)
Velocity Index						
Non users	126.68 (47.07)	137.39 (43.90)	10.37 (3.64, 17.09)	Reference	9.96 (3.15, 16.77)	Reference
Rosuvastatin users	140.33 (57.35)	154.69 (50.87)	14.36 (7.38, 21.34)	4.41 (-5.35, 14.17)	12.52 (5.60, 19.44)	3.07 (-6.66, 12.80)

Abbreviations: ETP, endogenous thrombin potential. * Paired analysis. ‡ Between comparison analysis, adjusted for age and sex. ^ Paired analysis to eight participants who reported an infection at time of end of study excluded. # Between comparison analysis, adjusted for age and sex to 8 participants who reported an infection at time of end of study excluded.

Table 3. Mean difference in endogenous thrombin potential between rosuvastatin users and non-users (reference) at the end of the study, adjusted for coagulation factors

	Mean difference (95% CI)‡		
ETP (nM*min) at the end of the study			
No coagulation factor	-89.46	(-153.18,	-25.74)
+ factorVIII	-87.66	(-148.43,	-26.89)
+factorXI	-73.13	(-133.58,	-12.68)
+factorVII	-59.93	(-120.02,	0.17)
+DD	-87.20	(-151.41,	-22.99)
+factors VIII, XI, VII, DD	-54.98	(-111.99,	2.03)

‡ comparison between rosuvastatin treatment and no treatment at the end of the study, adjusted for age and sex. DD=D dimer CI= confidence interval

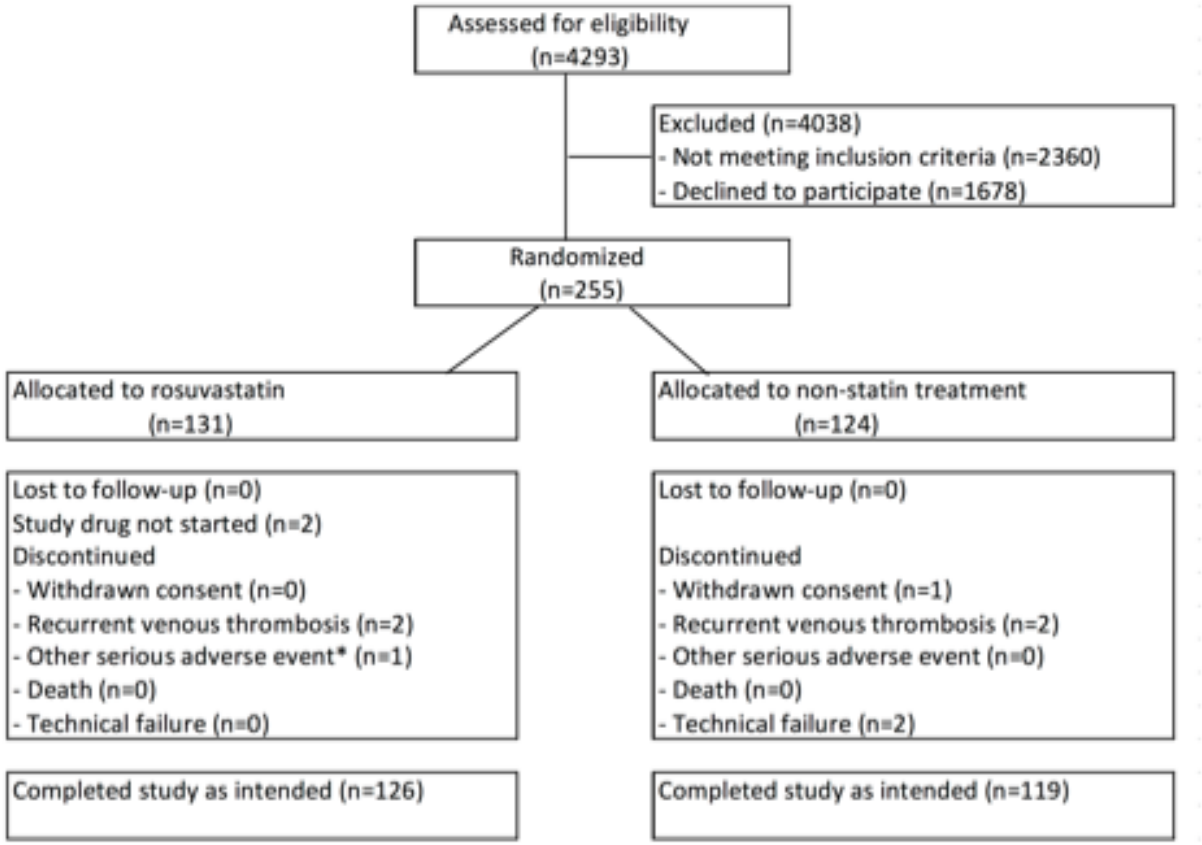


Figure 1. Trial profile. study enrolment, randomization, follow-up and reasons for withdrawal (*one participant admitted to hospital with a diagnosis of acute asthma exacerbation)

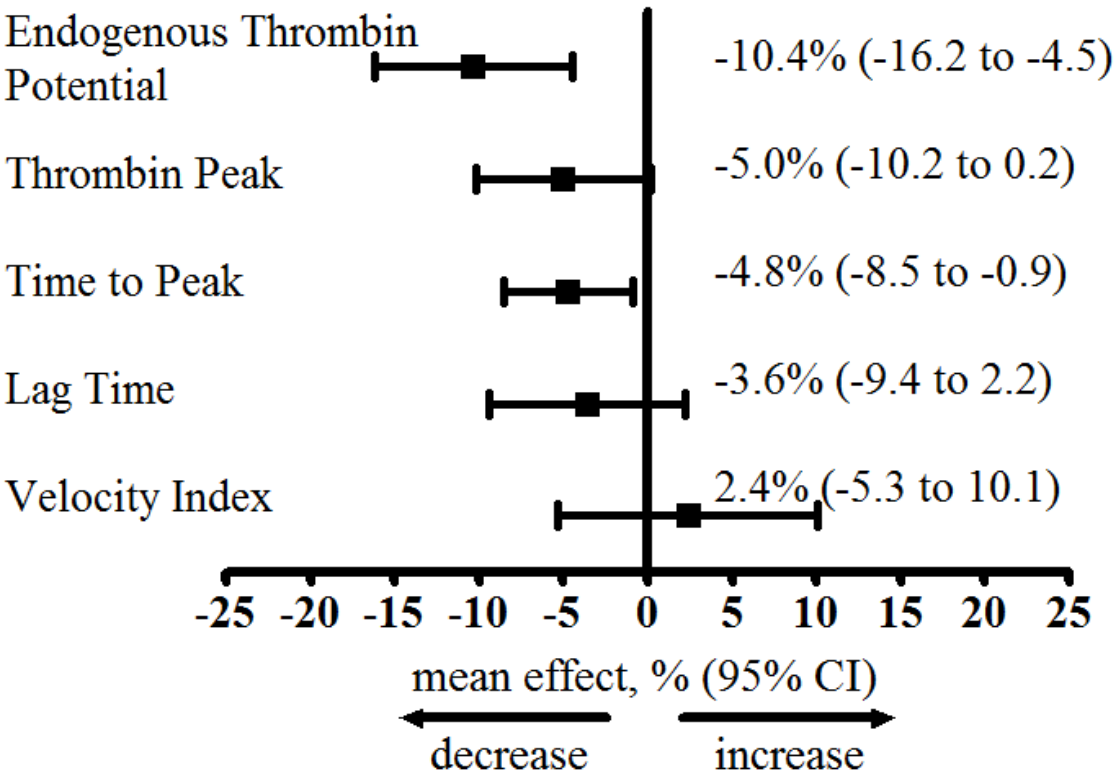


Figure 2. Relative effect of rosuvastatin treatment on thrombin generation. This figure illustrates the changes in endogenous thrombin potential, from baseline to the end of treatment, compared between rosuvastatin users versus non-statin users.

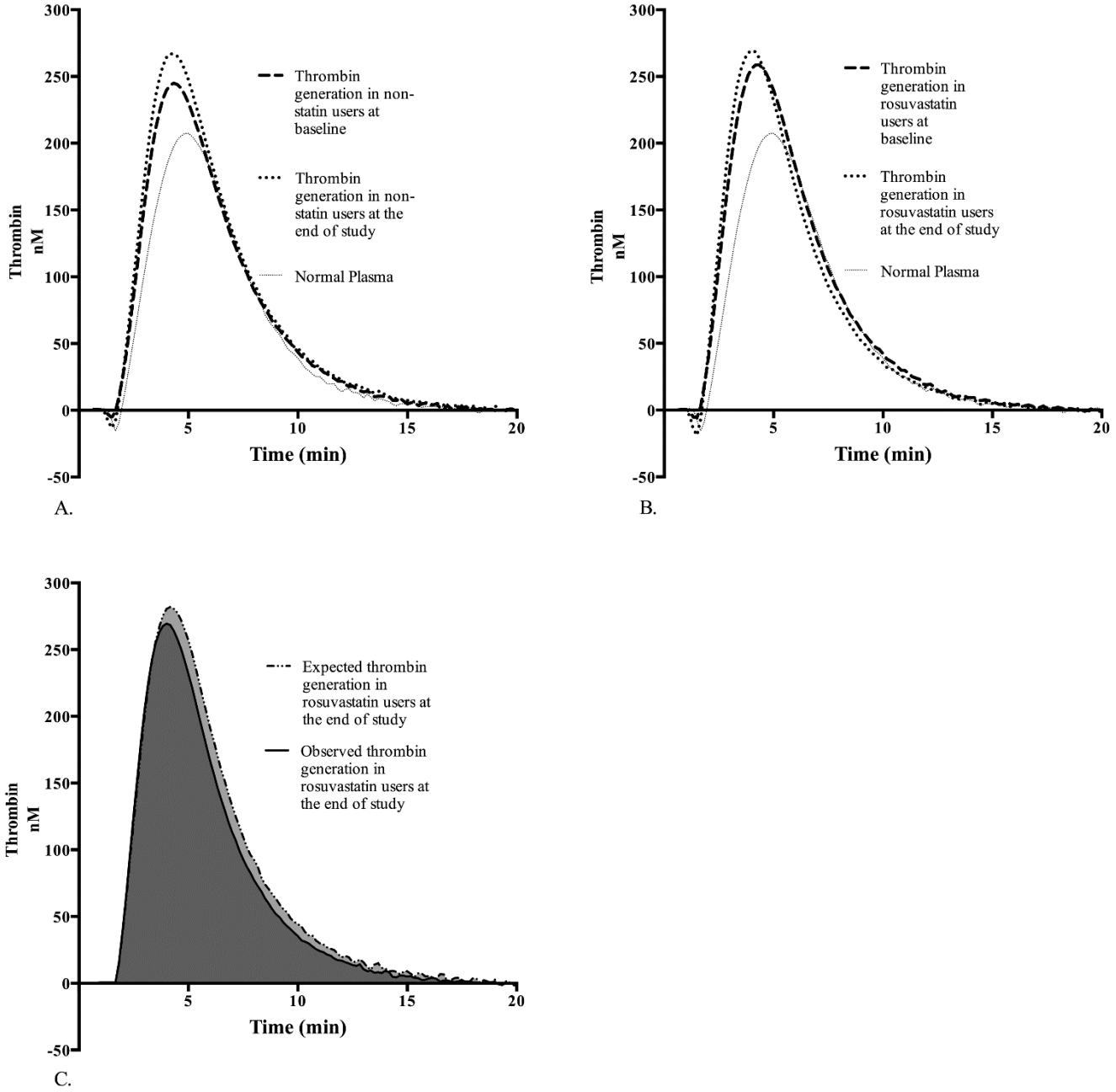
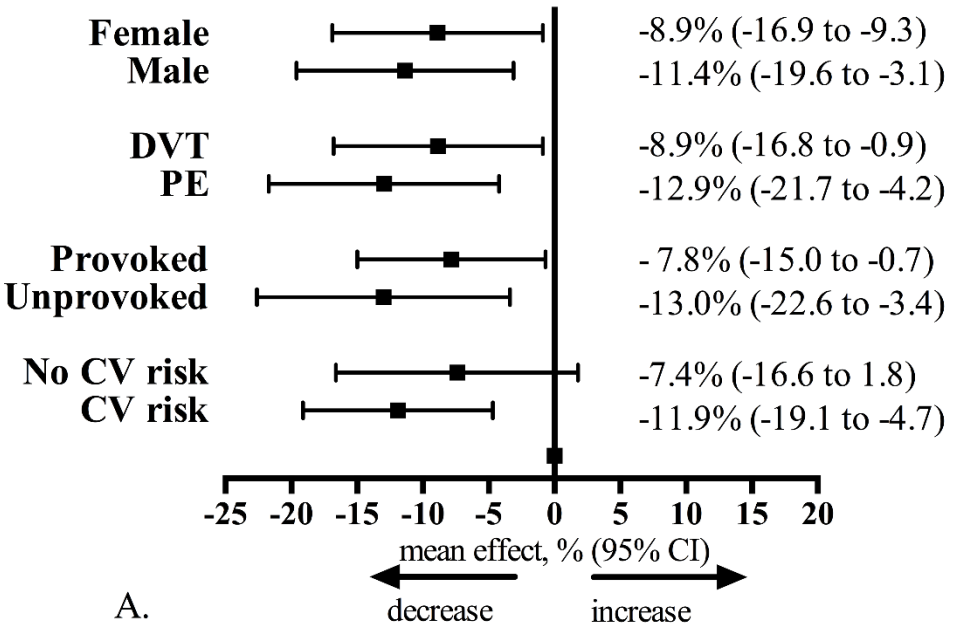


Figure 3. Thrombin generation curves (A) mean values of thrombin generation over time in non-statin users at baseline and at the end of study. (B) mean values of thrombin generation over time in rosuvastatin users at baseline and at the end of study. (C) expected mean thrombin generation values (if rosuvastatin would not have a treatment effect on thrombin generation) and observed mean thrombin generation values by the end of study in patients receiving rosuvastatin.



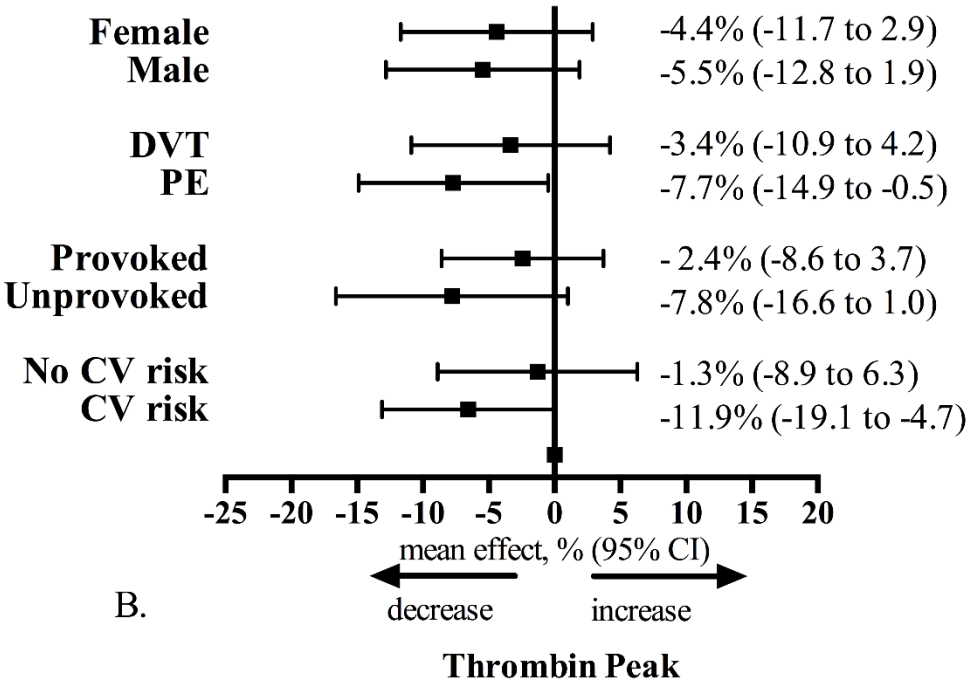


Figure 4. Relative effect of rosuvastatin treatment on thrombin generation potential by subgroups. The relative effect of rosuvastatin treatment on endogenous thrombin potential (A) and on thrombin peak (B) in pre-specified subgroups: sex (female/male), type of VTE (DVT/PE), VTE classification (provoked/unprovoked) and presence of cardiovascular (CV) risk factors (no CV risk/CV risk) compared with non-statin treatment.

Chapter 4

Association of Apolipoproteins C-I, C-II, C-III and E with Coagulation Markers and Venous Thromboembolism Risk.

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Summary

Background: Apolipoproteins C-I, C-II, C-III and E have been associated with risk of arterial thrombotic diseases. **Objectives?** We investigated whether these apolipoproteins have prothrombotic properties and are associated with risk of venous thromboembolism (VTE). **Patients and methods:** A total of 127 VTE patients and 299 controls were randomly selected from the Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis study (1999–2004), in the Netherlands. The apolipoproteins were quantified using mass spectrometry (LC/MS/MS) and their levels were analysed as continuous variable (per SD increase). **Results:** In controls, increases in levels of apolipoproteins were associated with increases in levels of vitamin K dependent factors, factor XI, antithrombin and clot lysis time. Additionally, increasing apolipoproteins C-III and E levels were associated with higher factor VIII and von Willebrand factor levels. Levels of C-reactive protein were not associated with any apolipoprotein. The age and sex adjusted odds ratios of apolipoproteins E, C-III, CII and CI to the risk of venous thrombosis were 1.21 (95% CI, 0.98-1.49), 1.19 (0.99-1.44), 1.24 (0.95-1.61) and 1.06 (95% CI, 0.87-1.30) per SD increase, respectively. These odds ratios did not attenuate after adjustments for statin use, estrogen use, BMI, alcohol use, and self-reported diabetes. **Conclusions:** Levels of apolipoproteins C-I, C-II, C-III and E are associated with those of several coagulation factors. However, whether these apolipoproteins are also associated with an increased risk of VTE remains to be established.

Keywords: Thrombosis; Proteomics; Lipids and Cholesterol; Coagulation; Risk Factors

INTRODUCTION

Venous thromboembolism (VTE) and arterial thrombosis are distinct diseases linked by several clinical aspects. Patients with VTE have a 1.6 to 3 fold- increase in the risk of subsequent arterial cardiovascular events [1, 2], and drugs such as aspirin and statins prevent the occurrence of both arterial and venous thrombosis [3-5]. Furthermore, the two diseases share risk factors, such as age, sex, lifestyle and body mass index (BMI) [6-8].

Disorders of lipid metabolism are risk factors for arterial thrombosis that can potentially play a role in the risk of VTE, as these disorders affect hemostasis and lead to a hypercoagulable state [9, 10]. However, an association between lipid disorders and VTE has not been confirmed so far. Lipid levels were not associated with a risk of VTE in several clinical studies [6, 11, 12], and a relationship between apolipoproteins (apo) and VTE risk remains controversial [11, 13-16]. Although higher levels of apoB and lower levels of apoA-I were reported to increase the risk for VTE in selected populations [14, 16], these observations were not confirmed in larger cohorts [11, 13]. A recent report from a large, unselected population, has demonstrated that decreasing levels of apoA-I and B were associated with increased risk of VTE [12].

Recently, apoC-II, C-III and E have emerged as potential risk factors for cardiovascular disease [17-20] and as target for new lipid lowering agents [21]. Whether these newly described risk factors for cardiovascular disease also play a role in the risk of VTE has not been evaluated thus far. In this study, we apply recent advances in liquid chromatography tandem-mass spectrometry (LC/MS/MS) [22] to perform a comprehensive analysis of the association between serum apoC-I, C-II, C-III and E and levels of hemostatic factors (proteins C and S, antithrombin, TFPI activity, fibrinogen, factors II, VII, VIII, IX, X, XI, von Willebrand and clot lysis time) and inflammatory markers (C-reactive protein). We also investigate the association of these serum apos with the risk of VTE.

MATERIAL AND METHODS

Study population

For this study patients and random digit-dialing (RDD) controls were randomly selected from a population-based case-control study, i.e., the Multiple Environmental and Genetic

Assessment of Risk Factors for Venous Thrombosis (MEGA) study and from a cohort study, the MEGA follow-up.

Details of the MEGA study were described previously [23]. Briefly, between March 1999 and August 2004, 4956 consecutive patients aged 18-70 years with a first acute VTE (deep vein thrombosis or pulmonary embolism) were included. A questionnaire on putative risk factors for VTE was filled in and blood was sampled on the day of enrolment for the study. Participants were asked to provide blood samples up to June 2002, and 2377 patients and 1459 RDD controls provided blood samples. Blood samples were collected from at least 3 months after discontinuation of anticoagulation, or during anticoagulant therapy if this was continued for more than 1 year. None of the included patients with VTE had a recurrence before blood sampling.

RDD controls were frequency matched for sex and age to the cases. RDD controls were aged 18-70 y and had no history of VTE. Patients were followed until 2007-2009 when the vital status of all MEGA follow-up participants was accessed, as described previously (MEGA follow-up study) [24]. The MEGA study was approved by the Ethics Committee of the Leiden University Medical Center, and written informed consent was obtained from all participants at the date of the inclusion in the study. This study was conducted in accordance with the Declaration of Helsinki.

A total of 127 patients with a first VTE event were selected from the MEGA study and MEGA follow-up study, of whom 63 (50%) had had one VTE event and 64 (50%) recurrent VTE. Patients with recurrent VTE were oversampled *a-priori* as we considered them to be more likely to have aberrant apo levels leading to first and recurrent VTE. Otherwise the sampling was completely random. The control group comprised of 300 controls randomly chosen from the RDD control population of the MEGA study. In all groups apoC-I, C-II, C-III and E were measured. Figure 1 illustrates the flow chart of the participants' selection for the study.

Clinical outcomes

The information about the diagnosis of VTE was obtained from hospital discharge reports and general practitioners. The diagnosis of deep vein thrombosis was confirmed with Doppler ultrasonography and the diagnosis of pulmonary embolism was confirmed with a ventilation perfusion lung scan, computed tomography of the chest or angiogram.

Between June 2008 and July 2009, participants were invited to answer questions

regarding recurrent VTE by mail or by telephone interview. Information about recurrences was also retrieved from the anticoagulation clinics where patients were initially included for their first event and at the clinic nearest to their address in case they moved house. Discharge letters were requested from the clinician who had diagnosed the recurrence. A decision rule regarding certainty of the diagnosis was made according to the information collected per patient, as described previously [25]. Reported recurrences were classified as certain when 1) a discharge letter stated a diagnosis of a recurrent event based on clinical and radiological data, or 2) both the anticoagulation clinic and the patient reported a recurrent event that was at a clearly different location than the first event or occurred more than one year since the first event, or 3) a registered death from a recurrent event at least six months after the first event was found. For this analysis, we considered certain recurrences as outcome event only.

Laboratory procedures

ApoC-I, C-II, C-III and E were measured on stored (-80 °C) and once previously thawed fasting serum samples. To determine the apo profile, a mass spectrometric method was developed for multiplexed quantification of apolipoproteins [26]. This multiplex mass spectrometry-based analytical method was validated according to Clinical and Laboratory Standards Institute (CLSI) protocols and its **total coefficient of variation (CV) ranges from 2.5% to 5.9% for the apolipoproteins [27]**.

In contrast to classical high density lipoprotein –cholesterol (HDLc) and low density lipoprotein –cholesterol (LDLc) tests and lipoprotein particle counting methods, the quantitative proteomics test allows quantitation of unequivocally characterized apos with an analytical performance that meets test requirements derived from biological variation. The peptides in the serum digest are separated by liquid chromatography (LC) and detected by tandem mass spectrometry (MS/MS). In MS/MS, ions of the selected peptides are pre-filtered by their molecular mass-to-charge ratio before fragmentation by a collision gas and detection of the generated, highly specific, product ions, relative to stable isotope labelled internal standard peptides. The trace of a precursor-to-product ion transition is used for protein identification and/or quantification.

Serum lipids and lipoprotein fractions were measured using reagents and calibrators from Roche Diagnostics on Modular P analyzers. Traceability of serum lipid and lipoprotein test results to the CDC Reference Measurement System is guaranteed and monitored through stringent vigilance by the Dutch SKML EQA-programme [28].

Procoagulant factors (fibrinogen, factors II, VII, VIII, IX, X and XI, and von Willebrand factor), natural anticoagulants (antithrombin, protein C, and total protein S), clot lysis time, and high sensitive C-reactive protein (hsCRP) levels were determined according to previously described methods [29, 30]. Briefly, measurements of antithrombin and protein C levels were performed with a chromogenic assay and factors II, VII, VIII, X and XI activities were measured with a mechanical clot detection method on a STA-R coagulation analyzer following the instructions of the manufacturer (Diagnostica Stago, Asnieres, France). Total protein S levels and levels of factor IX antigen were determined by ELISA (Diagnostica Stago). Fibrinogen activity was measured on the STA-R analyzer. Von Willebrand factor antigen was measured by immunoturbidimetry (STA Liatest®, Diagnostica Stago), following the instructions of the manufacturer. CRP was measured by automated particle-enhanced immunoturbidimetric assay (Tina-quant® CRP detection method; Roche Diagnostics, West Sussex, UK).

Total tissue factor pathway inhibitor (TFPI) activity in citrated plasma was determined using the Actichrome TFPI activity assay (Sekisui Diagnostics, Stamford, CT, USA). Briefly, TFPI activity is assessed by measuring TFPI inhibition of the catalytic tissue factor (TF)-factor VIIa (FVIIa) complex; one unit of TFPI activity corresponds to 55 ng/mL plasma TFPI. All laboratory analyses were performed without knowledge of whether the sample was from a patient or a control subject.

Statistical analysis

Baseline characteristics are presented as counts and percentages if they are categorical variables or mean +/- standard deviation (SD) if they are continuous variables.

To assess the association of apoC-I, C-II, C-III and E with hemostatic factors and inflammatory markers, the apo-levels were considered as per standard deviation (SD) increase and linear regression models were performed. Data that were not normally distributed were log-transformed. The models were adjusted for age (continuous), sex (dichotomous), statin use (dichotomous), estrogen use (dichotomous), alcohol intake (dichotomous), body mass index (continuous), and self-reported diabetes (dichotomous).

The association between apoC-I, C-II, C-III and E and VTE was assessed by logistic regression analysis. Three adjusted logistic regression models were used: age and sex-adjusted model; age, sex and statin use –adjusted model and age, sex, statin-use, estrogen use, alcohol intake, body mass index, and self-reported diabetes adjusted model.

In each logistic regression model, odds ratios (OR) and their 95% CI were used to estimate the association with all VTE (n=127), or one VTE event only (n= 63), using the levels of apolipoproteins as continuous variables.

As 64 patients also had a recurrent VTE, differences in the outcome between patients with only one VTE event (n=63) and recurrent VTE (n=64) were studied in a case-control analysis within the MEGA follow-up study. For this purpose, an age and sex-adjusted logistic regression model was applied to estimate the odds ratio of recurrent VTE, as compared with first VTE only, for apo levels as continuous values.

All statistical analyses were performed with SPSS version 23.0 for Windows (SPSS Inc, IBM, Armonk, NY, USA). Graphs were plotted using GraphPad Prism version 6.0 for MAC (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Clinical characteristics

Clinical characteristics of the participants are shown in Table 1. The main site of thrombosis was deep vein thrombosis (65%). There were no substantial clinical differences between patients and RDD controls selected for the current analysis and the total patients and RDD control participants of MEGA study (data not shown).

Association of apoC-I, C-II, C-III and E with hemostatic and inflammatory markers

Graphic material on the correlation between apos C-I,-II, -III, E and vitamin K dependent coagulation factors and FVIII and VWF is provided as Supplementary Figures 1-8.

In controls, the mean levels (and SD) of apoC-I, C-II, C-III and E were 21.38 mg/L (5.33), 39.89 mg/L (27.64), 103.59 mg/L (37.11), 30.71 mg/L (13.03), respectively. The associations of apoC-I, C-II, C-III and E with hemostatic factors and inflammatory markers in control subjects are shown in detail in Table 2.

Increases in the levels of all measured apos (apoC-I, C-II, C-III and E) were associated with higher levels of FII, FVII, FIX, FX, FXI, natural anticoagulants (protein C, protein S, antithrombin) and clot lysis time. Additionally, an increase in the levels of FVIII and VWF was observed with increases in the levels of apoC-III and apoE (Table 2). There was no association of apoC-I, C-II, C-III or E with levels of CRP.

Association of apoC-I, C-II, C-III and E with venous thromboembolism

In VTE patients, the mean levels (and SD) of apoC-I, C-II, C-III and E were 21.59 mg/L (6.29), 43.08 mg/L (32.22), 110.53 mg/L (46.04), 32.92 mg/L (12.69), respectively. Figure 2 illustrates the association between apo levels and first VTE risk. There was no association between first VTE and levels of apoC-I. The sex and age adjusted OR and 95% CI of first VTE was 1.24 (95% CI: 0.95, 1.61) with apoC-II, 1.19 (OR 95% CI: 0.99, 1.44) with apoC-III and 1.21 (95% CI: 0.98, 1.49) with apoE, as compared with RDD controls. The observed associations remained similar after further adjustments for statin use, estrogen use, BMI, alcohol use, and self-reported diabetes. Results were also similar when considering patients with only one VTE event. Age and sex adjusted odds ratios of venous thromboembolism for apolipoproteins C-I, C-II, C-III, and E levels when comparing patients with only one VTE event with RDD controls were 1.03 (95% CI 0.78 – 1.37), 1.26 (95% CI 0.88 – 1.81), 1.07 (95% CI 0.81 – 1.41) and 1.21 (95% CI 0.93 – 1.57) respectively.

The sex- and age-adjusted ORs and 95% CIs of recurrent VTE with apoC-I, C-II, C-III and E levels (per SD increase) were 1.03 (95% CI: 0.77, 1.39), 1.14 (95% CI: 0.74, 1.78), 1.22 (95% CI: 0.90, 1.66) and 0.99 (0.69-1.42), respectively, as compared with first VTE only.

DISCUSSION

In individuals without VTE, levels of apoC-I, C-II, C-III and E were associated with levels of several hemostatic factors, in particular with vitamin K dependent pro- coagulant factors and natural anticoagulants. The association of these apos with coagulation factors remained significant after adjustment for confounding factors. These observations are consistent with previous results from the MEGA study, in which a positive association of apoA-I and B with vitamin K dependent factors and coagulation inhibitors was reported [12].

Additionally, higher levels of both apoC-III and E were associated with increases in levels of FVIII and VWF, which are well known risk factors for VTE [31-33]. It is also worth noticing that apoC-III and apoE have consistently been associated with cardiovascular diseases [34]. Previous studies demonstrated that apoE (per SD increase)

is associated with 15 to 30% increased risk of ischemic heart disease [20, 35] and 2-fold increased risk of cardiovascular mortality.[36] Therefore, our findings provide additional evidence for an association between the proteomic signature and a worse or impaired lipid/coagulation pattern due to cross-talks between these systems.

Multiple mechanisms are potentially at the basis of this association. As the major source of serum apoC-I, C-II, C-III and E are the hepatocytes, it is possible that the synthesis of apos and hepatocyte-derived coagulation factors and natural anticoagulants is regulated by common mechanisms [33, 34]. Additionally, biochemical studies have shown that triglycerides-rich lipoproteins, which contain apoC-I, C-II, C-III and E, stimulate coagulation *in vitro* by binding vitamin K-dependent coagulation factors, promoting enzymatic activity of prothrombinase complex and enhancing thrombin activation [37, 38]. Although these hypotheses may explain the association between apoC-I, C-II, C-III and E and hepatocyte-derived coagulation factors, it does not explain the association of apoC-III and E with coagulation factors secreted by the endothelium and not dependent of vitamin K, such as VWF and FVIII [35, 36]. Biological mechanisms linking apoE and FVIII have been described [39-42]. In animal models of atherogenesis, the absence of FVIII had a protective effect against the development of atherosclerosis in *APOE* knock-out mice [39, 40]. Moreover, low-density lipoprotein receptor-related protein-1 (LRP-1) is a common receptor for both apoE and FVIII [41]. The receptor is also capable of mediating the degradation of FVIII [42] and contributes to variations in FVIII plasma levels [43]. Therefore, the observed association between apoC-I, C-II, C-III and E and coagulation factors may be explained by various mechanisms, such as common pathways of synthesis in hepatocytes [33, 34] coagulation activation [37, 38] and competition binding between apoE and FVIII for a common receptor [43].

Since VTE patients are characterized by a high prevalence of classic cardiovascular risk factors [6], we further evaluated whether apoC-I, C-II, C-III and E were associated with risk of VTE. Although our analyses pointed towards an association between apoC-II, C-III and E with VTE risk, numbers were small and confidence intervals included unity. Therefore, our results need larger studies to definitely conclude on the relation between these apos and VTE risk.

Some aspects in this study need to be considered in order to interpret our results. First, the sample was randomly selected from a large population-based study, the MEGA study, but was not large enough for strong conclusions on the association with VTE risk.

Second, we cannot rule out that some associations between apos and hemostatic factors occurred by chance or due to residual confounding such as hepatic function or diet, variables on which we have no information in MEGA. However, similar associations were described previously both in basic research and in population-based studies [9, 20, 37-44] and can be explained biologically. Third, despite patients with recurrent VTE being overrepresented, the study sampling was otherwise completely random. As a result, clinical characteristics of patients and RDD controls selected for this study were similar to that reported for the total MEGA study population [12]. Furthermore, the availability of the blood samples was based on logistical reasons only, where blood sampling was performed for all participants included up to June 2002 [45]. It is possible, though, that a participant's clinical condition contributed to his/her decision to partake on blood sampling. Forth, it is possible to argue that since apos were tested after the VTE event, reverse causality was present. Indeed, after VTE, patients may have modified certain lifestyle factors, which could have affected their apo profile. However, results from randomized trials have shown that changes in diet hardly affect lipid levels without concomitant statin use or aerobic-exercise programs.[46, 47] Finally, since the investigation of apoC-I, C-II, C-III and E for associations with coagulation factors, inflammatory markers and VTE is unprecedented, our findings require replication.

Conclusion

In conclusion, we have shown that serum apoC-I, C-II, C-III and E were associated with multiple plasma coagulation factors and natural anticoagulants. Particularly, higher levels of apoC-III and E were associated with an increase in levels of FVIII and VWF. Whether these apolipoproteins are also associated with an increased risk of VTE remains to be established.

Authors' contributions

Fernanda A. Orsi performed the statistical analyses and drafted the manuscript. Willem M. Lijfering designed and performed the statistical analyses and revised the manuscript. Arnoud Van der Laarse and L. Renee Ruhaak were responsible for the laboratory analyses and revised the manuscript. Frits R. Rosendaal was responsible for the MEGA study concept, designed the analyses and revised the manuscript, Suzanne C. Cannegieter designed the analyses and revised the manuscript and Christa Cobbaert was

responsible for the development of the laboratory methods, designed the analyses and revised the manuscript.

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Disclosure

The authors declare no competing financial interests.

List of Abbreviations

Apo Apolipoprotein
CI confidence interval
CLT clot lysis time
F Factor
HDLc high density lipoprotein –cholesterol
Hs-CRP high sensitivity C-reactive protein
LC/MS/MS liquid chromatography detected tandem mass spectrometry
LDLc low density lipoprotein –cholesterol
PC Protein C
PS Protein S
RDD random digit-dialing
TFPI Tissue factor pathway inhibitor
VTE Venous Thromboembolism
VWF von Willebrand factor

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Table 1 Clinical Characteristics of Patients and RDD Controls Randomly Selected from the Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis Case-Control Study, the Netherlands, 1999–2004

Characteristic[a]	Patients			RDD controls
	All (n=127)	First VTE (n=63)	Recurrent VTE (n=64)	(n=299)
Age at enrollment[b], mean (SD)	46 (13)	45 (12)	47 (13)	47 (13)
Men, n (%)	59 (46)	20 (32)	39 (61)	138 (46)
Statin use, n (%)	3 (2.4)	3 (4.8)	0 (0)	22 (7)
BMI, kg/m^2, mean (SD)	27.4 (4.8)	27.6 (5.2)	27 (4.4)	25.3 (4.2)
Estrogen use, n (% in women)	43 (64.2)	26 (41.3)	17 (26.6)	40 (25.3)
Diabetes (self-reported), n (%)	3 (2.4)	1 (1.6)	2 3.1	5 (1.7)
Alcohol use, n (%)				
No	26 (21)	15 (25)	11 (17)	35 (12)
Yes	96 (79)	45 (75)	52 (83)	263 (88)

Abbreviations: BMI, body mass index; RDD, random digit-dialing.

[a] There were some missing data on BMI, estrogen use, statin use, alcohol use and diabetes.

[b] Age is expressed in years.

Table 2 Association of Apolipoproteins With Levels of Hemostatic Factors and C-Reactive Protein in RDD Controls Randomly Selected From the Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis Case-Control Study, the Netherlands, 1999–2004

Parameters	**ApoC-I** (n=292)			**ApoC-II**[f] (n=286)			**ApoC-III** (n=292)*			**ApoE** (n=292)*		
	per SD increase (5.33 mg/L)	(95%CI)		per SD increase (0.97 mg/L)	(95%CI)		per SD increase (37.11 mg/L)	(95%CI)		per SD increase (13.03 mg/L)	(95%CI)	
Anticoagulant factors												
Protein C (IU/dL)[e]	8.21	(6.02;	10.40)	6.25	(3.77;	8.72)	8.00	(5.73;	10.26)	7.34	(5.00;	9.68)
Protein S antigen (IU/dL)[e]	5.51	(3.52;	7.50)	5.26	(3.10;	7.42)	5.74	(3.71;	7.78)	3.86	(1.72;	5.99)
Antithrombin (IU/dL)	2.78	(1.56;	4.00)	2.12	(0.77;	3.46)	2.41	(1.19;	3.64)	1.74	(0.45;	3.02)
TFPI activity (IU/mL)	0.07	(0.01;	0.12)	0.04	(-0.02;	0.10)	0.00	(-0.05;	0.06)	0.04	(-0.01;	0.10)
Procoagulant factors												
Fibrinogen (g/L)	-0.07	(-0.14;	0.00)	0.02	(-0.06;	0.10)	0.01	(-0.07;	0.08)	0.06	(-0.01;	0.14)
Factor II (IU/dL)[e]	4.47	(3.07;	5.86)	3.10	(1.55;	4.65)	4.83	(3.41;	6.24)	3.42	(1.92;	4.92)
Factor VII (IU/dL)[e]	8.10	(5.56;	10.63)	4.90	(2.07;	7.73)	9.87	(7.35;	12.39)	8.80	(6.17;	11.43)
Factor VIII (IU/dL)	1.07	(-3.72;	5.85)	2.83	(-2.37;	8.02)	6.60	(1.91;	11.30)	5.12	(0.23;	10.02)
VWF (IU/dL)	2.07	(-2.72;	6.85)	3.01	(-2.20;	8.21)	5.74	(1.02;	10.45)	6.08	(1.19;	10.96)
Factor IX antigen (IU/dL)[e]	3.62	(1.62;	5.61)	2.80	(0.63;	4.96)	5.45	(3.46;	7.43)	2.74	(0.64;	4.84)
Factor X (IU/dL)[e]	7.06	(5.22;	8.90)	4.81	(2.73;	6.90)	7.96	(6.11;	9.80)	4.70	(2.67;	6.73)
Factor XI (IU/dL)	3.67	(1.52;	5.82)	2.26	(-0.07;	4.58)	2.14	(-0.02;	4.31)	3.68	(1.46;	5.89)
Fibrinolytic factor												
Clot lysis time (min)[e]	5.91	(3.71;	8.11)	3.40	(0.95;	5.85)	5.95	(3.69;	8.20)	6.36	(4.07;	8.64)
Inflammatory marker												
hsCRP (mg/L)[f]	-0.10	(-0.21;	0.02)	0.03	(-0.10;	0.16)	0.02	(-0.10;	0.14)	0.09	(-0.03;	0.21)

Abbreviations: ApoC-I, apolipoproteinC-I; ApoC-II, apolipoproteinC-II; ApoC-III, apolipoproteinC-III; ApoE, apolipoproteinE; CI, confidence interval; TFPI, tissue factor pathway inhibitor; VWF, von Willebrand factor. [a] Adjusted for age, sex, statin use, estrogen use, BMI, alcohol use, and self-reported diabetes. [e] Individuals using VKA are excluded when analysing vitamin K dependent coagulation factors (3 RDD were using anticoagulants) [f] values were log-transformed.

1

Legends to figures

Figure 1. Flow chart of participants selection. Patients and RDD controls were randomly selected from the Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis case-control study, the Netherlands, 1999–2004. Abbreviations: RDD, random digit dialing; VTE, venous thromboembolism; apo, apolipoprotein.

Figure 2. Odds ratio of venous thromboembolism for apolipoproteins C-I, C-II, C-III, and E levels when comparing patients and RDD controls randomly selected from the Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis case-control study, the Netherlands, 1999–2004. The models were adjusted for: A) age and sex; B), age, sex and statin use; C) and age, sex, statin use, estrogen use, alcohol intake, body mass index, and self-reported diabetes adjusted model. Data shows odds ratio (OR) and 95% confidence interval (CI). Abbreviations: RDD, random digit dialing; VTE, venous thromboembolism; apo, apolipoprotein.

Supplementary Figure 1. The scatter plot matrix graphic illustrates the correlation between apoC-I and vitamin K dependent coagulation factors (protein C, protein S, FII, FVII, FIX, FX)

Footnotes: Abbreviations: ApoC-I, apolipoproteinC-I. Individuals using VKA are excluded when analysing vitamin K dependent coagulation factors (3 RDD were using anticoagulants).

Supplementary Figure 2. The scatter plot matrix graphic illustrates the correlation between apoC-II and vitamin K dependent coagulation factors (protein C, protein S, FII, FVII, FIX, FX).

Footnotes: Abbreviations: ApoC-II, apolipoproteinC-II. Individuals using VKA are excluded when analysing vitamin K dependent coagulation factors (3 RDD were using anticoagulants). ApoC-II values were log-transformed.

Supplementary Figure 3. The scatter plot matrix graphic illustrates the correlation between apoC-III and vitamin K dependent coagulation factors (protein C, protein S, FII, FVII, FIX, FX).

Footnotes: Abbreviations: ApoC-III, apolipoproteinC-III. Individuals using VKA are excluded when analysing vitamin K dependent coagulation factors (3 RDD were using anticoagulants).

Supplementary Figure 4. The scatter plot matrix graphic illustrates the correlation between apoE and vitamin K dependent coagulation factors (protein C, protein S, FII, FVII, FIX, FX).

Footnotes: Abbreviations: ApoE, apolipoproteinE. Individuals using VKA are excluded when analysing vitamin K dependent coagulation factors (3 RDD were using anticoagulants).

Supplementary Figure 5. The scatter plot matrix graphic illustrates the correlation between apoC-I and factor VIII and VWF.

Footnotes: Abbreviations: ApoC-I, apolipoproteinC-I.

Supplementary Figure 6. The scatter plot matrix graphic illustrates the correlation between apoC-II and factor VIII and VWF.

Footnotes: Abbreviations: ApoC-II, apolipoproteinC-II. ApoC-II values were log-transformed.

Supplementary Figure 7. The scatter plot matrix graphic illustrates the correlation between apoC-III and factor VIII and VWF.

Footnotes: Abbreviations: ApoC-III, apolipoproteinC-III.

Supplementary Figure 8. The scatter plot matrix graphic illustrates the correlation between apoE and factor VIII and VWF.

Footnotes: Abbreviations: ApoE, apolipoproteinE.

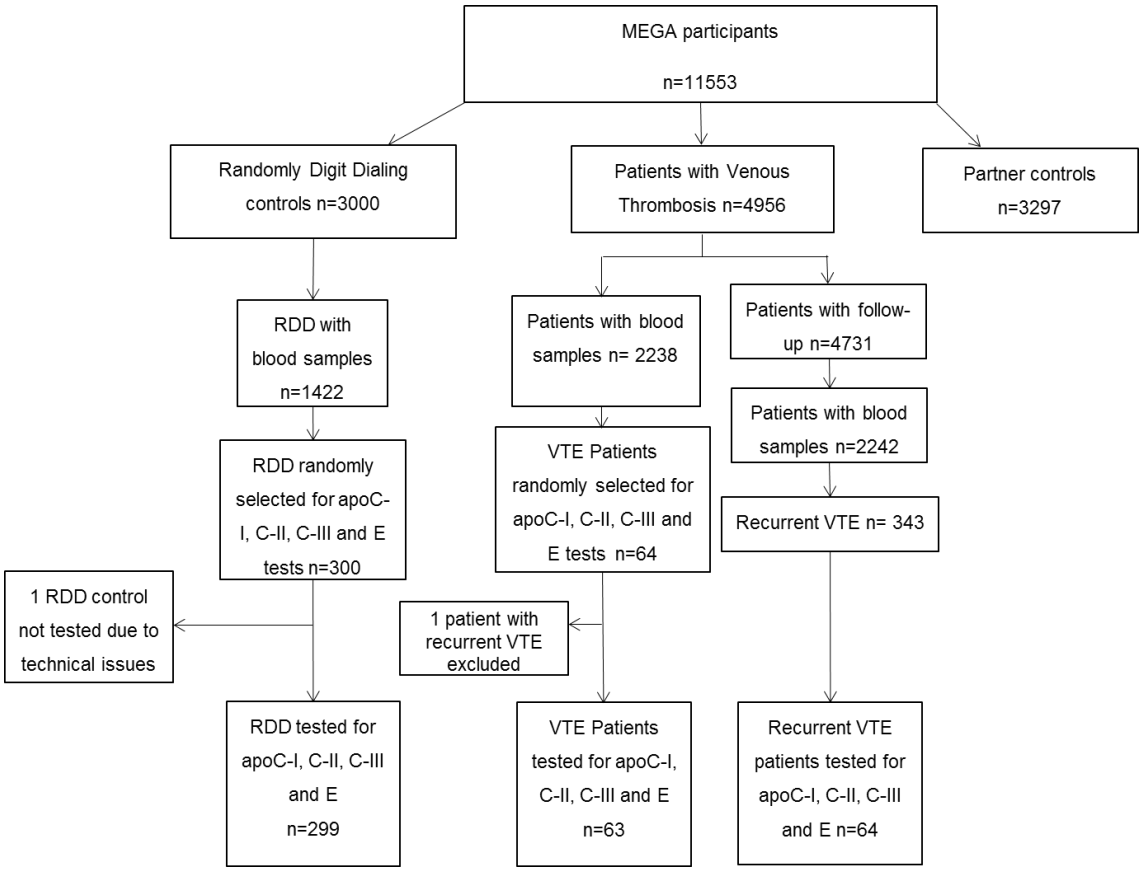


Figure 1. Flow chart of participants selection. Patients and RDD controls were randomly selected from the Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis case-control study, the Netherlands, 1999–2004. A total of 127 patients with a first VTE event were selected from the MEGA and MEGA follow-up studies, 63 had only one VTE event and 64 had recurrent VTE. Abbreviations: RDD, random digit dialing; VTE, venous thromboembolism; apo, apolipoprotein.

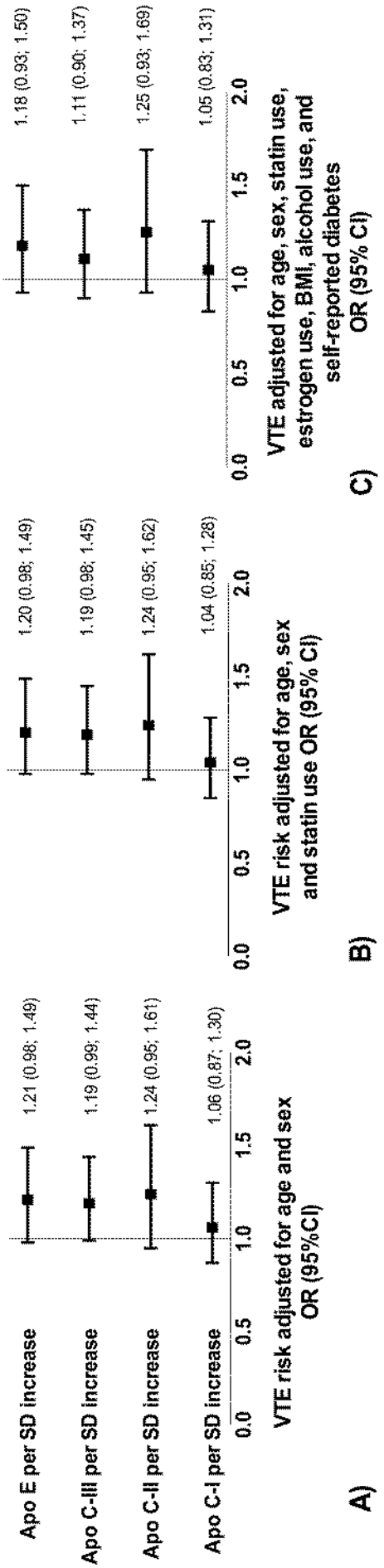
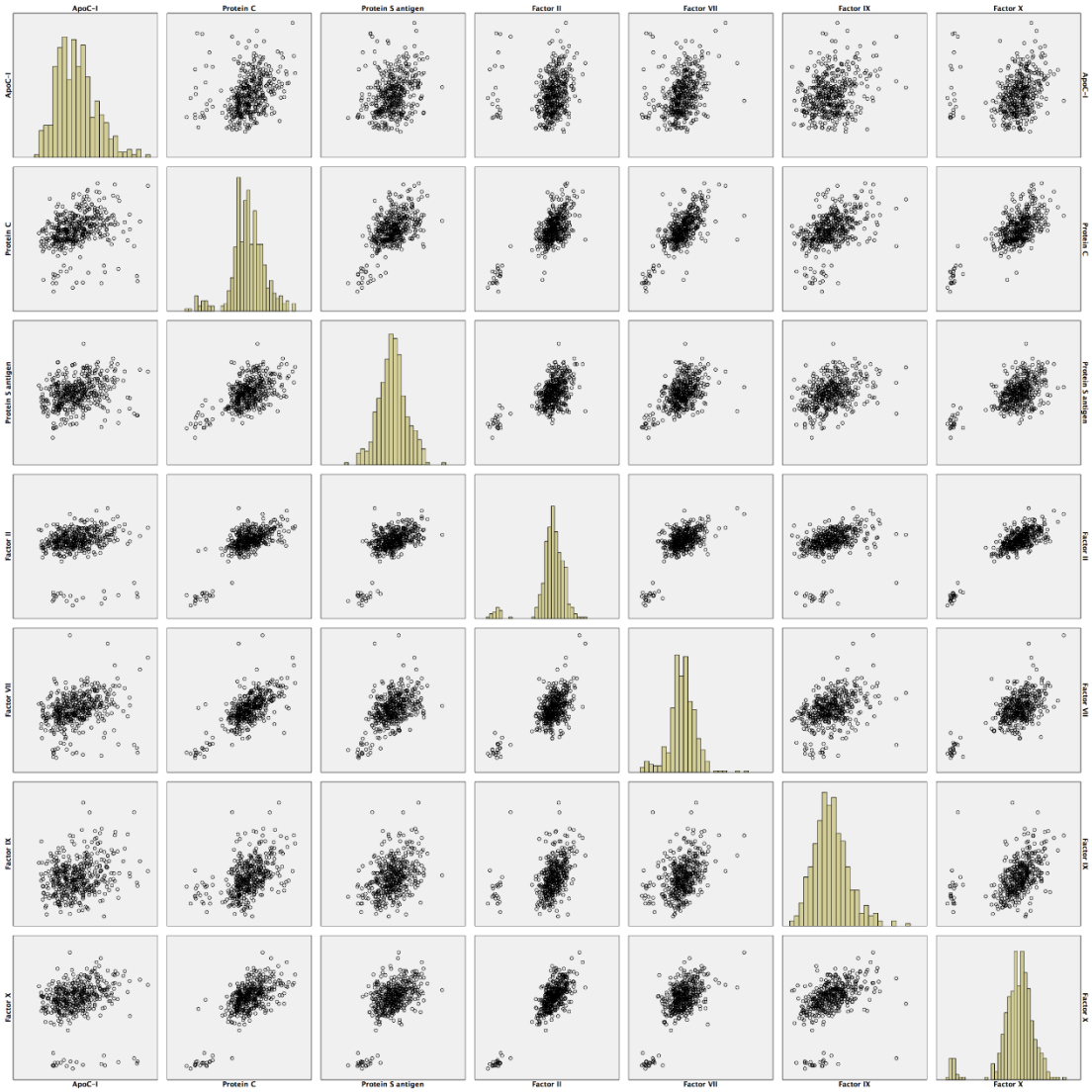
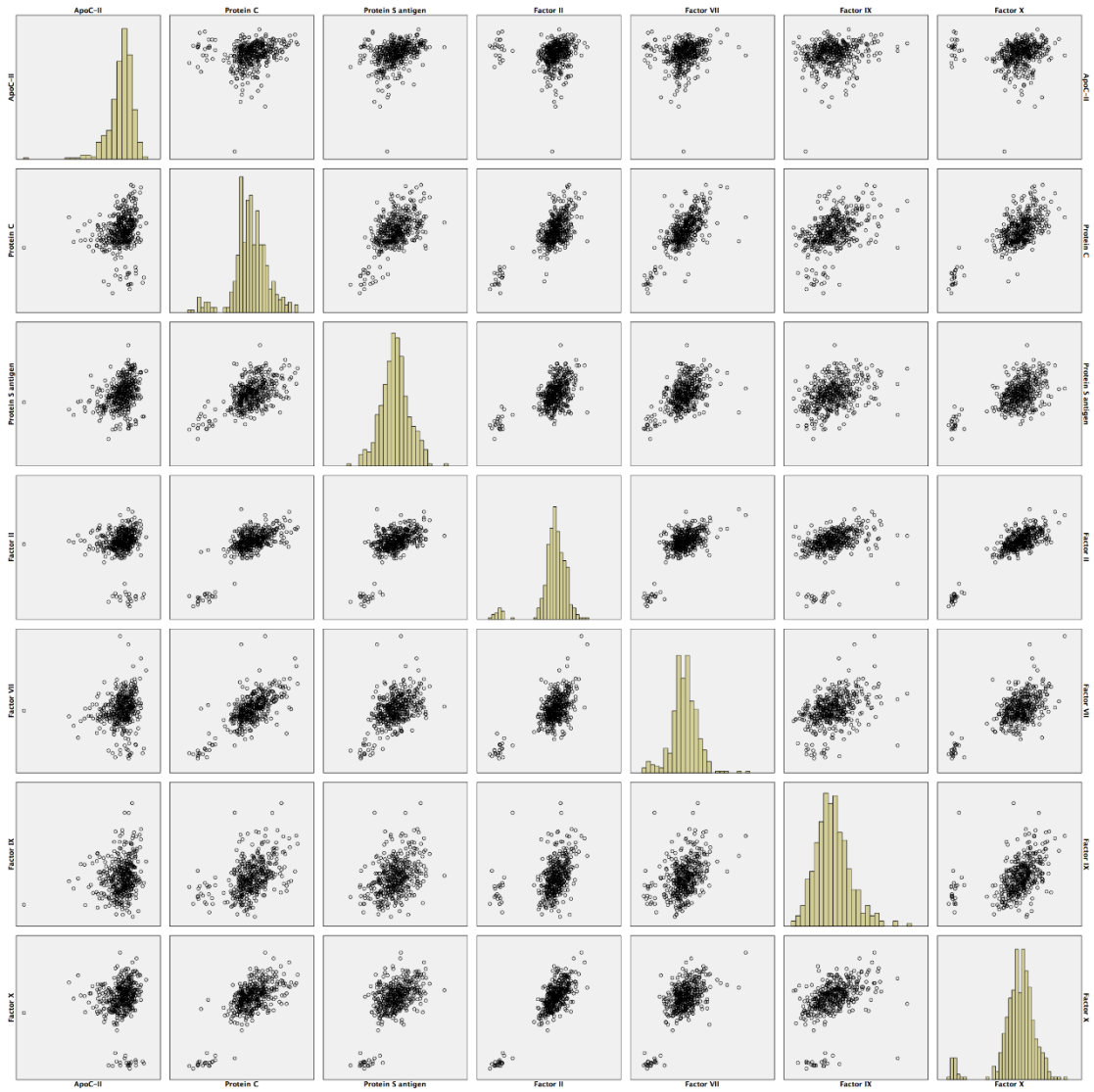


Figure 2. Odds ratio of venous thromboembolism for apolipoproteins C-I, C-II, C-III, and E levels when comparing patients with first VTE (n=127) and RDD (n=299) controls randomly selected from the Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis case-control study, the Netherlands, 1999–2004. The models were adjusted for: A) age and sex; B), age, sex and statin use; C) and age, sex, statin use, estrogen use, alcohol intake, body mass index, and self-reported diabetes adjusted model. Data shows odds ratio (OR) and 95% confidence interval (CI). Abbreviations: RDD, random digit dialing; VTE, venous thromboembolism; apo, apolipoprotein.



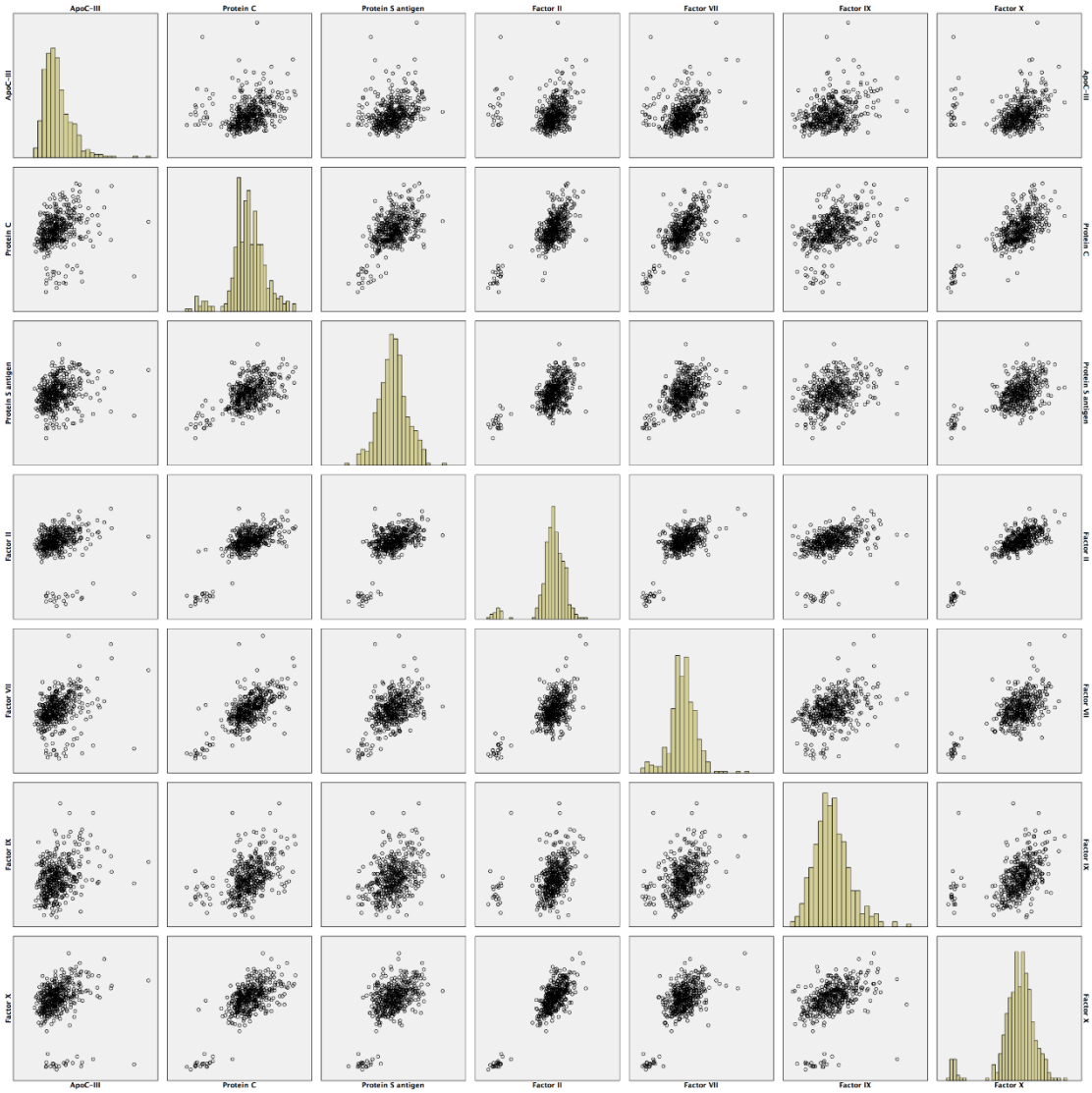
Supplementary Figure 1. The scatter plot matrix graphic illustrates the correlation between apoC-I and vitamin K dependent coagulation factors (protein C, protein S, FII, FVII, FIX, FX)

Footnotes: Abbreviations: ApoC-I, apolipoproteinC-I. Individuals using VKA are excluded when analysing vitamin K dependent coagulation factors (3 RDD were using anticoagulants).



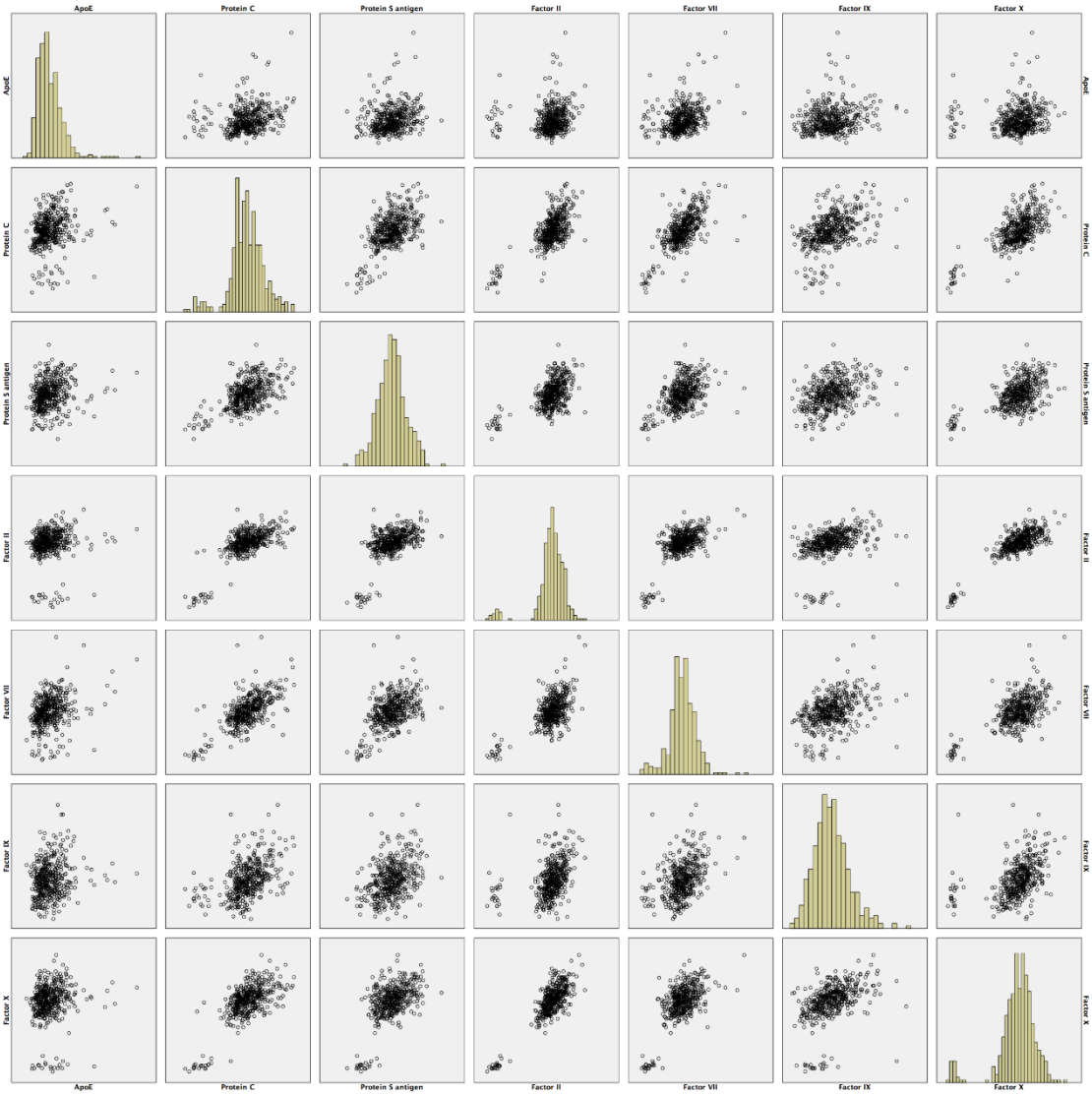
Supplementary Figure 2. The scatter plot matrix graphic illustrates the correlation between apoC-II and vitamin K dependent coagulation factors (protein C, protein S, FII, FVII, FIX, FX).

Footnotes: Abbreviations: ApoC-II, apolipoproteinC-II. Individuals using VKA are excluded when analysing vitamin K dependent coagulation factors (3 RDD were using anticoagulants). ApoC-II values were log-transformed.



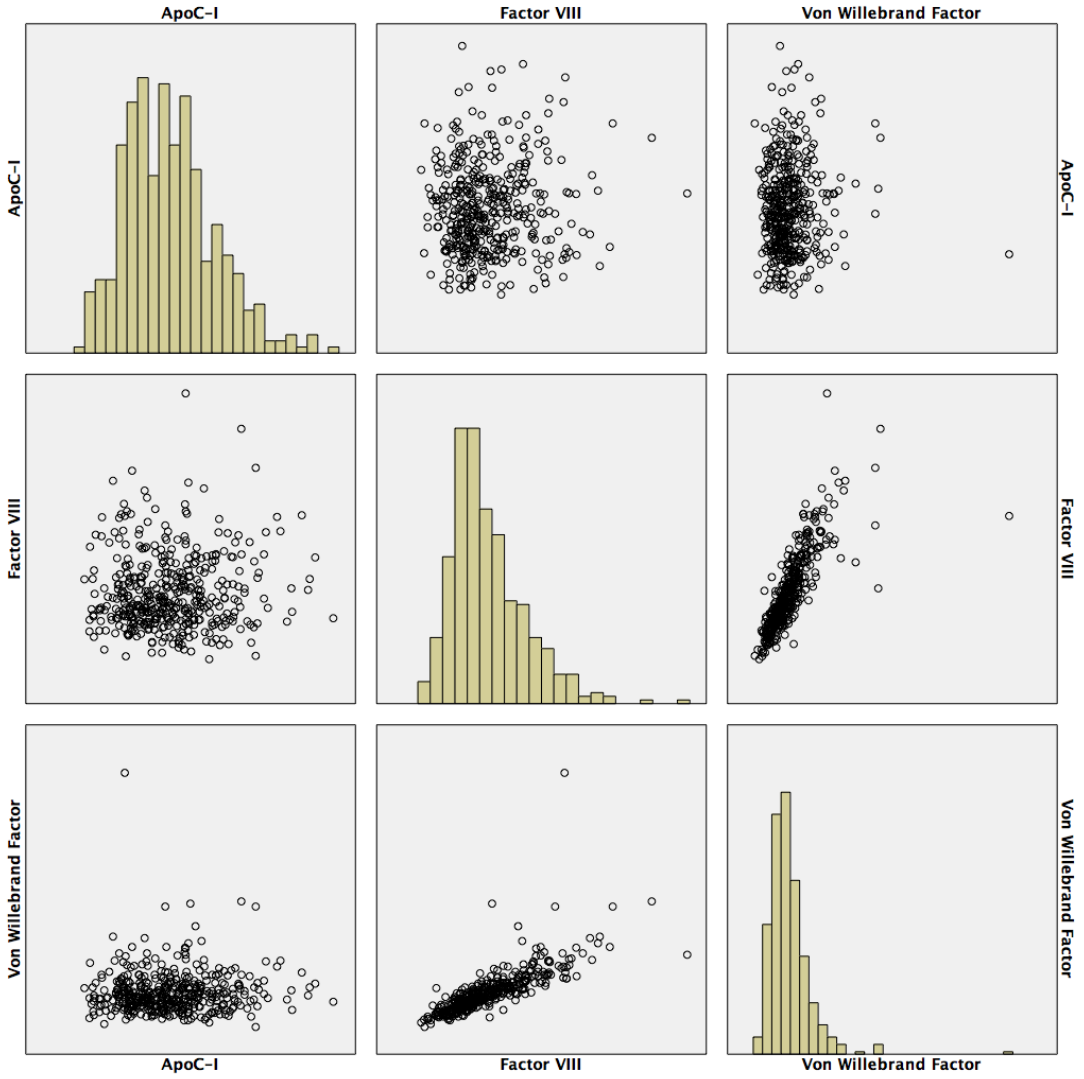
Supplementary Figure 3. The scatter plot matrix graphic illustrates the correlation between apoC-III and vitamin K dependent coagulation factors (protein C, protein S, FII, FVII, FIX, FX).

Footnotes: Abbreviations: ApoC-III, apolipoproteinC-III. Individuals using VKA are excluded when analysing vitamin K dependent coagulation factors (3 RDD were using anticoagulants).



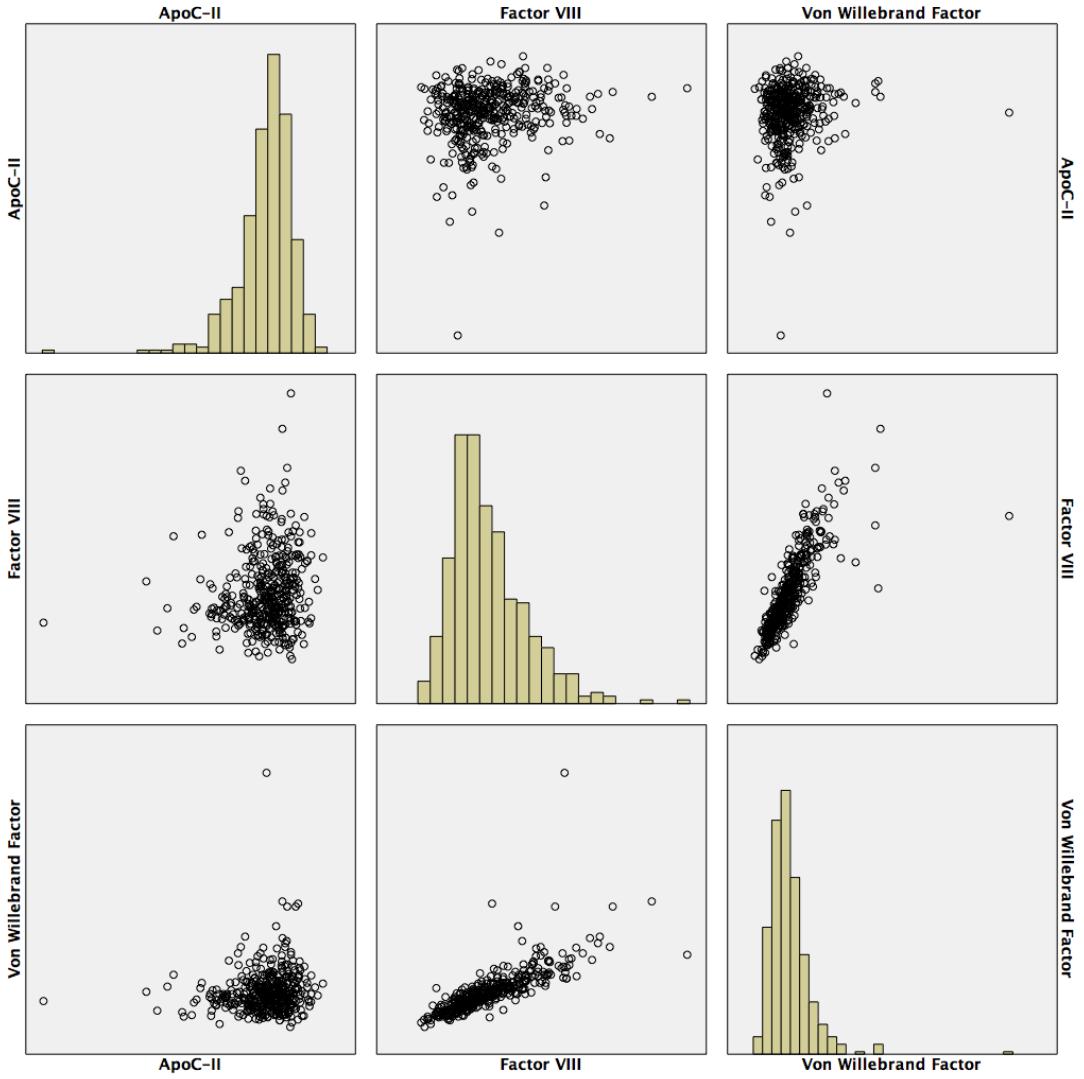
Supplementary Figure 4. The scatter plot matrix graphic illustrates the correlation between apoE and vitamin K dependent coagulation factors (protein C, protein S, FII, FVII, FIX, FX).

Footnotes: Abbreviations: ApoE, apolipoproteinE. Individuals using VKA are excluded when analysing vitamin K dependent coagulation factors (3 RDD were using anticoagulants).



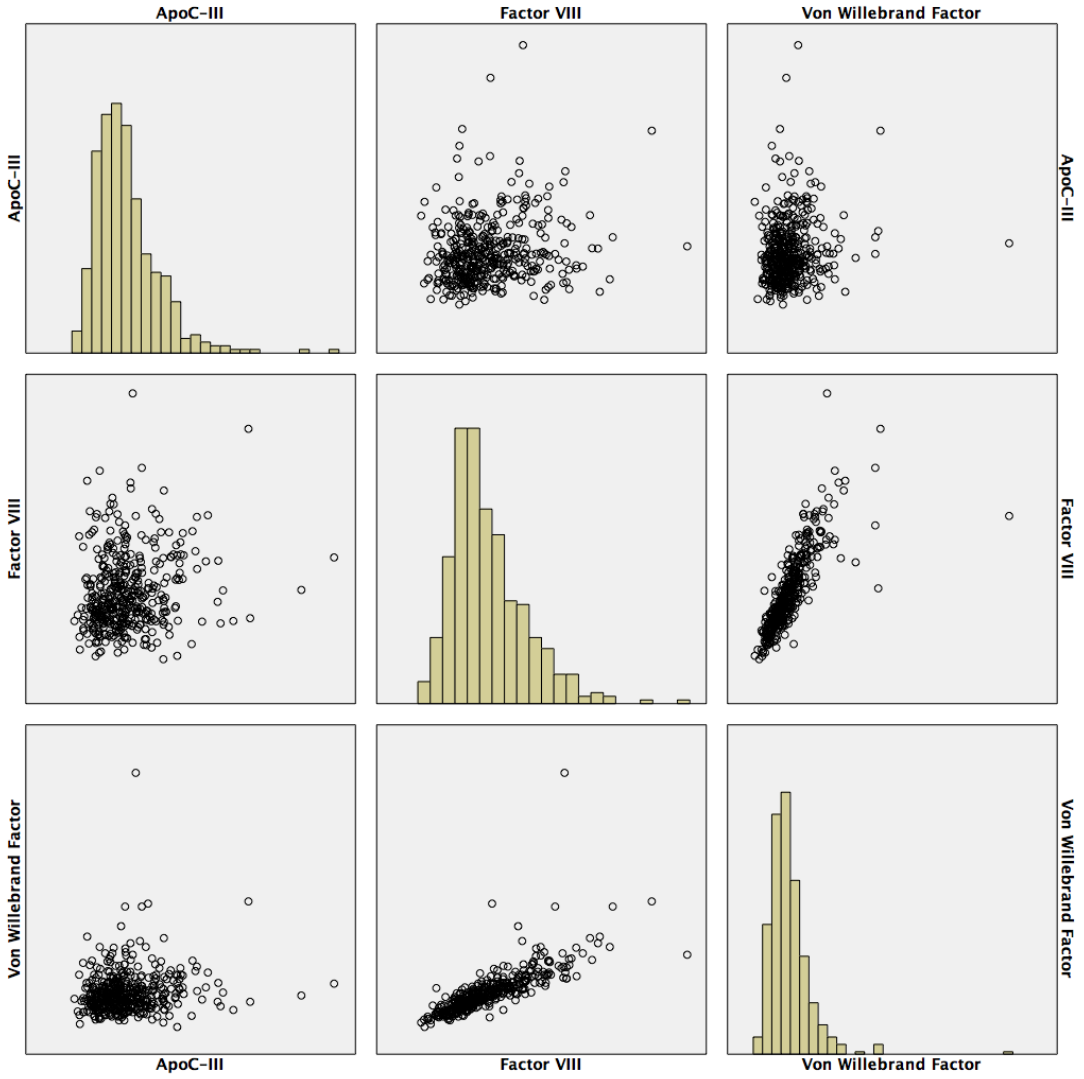
Supplementary Figure 5. The scatter plot matrix graphic illustrates the correlation between apoC-I and factor VIII and VWF.

Footnotes: Abbreviations: ApoC-I, apolipoproteinC-I.



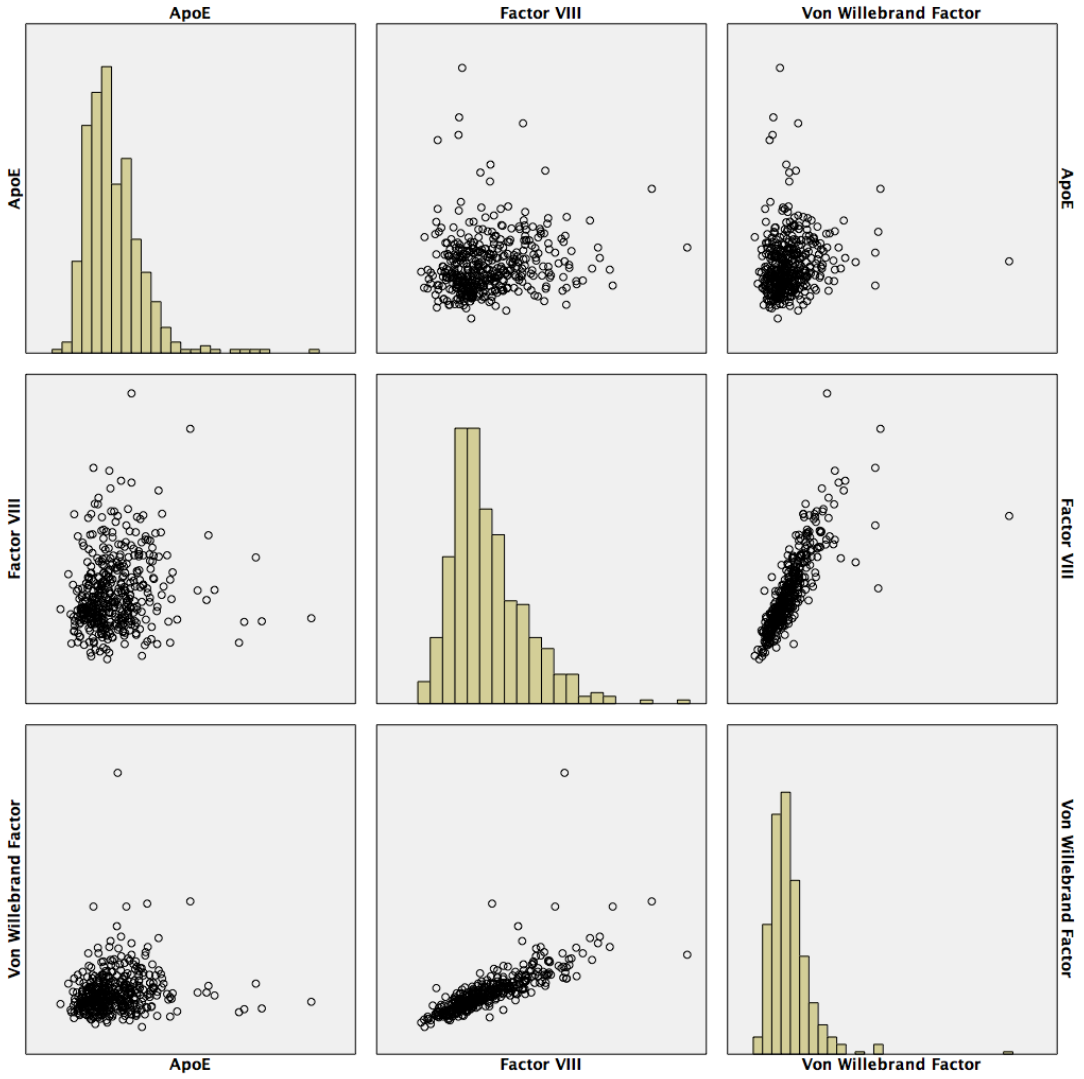
Supplementary Figure 6. The scatter plot matrix graphic illustrates the correlation between apoC-II and factor VIII and VWF.

Footnotes: Abbreviations: ApoC-II, apolipoproteinC-II. ApoC-II values were log-transformed.



Supplementary Figure 7. The scatter plot matrix graphic illustrates the correlation between apoC-III and factor VIII and VWF.

Footnotes: Abbreviations: ApoC-III, apolipoproteinC-III.



Supplementary Figure 8. The scatter plot matrix graphic illustrates the correlation between apoE and factor VIII and VWF.

Footnotes: Abbreviations: ApoE, apolipoproteinE.

Chapter 5

Oral Glucocorticoid Treatment and Risk of First and Recurrent Venous Thromboembolism: A Self-Controlled Case-Series and a Cohort Study

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Summary

Background: Experimental and clinical studies show that glucocorticoids increase coagulation factor levels and possibly venous thromboembolism (VTE) risk. **Objective**: To assess the risk of first and recurrent VTE in patients using oral glucocorticoids. **Patients/Methods:** Design: Nested cohort study. Setting: MEGA follow-up study, the Netherlands. Patients: 4731 patients with VTE of whom 2547 were linked to the Dutch Foundation for Pharmaceutical Statistics register where prescriptions of oral glucocorticoids were identified by ATC6 code. A total of 363 patients received a prescription of oral glucocorticoid. Measurements: The risk of first VTE during periods of exposure with oral glucocorticoids was estimated by use of the self-controlled case series (SCCS) method. The risk of recurrent VTE associated with oral glucocorticoid use was examined with Cox regression with adjustments for age and sex. **Results**: The incidence rate ratio (IRR) for a first VTE in the aggregated period of glucocorticoid treatment was 3.5 (95%CI 2.6-4.8). IRR of a first VTE was 2.5 (95%CI, 1.1-5.7) in the week before treatment started, was increased to 5.3 (95% CI, 2.9-9.5) in the following first 7 days with treatment, remained elevated afterwards until six months with treatment (IRR 3.7; 95%CI, 2.6-5.2) and was reduced to 1.6 (95% CI, 0.8-3.1) after 6 months with oral glucocorticoids, as compared to baseline periods without treatment. The hazard ratio (HR) for recurrence was 2.7 (95%CI, 1.6-4.8) during current treatment periods as compared with no treatment periods. Limitations: The absolute risk for first VTE in patients using oral glucocorticoids could not be assessed by SCCS method. **Conclusion**: Patients taking oral glucocorticoids have an increased risk of first and recurrent VTE events. Oral glucocorticoid use increases the disease-associated risk for first VTE.

INTRODUCTION

Glucocorticoids are potent anti-inflammatory drugs used for a variety of chronic diseases and acute conditions, as allergy, pulmonary, dermatological and rheumatic disorders and malignancies [1]. In contrast with their efficacy against several localized and systemic diseases [2], long term use of glucocorticoids can cause severe adverse events, such as diabetes, osteoporosis, hypertension and arterial cardiovascular diseases [3].

The risk of venous thromboembolism (VTE) has also been reported to be 2 to 3-fold increased with the use of glucocorticoids [4-6]. However, several associated conditions may account for this association [7], such as chronic diseases [8-13], periods of disease exacerbation or flares [11, 14, 15], comorbidities [16] and toxicity of concomitant medications [17]. Moreover, previous studies mainly addressed the relative risk of first VTE with glucocorticoid use [4-6], whereas clinical implications of medication treatment depend also on the absolute risk. Since the absolute risk for recurrent VTE is much higher than that for first VTE, it is worthwhile to study the effect of glucocorticoids on recurrent VTE as well. As far as we know, such studies have not been performed yet.

This study characterized individuals who use oral glucocorticoids and their risk of first VTE employing the self-controlled case-series (SCCS) method, which eliminates between-person confounding and time-invariant confounding by design [18]. We also assessed the absolute risk of recurrent VTE with oral glucocorticoid therapy in a standard follow-up design.

METHODS

Population description and clinical outcomes

This study enrolled patients and random digit-dialing (RDD) controls from the population-based case-control study of the Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis (MEGA) and from the MEGA follow-up study. The MEGA study was approved by the Ethics Committee of the Leiden University Medical Center, and written informed consent was obtained from all participants at the date of the inclusion in the study.

Between February 1999 and August 2004, 4956 consecutive patients, aged from 18 to 70 years-old, with a first acute VTE (deep vein thrombosis or pulmonary embolism) were identified at six anticoagulation clinics in the Netherlands [19, 20]. An extensive questionnaire on putative risk factors for VTE was filled in and blood was sampled on the day of enrolment for the study. The information about the diagnosis of VTE was obtained from hospital discharge reports and general practitioners. The diagnosis of deep vein thrombosis was confirmed with Doppler ultrasonography and the diagnosis of pulmonary embolism was confirmed with a ventilation perfusion lung scan, computed tomography of the chest or angiogram. Unprovoked VTE was defined as deep vein thrombosis (DVT) or pulmonary embolism (PE) without surgery, trauma, plaster cast, pregnancy or immobilization in the 3 months immediately before the event, prolonged travel in the 2 months immediately before the event, active malignancies in the 5 years prior the event or hormone use (oral contraceptives or hormone replacement therapy) at the time of the event. Patients who had one or more of these risk factors at time of their thrombotic event were classified as having had a provoked VTE.

A total of 4731 patients agreed to participate in a follow-up study (MEGA follow-up study). These patients were followed from their first episode of acute VTE until 2007-2009 when the vital status of all MEGA follow-up participants was acquired from the Dutch population registers, as described previously [21].Questionnaires concerning recurrent VTE were sent by mail between June 2008 and July 2009. Questions were asked by telephone interview when questionnaires were not returned. During the same period information about recurrences was retrieved from the anticoagulation clinics where patients were initially included for their first event and in case they moved house, also at the clinic nearest to their new address. Deaths due to recurrent VTE were counted as fatal recurrent events. Discharge letters were requested from the clinician who had diagnosed

the recurrence. A decision rule regarding certainty of the diagnosis was made according to the information collected per patient. Possible recurrences were classified into certain recurrences and uncertain recurrences. Details of this decision rule have been described previously [21]. In short, reported recurrences were classified as certain when 1) a discharge letter stated a diagnosis of a recurrent event based on clinical and radiological data, or 2) both the anticoagulation clinic and the patient reported a recurrent event that was at a clearly different location than the first event or occurred more than one year since the first event, or 3) a registered death from a recurrent event at least six months after the first event was found. For this analysis, we considered certain recurrences as outcome event only.

Exposure (use of oral glucocorticoids)

Information on oral glucocorticoid treatment was obtained by linking patients from the MEGA study to the Stichting Farmaceutische Kengetallen [SFK (Dutch Foundation for Pharmaceutical Statistics)] register (http://www.sfk.nl/english) [22]. In the Netherlands, oral glucocorticoids are only available by prescription, and over 95% of the community pharmacies are represented in this register. SFK contains information about patient-specific drugs dispensed: the generic name of a drug, the dose per pill, the Anatomical Therapeutic Chemical (ATC6) classification, the date of prescription, the total number of pills prescribed and the daily doses.

Linkage was based on a combination of age, sex, 4-digit postal code and vitamin K antagonist use within the first month after the initial VTE. Between 1999 and 2004, the inclusion period for the MEGA study, low molecular weight heparins (as well as direct oral anticoagulants) were not regularly prescribed for long-term anticoagulation [20]. In total, 2547 (54%) patients of the MEGA study could be individually linked with SFK. Daily doses were missing in 2% of prescriptions and were imputed considering the average daily dose prescribed in the immediately previous and future prescriptions for each patient. A gap period between prescriptions lasting less than four weeks was considered as continuing treatment. A new prescription starting after more than four weeks from the end of the previous prescription was considered a new period of treatment.

Study design

We investigated the risk of first VTE with oral glucocorticoid treatment using the SCCS design, in which patients who experienced both the outcome and exposure of interest are included in the analysis and there is no censoring by the outcome of interest [23]. The risk for the outcome is estimated comparing the within-patients rate of events during periods of exposure and periods of non-exposure. Using this approach, patients with a first VTE and at least one prescription of glucocorticoids during the inclusion period for the MEGA study (February 1999 to September 2004) were included in the analysis.

Next, in a longitudinal study design, we investigated the association of recurrent VTE with glucocorticoid treatment. Patients with a first VTE who could be linked to SFK register were included in the analysis. Duration of follow-up was estimated as the time at risk from the date of the index (first) VTE to the end of follow-up. The end of follow-up was defined as the date of a recurrent event and in the absence of a recurrence, the date of filling in the follow-up questionnaire or the last date they were known to be recurrence-free. Details of the end of follow-up assessment was described previously [21]. We considered certain recurrent events only, patients with uncertain recurrent events were censored from this uncertain recurrent event onwards. The association between oral glucocorticoids and recurrent VTE was examined using two different approaches. First, we evaluated the recurrence rates of VTE in patients whose first event was associated, or not, with an oral glucocorticoid treatment. Next, we investigated whether a current prescription of oral glucocorticoids affected the risk of recurrent VTE, as an approach to evaluate the safety of oral glucocorticoid treatment in patients with a prior VTE.

Statistical analysis

Figure 1 illustrates the study analyses. For the SCCS design we defined periods of exposure and non-exposure to oral glucocorticoids, as illustrated in Figure 1a. The follow-up time for each patient was firstly divided into two periods; the period when patients were unexposed to glucocorticoids (baseline period) and the total period of oral glucocorticoid treatment.

To address the issue of time-varying confounders, mainly represented by the exacerbation of the underlying disease, we chose to separate a period immediately before the start of treatment to allow the detection of the effect of disease activity on the risk of VTE and as an attempt to distinguish between disease effect (e.g. procoagulant state) and medication effects (e.g. procoagulant response), as previously described [24, 25]. Therefore, the total period of exposure to oral glucocorticoids was separated into five

periods: 1) the seven day-period immediately before a prescription was given, when we expected to observe the effect of the exacerbation of the disease on the risk of VTE, 2) the first 7 days with glucocorticoid treatment, 3) 8-30 days with oral glucocorticoid treatment, 4) 31-180 days with oral glucocorticoid treatment and 5) >180 days with oral glucocorticoid treatment. Periods 2 and 3 represent short-term use of oral glucocorticoid and periods 4 and 5 represent long-term glucocorticoid use.

We also differentiated between agents according to their degree of anti-inflammatory activity: hydrocortisone; prednisone, prednisolone and methylprednisolone; dexamethasone and betamethasone [26, 27].

To standardize the glucocorticoid doses, we first converted the daily dose into equivalent prednisolone dose [26, 28]. Next, we calculated the initial daily dose of the first glucocorticoid prescription and the cumulative dose in the prior 30 days with treatment (30-day cumulative dose). The initial daily dose was *a-priori* divided into four categories of clinical relevance [29, 30], as ≤7.5mg, 7.5-20mg, 21-39mg and ≥40mg equivalent prednisolone dose per day. The 30-day cumulative dose was categorized into three groups, according to the dose distribution in the study population, as ≤300mg, 300-2000mg and >2000mg equivalent prednisolone dose. Incidence rate ratios (IRR) and 95% confidence intervals (95%CI) for first VTE were estimated using conditional Poisson regression for the total period with glucocorticoid treatment and for the sub-periods, as compared with baseline period. IRRs for first VTE were also estimated with types of oral glucocorticoids and initial and 30-day cumulative prednisolone dose.

For the cohort study into recurrence risk, we first estimated absolute risks of a recurrence in patients with an otherwise provoked or unprovoked first VTE during a period of oral glucocorticoid treatment, as illustrated in Figure 1b. Incidence rates and 95%CI of recurrent VTE were estimated as the number of events over the accumulated follow-up time and with person time split for periods with and without glucocorticoid treatment, as illustrated in Figure 1c. Periods with glucocorticoid treatment were defined as the total period of glucocorticoid use by patient and then split into two periods; 1) first 180 days with treatment and 2) more than 180 days with treatment. Hazard ratios and 95% CI for recurrent VTE were estimated using Cox regression and were adjusted for age and sex. All statistical analysis were performed with the use of STATA software (version 14.1, College Station, TX, USA).

Role of the funding sources

The funding organizations are public institutions and had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the paper.

RESULTS

A total of 2547 patients with first VTE were linked to the SFK data register and were included in the study. Baseline demographic and clinical characteristics of these patients at the time of their first VTE are summarized in Table 1. The median age was 51 years old and 47% of the patients (n=1197) were male. Patients who could be linked to SFK registry had similar demographic and clinical characteristics as those who could not be linked to SFK, as shown in Appendix Table 1.

Risk of first venous thromboembolism with oral glucocorticoid treatment

Three hundred three patients with a first VTE received at least one outpatient prescription of oral glucocorticoids within the observation period and were included in the SCCS analysis. Table 1 summarizes the baseline demographic and clinical characteristics of these patients. The median number of prescriptions was 2 (interquartile range [IQR] 1-3) per patient and the median length of oral glucocorticoid treatment was 102 days (IQR 36-238 days) per prescription. Eighty-five patients (23%) had their first VTE event during a period of glucocorticoid use.

Table 2 shows the IRR of VTE during oral glucocorticoid treatment. The risk for a first VTE event was 3.5-fold (95%CI, 2.55-4.80) higher in the aggregated period of oral glucocorticoid treatment as compared with baseline periods (without treatment). Furthermore, oral glucocorticoid treatment enhanced the risk of first VTE associated with the condition that motivated this treatment. The IRR of a first VTE event was 2.5 (95% CI, 1.1-5.7) in the week before treatment started, reflecting the risk for VTE with the underlying condition, and was increased to 5.3 (95% CI, 2.9-9.5) after glucocorticoid treatment started (first 7 days with treatment). IRR of first VTE remained elevated (IRR 3.7; 95% CI, 2.6-5.2) afterwards until six months with treatment and was reduced to 1.6 (95% CI, 0.8-3.1) during prolonged treatment (after 6 months with oral glucocorticoids), as compared with baseline periods (Table 2).

The observed risk for VTE did not change with the potency of the oral glucocorticoid prescribed. For this analysis we did not consider treatments with cortisone, hydrocortisone or with combination of several types of glucocorticoids separately because the number of prescriptions in these groups was small. A dose-dependent relationship between oral glucocorticoid treatment and VTE risk was observed as the IRR increased from 3.4 (95% CI, 2.3-5.0) with 30-day cumulative doses below 300mg to 4.9 (95% CI, 1.7-14.0) with 30-day cumulative doses above 2000mg, as compared with baseline periods. A dose-dependent relationship between oral glucocorticoids and VTE risk was not observed, however, with increases in the initial dose (Table 2).

IRRs for DVT (3.9; 95% CI, 2.9-9.5) and PE (3.1; 95% CI, 2.0-4.9) were similar and the IRR for unprovoked VTE (2.4; 95% CI, 1.3-4.7) was lower than the IRR for provoked VTE (4.2; 95% CI, 2.9-6.0). To rule out the effect of malignancy on the risk of provoked VTE, we performed a sensitivity analysis removing 94 patients who had VTE and malignancy. This analysis did not substantially change the results (Table 3).

Risk of recurrent venous thromboembolism with oral glucocorticoid treatment at the time of the first event and during the follow-up

All 2547 patients with a first VTE event who were linked to the SFK data register were included in these analyses. During a median follow-up time of 2071 days (IQR 632-2658), 424 patients received 857 prescriptions of oral glucocorticoids. The median number of prescriptions was 1 (IQR 1-2) per patient and the median length of oral glucocorticoid treatment was 128 days (IQR 46-304 days) per prescription.

Three hundred sixty-seven patients had a recurrent VTE during the follow-up period. Of them, 13 patients (8%) had a VTE recurrence during a period of oral glucocorticoid use. As shown in Table 4, the absolute risk of recurrent VTE was 2.8%/year during periods without oral glucocorticoids (baseline periods) and increased to 7.5%/year during periods of oral glucocorticoid treatment. The risk for recurrent VTE was 2.7-fold increased (95% CI, 1.6-4.8) during treatment periods as compared to baseline periods and did not vary substantially according to the length of the treatment.

We also estimated the incidence rate of recurrent VTE separately for patients with provoked or unprovoked first VTE event during a period of glucocorticoid treatment. The results are shown in Table 5. The lowest absolute risk of recurrent VTE was observed in

patients with provoked first VTE who were not using glucocorticoids at the time of their event (2.2%/year). The absolute risk of recurrent VTE was elevated in patients with unprovoked VTE (i.e. 4.5%/year) and in those who had their first VTE during a period of oral glucocorticoid treatment, either if their event was otherwise classified as provoked (4.9%/year) or unprovoked (7.8%/year). The age and sex adjusted HRs for recurrent VTE were 1.6 (95% CI, 1.2-2.0) in patients with unprovoked VTE not associated with oral glucocorticoids, 2.1 (95% CI, 1.2-3.8) in those with glucocorticoid-related provoked VTE and 2.3 (95%CI, 1.2-7.0) in those with glucocorticoid-related unprovoked VTE, as compared with patients with a provoked first event not associated with oral glucocorticoids (Table 5).

DISCUSSION

In this study, the risk for incident VTE was 3.5 fold higher during periods of outpatient treatment with oral glucocorticoids than in periods without this medication. These results are in line with observational data from a large population based case-control study [6] and from a recent SCCS study [5] that reported a roughly 3-fold increase in the risk for VTE. An advantage of our study is that the SCCS method automatically adjusts for fixed confounders such as age, frailty and socioeconomic status. However, as with the other observational studies [4-6], our results are susceptible to time-varying confounders, mainly represented by the exacerbation of the underlying disease. To address this issue, we separated the total period of exposure to oral glucocorticoids into five periods: one period immediately before the exposure, when we expected to observe the effect of the exacerbation of the disease on the risk of VTE and 4 periods with glucocorticoid treatment, which represented short and long-term glucocorticoid use. Relative to the baseline period, the risk for incident VTE increased by 2.5-fold one week before the prescription of an oral glucocorticoid and by 4 to 5-fold in the following weeks after the treatment started. VTE risk was only reduced after 6 months with treatment, when we expected a controlled underlying disease. These results demonstrate that although there is a risk for VTE with the underlying disease, particularly when the condition is severe enough to require a glucocorticoid treatment, this risk is further increased when oral glucocorticoids have been started and remains elevated during treatment period up to six months. Therefore, our findings suggest that outpatient oral glucocorticoid use worsens the disease-associated risk of VTE. Pathophysiological studies support such an

association from a causal point of view as it has been shown previously in a randomized clinical trial that oral glucocorticoids induce a procoagulant state in healthy individuals [31].

In addition to the time-dependent relationship, we observed a dose-response association between oral glucocorticoids and VTE risk. This also suggests a direct effect of glucocorticoid treatment. Initial doses as high as 40mg per day and cumulative dose above 2000mg in 30 days increased the risk for VTE 4-5 times as compared with no glucocorticoid use. However, VTE risk was still increased more than 3 times with doses as low as 7.5 mg per day. These findings are confirmatory to Waljee et al [5] who have previously described a 3-fold increase in the risk for VTE with doses below 20mg per day. Although the association between low-dose glucocorticoids and VTE risk may appear counterintuitive, concerns on safety of low dosages exist [29] and some adverse events, including weight gain, eye cataract, acne and skin bruising also occur in patients taking low dosages of the drug [32]. As low-dose glucocorticoids are often prescribed for less severe conditions, these results further suggest that oral glucocorticoids have a direct effect on the risk of VTE that seems independent of the underlying disease severity.

In our study, oral glucocorticoids were associated not only with the risk for a first VTE event but also with the risk of recurrent VTE. Having a first VTE event while taking oral glucocorticoids doubled the risk of having a recurrent event, regardless of the cause of the first event (provoked or unprovoked). Our results also showed that the prognosis of glucocorticoid-associated VTE was worse than that of unprovoked VTE, as the risk for recurrence was 1.6-fold increased with unprovoked VTE and 2-fold increased with glucocorticoid-associated VTE. Unprovoked VTE is a predictor of worse prognosis and may be an indication for continuous anticoagulation [33]. Therefore, a careful follow-up of patients with glucocorticoid-associated VTE may be necessary. We also observed that the risk for a recurrent VTE was more than 2 times increased during a period of oral glucocorticoid use, regardless of the duration of the treatment. This finding highlights that patients with a prior VTE are under higher risk for recurrence in the periods when a glucocorticoid treatment is indicated. This finding further reinforces that a careful follow-up during these periods may be necessary.

Some limitations to our study need to be considered in order to interpret the results. First, only 54% of the patients from the MEGA study could be linked to the SFK registry. However, there is no reason to assume that linked and non-linked patients were

different, as was also shown by the similar baseline characteristics of linked and not linked patients. Second, we could not detect the condition that motivated the prescription of oral glucocorticoids because the diagnoses were not available in the SFK registry. By using the SCCS method, we controlled for fixed confounding such as chronic disease and socio-economic status, however, time-varying confounding, such as disease flares, was not controlled for. We used information on the pre-exposure period and initial and cumulative doses as surrogate measurements for disease severity. This approach seems reasonable as the condition must be sufficiently severe before a treatment with oral glucocorticoids is prescribed, particularly at high dose, and must be under control when the dose is reduced [11]. However, it is not possible with our data to completely separate the effect of the drug from that of the underlying condition because these effects are coincident. It is worth noting, though, that no study design would be capable of completely separating these effects and SCCS is the design that comes closest to that purpose so far. Furthermore, other time-varying confounders, such as co-medication, could not be addressed. Third, we only evaluated data on outpatient oral glucocorticoid treatment, so treatments conducted during hospital stays *were not available. Furthermore*, treatments with non-oral glucocorticoids (inhaled, injections, *intestinal-acting) were not evaluated because the systemic bioavailability of these drugs is heterogeneous [34, 35], particularly when compared with that of oral glucocorticoids [36]. Fifth, we only adjusted for age and sex in the Cox regression analysis because of small numbers and it is possible that other confounders played a role in the results on recurrent VTE.*

In conclusion, patients receiving oral glucocorticoids have a more than three-fold increase in risk of a first VTE event and those with a prior VTE have a two-fold increase in the risk of VTE recurrence. As oral glucocorticoids are commonly prescribed for a wide range of conditions, awareness of the drug associated risk for VTE may improve treatment strategies to prevent this complication. Although there is not enough evidence to support changes in current indication for oral glucocorticoid treatment, patients with higher risk for VTE, including those with a prior VTE event, should be treated and followed with caution. Given the risk for incident and recurrent VTE associated with oral glucocorticoids, future clinical trials are warranted to examine whether a prophylactic anticoagulation is beneficial for patients starting oral glucocorticoid treatment, in particular for those at higher risk for VTE.

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Disclosures

None.

Legends to Figures and Tables

Figure 1. Study analysis.

A) The observation period coincided with the inclusion period for the MEGA study (between February 1999 and September 2004). Periods with treatment were defined according to the day of prescription. Six risk periods were defined: 1) baseline period (without oral glucocorticoids); 2) seven-day preexposure period; 3) first 7 days with oral glucocorticoids treatment; 4) 8 - 30 days with oral glucocorticoids; 5) 31- 180 days with oral glucocorticoids and 6) more than 180 days with oral glucocorticoids. A new baseline period started the day after the end of prescription.

B) The follow-up started from the date of the first (1^{st}) VTE event until the date of a recurrent event and in the absence of a recurrence, the date of the end of the study (September 2009). The risk for recurrent VTE was estimated in patients with an otherwise provoked or unprovoked first VTE during a period of oral glucocorticoid (GC) treatment.

C) The follow-up started from the date of the first (1^{st}) VTE event until the date of a recurrent event and in the absence of a recurrence, the date of the end of the study (September 2009). The follow-up period was split into periods with and without oral glucocorticoid treatment to evaluate the association between current oral glucocorticoid treatment and recurrent VTE.

Table 1 Demographic and clinical characteristics of patients with first venous thromboembolism and a prescription of oral glucocorticoid during the observation period.

Table 2 Results from the Self-Controlled Case Series analysis for the use of oral glucocorticoid and risk of first venous thromboembolism.

Table 3 Results from the Self-Controlled Case Series analysis for the use of oral glucocorticoid and risk of different types of venous thromboembolism during the glucocorticoid treatment period.

Table 4 Risk of recurrent VTE related to periods of glucocorticoid use

Table 5 Risk of recurrent VTE related to glucocorticoid use at the day of the first venous thromboembolism

Appendix Table 1 Demographic and clinical characteristics of patients linked and not linked to SFK registry at time of first venous thromboembolism event.

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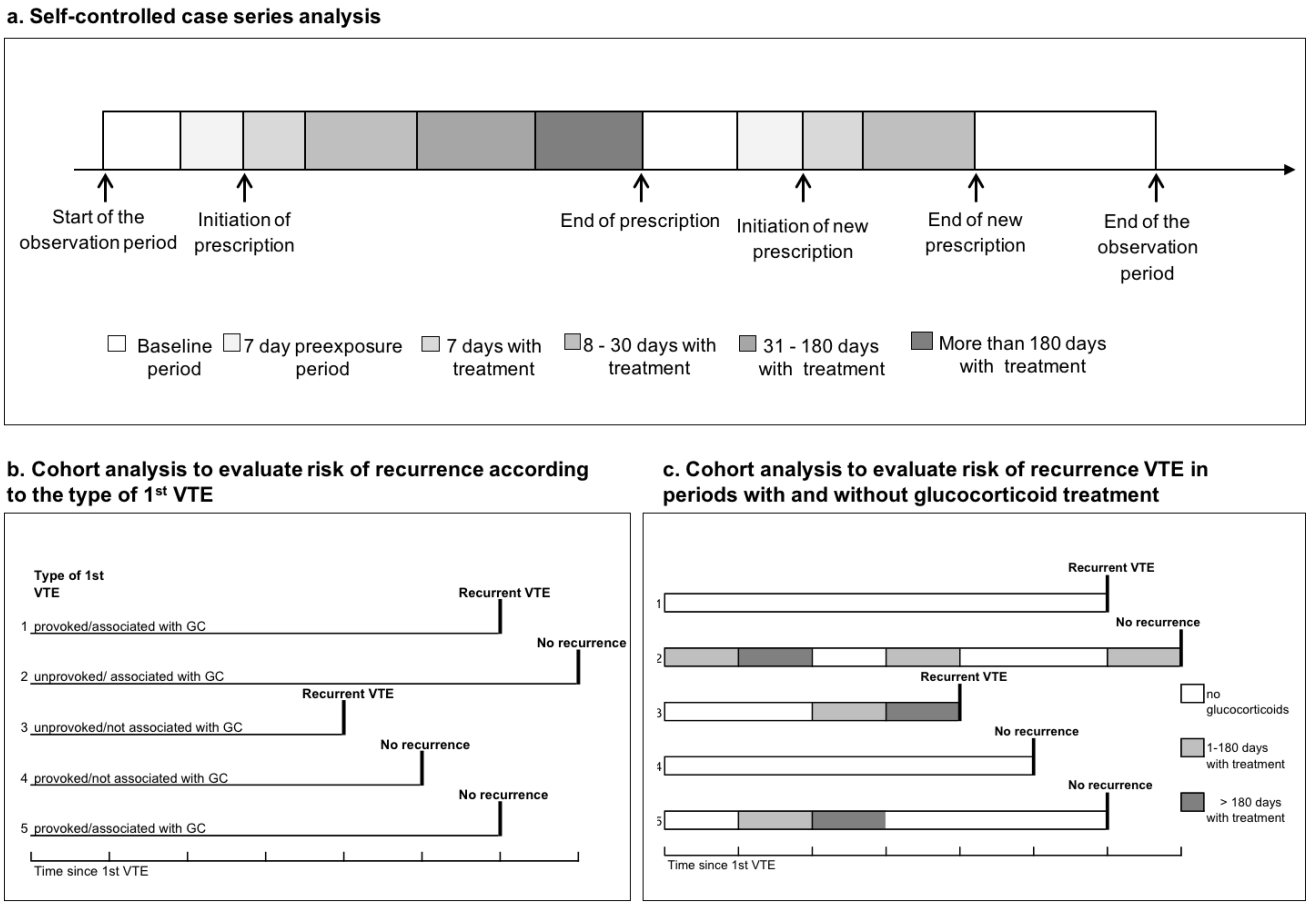

Figure 1: Study analysis

a. The observation period coincided with the inclusion period for the MEGA study (between February 1999 and September 2004). Periods with treatment were defined according to the day of prescription. Six risk periods were defined: 1) baseline period (without oral glucocorticoids); 2) seven-day preexposure period; 3) first 7 days with oral glucocorticoids treatment; 4) 8-30 days with oral glucocorticoids;5) 31- 180 days with oral glucocorticoids and 6) more than 180 days with oral glucocorticoids (not drawn to scale). A new baseline period started the day after the end of prescription.

b. The follow-up started from the date of the first (1st) VTE event until the date of a recurrent event and in the absence of a recurrence, the date of the end of the study (September 2009). The risk for recurrent VTE was estimated in patients with an otherwise provoked or unprovoked first VTE during a period of oral glucocorticoid (GC) treatment.

c. The follow-up started from the date of the first (1st) VTE event until the date of a recurrent event and in the absence of a recurrence, the date of the end of the study (September 2009). The follow-up periodwas split into periods with and without oral glucocorticoid treatmentto evaluate the association between current oral glucocorticoid treatment and recurrent VTE.

Table 1 Demographic and clinical characteristics of patients with first venous thromboembolism and a prescription of oral glucocorticoid during the observation period.

Patients with VTE and a prescription of oral glucocorticoids (n=363)	
Age, mean (SD)	54.2 (11.3)
Male, n(%)	168 (46%)
DVT only, n (%)	182 (50%)
PE±DVT, n (%)	181 (50%)
Provoked, n(%)	241 (66%)
Unprovoked, n(%)	103 (28%)
Inflammatory disease*	84 (23%)
rheumatoid arthritis	22 (26%)
multiple sclerosis	3 (4%)
emphysema	13 (15%)
chronic bronchitis	46 (55%)
Malignancy in the previous 5 years	94 (26%)
Time since diagnosis	
0 to 3 months	36 (39%)
> 3 months to □ 1 year	22 (24%)
>1 to □ 3 years	23 (25%)
>3 to □ 5 years	12 (13%)
Main sites of malignancy	
lung	18 (19%)
myeloma	8 (9%)
other hematological malignancies †	16 (17%)
gastrointestinal	11 (12%)
breast	12 (13%)
other female malignancies ‡	5 (5%)
male malignancies ±	7 (7%)
urinary	3 (3%)

Age is expressed in years. DVT, deep vein thrombosis; PE, pulmonary embolism. Provoked VTE was considered if: malignancy, trauma/surgery/ immobilization, plaster cast, estrogen use, pregnancy/puerperium, travel >4 h

* The diseases listed in this table were self-reported. Data on chronic disease or malignancy in the past 5 years were missing in 38 patients. Data on the date of malignancy diagnosis was missing in 1 patients.

† non-Hodgkin lymphoma, Hodgkin lymphoma, leukemia.‡ cervix, endometrium, ovarium. ± prostate or testis cancer. Gastrointestinal cancer were: colon, stomach, esophagus, liver or pancreas cancer. Urinary cancer were bladder or kidney cancer.

Table 2 Results from the Self-Controlled Case Series analysis for the use of oral glucocorticoid and risk of first venous thromboembolism

Oral glucocorticoid	**All VTE episodes (363 cases)**
	IRR (95%CI)
Any oral glucocorticoid	
No oral glucocorticoid	reference
Use of glucocorticoid	3.5 (2.5- 4.80)
Type of glucocorticoid drug	
No oral glucocorticoid	reference
Prednisone Prednisolone or Triamcinolon	3.2 (2.2- 4.5)
Dexamethasone or Betamethasone	3.4 (1.8- 6.6)
Initial daily equivalent prednisolone dose*	
0 mg	reference
<=7.5 mg	3.7 (2.1- 6.5)
7.5 - 20mg	3.1 (1.9- 4.9)
21 - 39mg	3.7 (1.9- 7.2)
>= 40mg	4.0 (2.1- 7.7)
30-day cumulative equivalent prednisolone dose at the time of VTE[#]	
0 mg	reference
<= 300mg	3.4 (2.3- 5.0)
300 - 2000mg	3.5 (2.3- 5.3)
>2000mg	4.9 (1.7- 14.0)
Days since prescription start date	
No oral glucocorticoid	reference
7 days before prescription	2.5 (1.1- 5.7)
First 7 days with glucocorticoid treament	5.3 (2.9- 9.5)
8 - 30 days with glucocorticoid treament	3.8 (2.3- 6.3)
31 - 180 days with glucocorticoid treament	3.7 (2.4- 5.3)
> 180 days with glucocorticoid treament	1.6 (0.8- 3.1)

*Initial daily doses were obtained from the first prescription of the treatment [#]Cumulative dose was calculated by summing the consecutive daily doses in the last 30 days of the treatment. CI denotes confidence interval; IRR, incidence rate ratio

Table 3 Results from the Self-Controlled Case Series analysis for the use of oral glucocorticoid and risk of different types of venous thromboembolism during the glucocorticoid treatment period.

Type of VTE(n=363)	**IRR (95%CI)**
DVT only (n=182)	3.9 (2.5-6.0)
PE +/- DVT (n=181)	3.1 (2.0-4.9)
Unprovoked (n=103)	2.4 (1.3-4.7)
Provoked (n=241)	4.2 (2.9-6.0)
Provoked without malignancy (n=147)	5.0 (3.1-8.2)

DVT, deep vein thrombosis; PE, pulmonary embolism; Provoked VTE was considered if: malignancy, trauma/surgery/ immobilization, plaster cast, estrogen use, pregnancy/puerperium, travel > 4 h; Unprovoked VTE was considered if no risk factor was present at the time of the event. We considered VTE provoked by malignancy if there was a diagnosis of neoplasia in the 5 years time prior to the first VTE event. Data on malignancy was missing in 4 patients

Table 4 Risk of recurrent VTE related to periods of glucocorticoid use.

Oral Glucocorticoids	Observation years	Recurrent events	Incidence rate per 1000 person-years (95%CI)	Hazard ratio (95%CI)	Hazard ratio (95%CI)*
Aggregated period of oral glucocorticoid treatment					
No oral glucocorticoid	12435	354	28.5 (25.7- 31.6)	reference	reference
Oral glucocorticoid treatment	172	13	75.4 (43.8- 129.8)	2.5 (1.4- 4.4)	2.7 (1.6- 4.8)
Periods with treatment					
No oral glucocorticoid	12435	354	28.5 (25.7- 31.6)	reference	reference
≤180 days with glucocorticoid treatment	103	7	67.8 (32.3- 142.2)	2.5 (1.2- 5.2)	2.6 (1.2- 5.4)
>180 days with glucocorticoid treatment	69	6	86.7 (39.0- 193.1)	2.6 (1.2- 5.8)	2.9 (1.3- 6.6)

*Adjusted for age and sex

Table 5 Risk of recurrent VTE related to glucocorticoid use at the day of the first venous thromboembolism

Oral Glucocorticoids	Observation years	Recurrent events	Incidence rate per 1000 person-years (95%CI)	Hazard ratio (95%CI)	Hazard ratio (95%CI)*
Provoked 1st VTE/no glucocorticoids	8557	185	21.6 (18.7- 25.0)	reference	reference
Unprovoked 1st VTE/ no glucocorticoids	3447	156	45.3 (38.7- 52.9)	2.1 (1.7-2.6)	1.6 (1.2- 2.0)
Provoked 1st VTE/ glucocorticoids	245	12	49.0 (27.8- 86.2)	2.2 (1.3-4.0)	2.1 (1.2- 3.8)
Unprovoked 1st VTE/ glucocorticoids	64	5	78.4 (32.6- 188.3)	3.4 (1.4-8.3)	2.3 (1.2- 7.0)

1st VTE. first venous thromboembolism; CI. confidence interval
*Adjusted for age and sex

Appendix Table 1 Demographic and clinical characteristics of patients linked and not linked to SFK registry at time of first venous thromboembolism event

	Patients linked to SFK		**Patients not linked to SFK**	
N (%)	2547	(54%)	2184	(46%)
Age, mean (SD)	51	(18-70)	47	(18-70)
Male, n(%)	1197	(47%)	967	(44%)
DVT only, n (%)	1490	(59%)	1257	(58%)
PE plus DVT, n (%)	1057	(41%)	927	(42%)
PE only, n (%)	826	(32%)	723	(33%)
Provoked, n(%)	1732	(68%)	1565	(72%)
Malignancy in the previous 5 years	247	(10%)	174	(8%)
Trauma/surgery/ immobilization	1033	(41%)	869	(40%)
Plaster cast	107	(4%)	112	(5%)
Estrogen use (women)	663	(26%)	687	(31%)
Pregnancy/puerperium (women)	86	(3%)	87	(4%)
Travel > 4 h	367	(14%)	350	(16%)
Unprovoked, n (%)	742	(29%)	559	(26%)

Age is expressed in years. DVT, deep vein thrombosis; PE, pulmonary embolism; SFK, Stichting Farmaceutische Kengetallen (Foundation for Pharmaceutical Statistics) registry

Summary and Discussion

SUMMARY AND GENERAL DISCUSSION

The aim of this thesis was to investigate the association of commonly prescribed drugs, such as statins and glucocorticoids, with changes in hemostasis and VTE risk. In this chapter we provide an overview of our main findings. Furthermore, we considere the clinical implications and discuss directions for future research.

STATINS TO MODULATE HYPERCOAGULABILITY AND THROMBOTIC RISK

In **Chapter 2**, we performed a narrative review on the effect of statins on hemostasis and VTE risk. We observed that previous data have suggested that statins might decrease VTE risk. The JUPITER trial, which is thus far the only statin trial performed with VTE as the outcome of interest, showed that rosuvastatin may reduce the risk of VTE in 40% in apparently healthy men over the age of 50 years and women over 60 years old [1]. The authors called "apparently healthy persons" those individuals with no history of cardiovascular disease, low levels of low-density lipoprotein (LDL) cholesterol and increased levels of high-sensitivity C-reactive protein level [1]. We also described that meta-analyses of observational studies reported a VTE risk reduction of 14 - 54% and meta-analyses of randomized controlled trials (RCT) reported a 11 – 25% reduction in VTE risk among statin users [2-4]. Furthermore, a meta-analysis of observational studies showed that statins may reduce the overall risk of recurrent VTE by 27% [5].

Although these findings might encourage the use of statins for VTE prevention, the results must be interpreted with caution for several reasons. First, data from the JUPITER trial may be questioned because the results on VTE came from a secondary analysis of the original trial, in which the primary outcome was the occurrence of a first major cardiovascular event (myocardial infarction or unstable angina, stroke, arterial revascularization procedure, or confirmed death from cardiovascular causes); moreover, the results may have run into statistical problem of small numbers, since in the statin treatment arm only 34 participants developed a venous thrombotic event, and chance, or a type I error, may therefore have influenced this result. Second, meta-analysis of observational studies has limitations, related to the studies included, that cannot be fully addressed, such as confounding and particularly survivor bias [6, 7]. As examples of potential bias in observational studies, the underlying disease severity in patients selected

for statin therapy may be different, even milder than that in patients for whom statin was not indicated (healthy user effect) [8], the inclusion of prevalent statin users results in missing the events that occurred in statin users before the inclusion, early after starting treatment (survivor bias), and in addition, observational studies are not able to control for those patients who do or do not adhere to statin treatment (adherence bias) [6]. Third, meta-analyses of RCTs included non-published data, which could have led to biased estimates, and were influenced by the results of the JUPITER trial. Fourth, the meta-analysis that pointed to a potential role of statin therapy in secondary VTE prophylaxis relied on a small number of observational studies, and a biased association between statin use and prevention of VTE recurrence cannot be ruled out. So far, the use of statins for secondary VTE prophylaxis has not been evaluated in well-designed intervention studies, such as RCTs.

More high quality evidence is needed to establish whether statin therapy is effective to prevent incident or recurrent VTE. However, the only trial currently being conducted for evaluating the effect of statins use on venous events, the SAVER trial (NCT02679664), aims to determine the feasibility of recruitment of VTE patients and to assess whether rosuvastatin can reduce post-thrombotic syndrome in VTE patients. The risk of recurrent VTE may be evaluate as a secondary endpoint if the recruitment is feasible. Therefore, the question on the role of statin therapy in VTE prevention will probably remain unanswered for the following years.

A possible explanation for the lack of conducting such costly studies is the fact that a clear biological mechanism behind the supposed effect of statins on the pathophysiology of VTE is not known. For this reason, the biological effect of statins on hemostasis was reviewed in **Chapter 2**, in which we observed that basic research and clinical studies suggest that statins are capable of decreasing platelet activity [9-21], downregulate both initiation and amplification phases of coagulation [12, 14, 22-32] and stimulate fibrinolysis [33-37]. However, the mechanisms underlying the statins effect on several phases of hemostasis are not explained. Thus, the reported findings raise suspicions of a problem similar to that encountered in the parable of "the blind men and the elephant", in which blind men reported different opinions about what was an elephant after touching only one part of the animal. This means that the inability of observing a problem as a whole may lead to opposing opinions on the same issue. As a result of such

imprecise findings, the potential antithrombotic property of statins is regarded with skepticism.

The best known mechanism by which statins may impair hemostasis is the direct downregulation of TF expression on endothelium cells and monocytes [38-42]. Several studies consistently demonstrated that statins affect hemostasis particularly by downregulating TF expression on endothelium cells and monocytes through a direct inhibitory effect on transcriptional activation of TF gene [38-42]. Although this mechanism can explain, in part, the downregulation of coagulation pathways, it cannot explain the impairment in platelet function and fibrinolysis. Furthermore, TF is not associated with the risk of VTE [43] and, therefore, a decrease in TF antigen or activity could not explain the supposed effect of statins on VTE prophylaxis. In this regard, the use of standard methods to measure the individual hemostatic potential, such as global assays of coagulation or established biomarkers of VTE risk, could be helpful to provide more accurate data on the capacity of statins to revert a hypercoagulability state or even cause hypocoagulability.

Therefore, in **Chapter 3** we investigated whether rosuvastatin use decreases thrombin generation potential in patients with a prior VTE in the STAtins Reduce Thrombophilia (START) trial. Thrombin generation potential is a global coagulation test that reproduces the kinetics of thrombin formation [44, 45], reflects the coagulation potential of an individual [46-48] and also predicts the risk of a first and recurrent VTE [49-51]. The START trial is a RCT aimed to investigate if statin improves the coagulation profile in patients with prior VTE. After anticoagulation withdrawal, patients with VTE were randomized to rosuvastatin 20mg/day for 4 weeks or no intervention. The thrombin generation potential was assessed at baseline and at end of study by means of the thrombin generation assay (TGA), which is a global coagulation test using the Calibrated Automated Thrombogram® (Diagnostica Stago, France).

The primary endpoint was the difference in change in endogenous thrombin potential (ETP) and peak between rosuvastatin users and non-users. Analyses were done by intention to treat and regression models were adjusted for age and sex. The study comprised 245 patients, 126 rosuvastatin users and 119 non-users. Mean age was 58 years, 61% were men, 49% had unprovoked VTE and 75% had cardiovascular (CV) risk factors. Endogenous thrombin potential (ETP) increased from baseline to end of study in non-statin users (mean change: 97.22nM*min; 95%CI 40.92 to 153.53) and decreased in

rosuvastatin users (mean change: -24.94nM*min; 95%CI -71.81 to 21.93). The mean difference in ETP change between treatments was -120.24nM*min (95%CI -192.97 to -47.51), yielding a 10.4% ETP reduction by rosuvastatin. Thrombin peak increased in both non-statin (mean change: 20.69nM; 95%CI 9.80 to 31.58) and rosuvastatin users (mean change: 8.41nM; 95%CI -0.86 to 17.69). The mean difference in peak change between treatments was -11.88nM (95%CI -26.11 to 2.35), yielding a 5% peak reduction by rosuvastatin. Other thrombin generation parameters, such as time to peak, lag time and velocity index, did not change substantially. All these results were not materially affected when we restricted the analyses to patients who did not develop an acute infection during follow-up, and when we adjusted our findings for age and sex. Predefined subgroup analyses with regard to sex and VTE cause (provoked or unprovoked) revealed similar results as in the main analysis, while the reduction in ETP by rosuvastatin appeared more pronounced in participants with CV risk factors and with pulmonary embolism than in those without CV risk factors and with deep vein thrombosis, respectively.

We concluded that rosuvastatin 20mg/day improves the coagulation profile in patients who had a VTE by reducing thrombin generation potential after anticoagulation withdrawal. Given that thrombin generation potential is associated with a first VTE [16, 28, 29, 31] and can predict the risk of recurrent VTE [25-27, 30], our finding could be interpreted as statins having the potential to decrease the risk of recurrence. Although our results do not answer the question whether this effect of rosuvastatin on changing the coagulation profile has clinical significance in terms of reducing VTE risk, they provide a basis for interventional studies necessary to establish the efficacy of rosuvastatin on VTE prophylaxis.

Another open question is whether the effect of statins on hemostasis is dependent of the drug-related lipid-lowering effect. Studies *in vitro* have shown that statins are capable of inhibiting TF expression and platelet activity by different mechanisms from that involved in the lipid-lowering process [11, 20, 52]. From a clinical perspective, it is not possible, however, to rule out that the effect of statin use on hemostasis is due to a decrease in the serum levels of lipids, since many lipoproteins have been related to coagulation activity [16, 53, 54]. Recently, apolipoproteins (apos) A and B, which are the major functional components of high and low density lipoprotein respectively, have been associated with altered coagulation profile [53]. In **Chapter 4**, we further evaluated the association of apos C-I,-II,-III and E with hemostasis and with VTE risk. Apos C-I, C-II,

C-III and E have been associated with risk of arterial thrombotic diseases and whether these apos have prothrombotic properties and are also associated with risk of VTE is not known. The study population comprised a total of 127 VTE patients and 299 controls randomly selected from the Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis study (1999–2004), in the Netherlands. The apos were quantified using mass spectrometry (LC/MS/MS) and their levels were analysed as continuous variable (per SD increase). In controls, increases in levels of apolipoproteins were associated with increases in levels of vitamin K dependent factors, factor XI, antithrombin and clot lysis time. Additionally, increasing apos C-III and E levels were associated with higher factor VIII and von Willebrand factor levels. Levels of C-reactive protein were not associated with any apolipoprotein. The age and sex adjusted odds ratios of apos E, C-III, CII and CI to the risk of venous thrombosis were 1.21 (95% CI, 0.98-1.49), 1.19 (0.99-1.44), 1.24 (0.95-1.61) and 1.06 (95% CI, 0.87-1.30) per SD increase, respectively. These odds ratios did not attenuate after adjustments for statin use, estrogen use, BMI, alcohol use, and self-reported diabetes. We concluded that apos C-I, C-II, C-III and E are associated with several coagulation factors. However, whether these apos are also associated with an increased risk of VTE remains to be established as numbers on VTE outcomes were small in our study. These findings are, however, in line with previous population studies that showed that apo A and B are associated with a mild increase in the risk of VTE (roughly 30-50%) despite being consistently associated with levels of coagulation markers [53]. Furthermore, only apos C-III and E were associated with levels of FVIII and VWF, which coagulation proteins are well known risk factors for VTE [55-57]. A potential direct effect of statins on coagulation factors release by the liver or by the endothelium may play a role in the association of statins use and hemostasis. Future clinical studies to investigate/ confirm the effects of statins and apos to modulate hypercoagulability and thrombotic risk are warranted.

ROLE OF GLUCOCORTICOIDS IN THROMBOTIC RISK: PLAYERS OR VIEWERS?

Systemic glucocorticoids are steroid hormones prescribed to decrease inflammation in diseases and conditions such as arthritis (rheumatoid arthritis), systemic lupus erythematosus, inflammatory bowel diseases, asthma, bronchitis, allergy and neoplasia [58-62]. These drugs are potent anti-inflammatory drugs widely used in patients with

different diseases of various severity, from autoimmune to neoplastic disorders and pulmonary diseases that, in contrast, can cause severe adverse events. Experimental studies show that glucocorticoids increase coagulation factor levels and possibly the risk of VTE. Although the risk of VTE has been reported to be two- to three-fold increased with the use of glucocorticoids [63-65], several confounders may account for the reported association, such as chronic diseases [58-62], periods of disease exacerbation or flares [59, 66, 67], comorbidities [68] and toxicity of concomitant medications [69]. The effect of the underlying disease on the risk of VTE is of particular concern. Firstly, because asthma, rheumatoid arthritis, inflammatory bowel diseases, lupus erythematosus, Sjögren syndrom, polymyositis, multiple sclerosis, immune thrombocytopenia and malignancy, which are widely treated with oral glucocorticoids, have been associated with a 3 to 9-fold increased risk of VTE [59-62, 66, 67, 70, 71]. Second, because the suppression of the underlying inflammatory process by glucocorticoid use might otherwise contribute to prevent thrombotic events [68]. The latter is in line with the observations that glucocorticoid use are associated with decreased VWF and platelet activities and increased natural anticoagulants in the context of inflammatory states [72, 73]. Such effects of glucocorticoids on coagulation markers are consistent with a downregulation in hemostasis.

Therefore, in **Chapter 5** we evaluated the risk of first and recurrent VTE associated with the use of oral glucocorticoids, addressing the issue of potential confounders by employing the self-controlled case-series (SCCS) method. The periods of exposure to oral glucocorticoids were divided into: 1) the seven day-period immediately before a prescription was given, 2) the first 7 days with glucocorticoid treatment, 3) 8-30 days with oral glucocorticoid treatment, 4) 31-180 days with oral glucocorticoid treatment and 5) >180 days with oral glucocorticoid treatment. In addition, we evaluated the effect of oral glucocorticoids on the risk of recurrent VTE in a cohort design. Patients with VTE from the MEGA study were linked to the Dutch Foundation for Pharmaceutical Statistics (SFK) register based on a combination of age, sex, digit postal code and vitamin K antagonist use within the first month after the initial VTE. Prescriptions of oral glucocorticoids in the period of the MEGA study were identified. The risk for the first VTE was estimated using SCCS method and comparing the within-patients rate of events during periods of exposure and periods of non-exposure to oral glucocorticoids. The association between oral glucocorticoids and recurrent VTE was examined using Cox regression models and two different approaches: 1) comparing the recurrence rates of

VTE in patients whose first event was associated, or not, with an oral glucocorticoid treatment and 2) comparing the recurrence rates of VTE in patients with and without current use of oral glucocorticoids.

A total of 2547 patients were linked to the SFK data register, from those 363 received at least one outpatient prescription of oral glucocorticoids. The risk for a first VTE event was 3.5-fold higher in the aggregated period of oral glucocorticoid treatment as compared with baseline periods (incident rate ratio [IRR] 3.5, 95%confidence interval [CI] 2.6-4.8). IRR of a first VTE event was 2.5 (95% CI, 1.1– 5.7) in the week before treatment started, 5.3 (95% CI, 2.9 – 9.5) during the first 7 days with treatment, 3.7 (95% CI, 2.6 – 5.2) until six months with treatment and 1.6 (95% CI, 0.8 – 3.1) after 6 months with oral glucocorticoids, as compared with baseline period. A dose-dependent relationship between oral glucocorticoid treatment and VTE risk was observed as the IRR increased from 3.4 (95% CI, 2.3 – 5.0) with 30-day cumulative doses below 300mg to 4.9 (95% CI, 1.7 – 14.0) with 30-day cumulative doses above 2000mg, as compared with baseline periods. IRRs for DVT (3.9; 95% CI, 2.9 – 9.5) and PE (3.1; 95% CI, 2.0 – 4.9) were similar and IRR for unprovoked VTE (2.4; 95% CI, 1.3 – 4.7) was lower than the IRR for provoked VTE (4.2; 95% CI, 2.9 – 6.0). The rates of recurrent VTE were elevated in patients with unprovoked VTE and in those who had their first VTE during a period of oral glucocorticoid treatment, either if the first event was otherwise classified as provoked or unprovoked. The adjusted HRs for recurrent VTE were 1.6 (95% CI, 1.2 – 2.0) in patients with unprovoked first VTE not using oral glucocorticoids at the time of their first event, 2.1 (95% CI, 1.2 – 3.8) in those who had a provoked first VTE while using oral glucocorticoids and 2.3 (95% CI, 1.2 – 7.0) in those with an unprovoked first VTE while using the drug, as compared with patients with a provoked first event not using oral glucocorticoids at the time of their first event. The risk for recurrent VTE was 2.7-fold increased (95% CI, 1.6 – 4.8) during treatment periods as compared to baseline periods and did not vary substantially according to the length of the treatment (below or above 180 days). We concluded that patients receiving oral glucocorticoids had a more than three-fold increase in the risk for first VTE event. The observed risk for VTE was associated with the underlying disease (pre-exposure period) but increased further after oral glucocorticoids were prescribed. The risk for a VTE recurrence was 2-fold increased during a period of oral glucocorticoid treatment. Additionally, patients using oral glucocorticoids at the time of their first VTE event had higher risk for subsequent events than those without glucocorticoids at that time. The results underscore that treatment

strategies to prevent VTE in patients treated with oral glucocorticoids are needed. As oral glucocorticoids are commonly prescribed for a wide range of conditions, awareness of the drug associated risk for VTE may improve treatment strategies to prevent this complication. Although there is not enough evidence to support changes in current indication for oral glucocorticoid treatment, patients with higher risk for VTE, including those with a prior VTE event, should be treated and followed with caution. Given the risk for incident and recurrent VTE associated with oral glucocorticoids, future clinical trials are warranted to examine whether prophylactic anticoagulation is beneficial for patients starting oral glucocorticoid treatment, in particular for those at higher risk for VTE. As an example, the incidence rate of VTE in patients with active inflammatory bowel disease during non-hospitalized periods is 9 per 1000 person-years (59). Assuming that the relative risk of VTE increases 3-fold when a glucocorticoid treatment is started and anticoagulation decreases the risk of VTE by 80% (74), a trial with 470 participants receiving anticoagulation and 470 receiving placebo would be statistically powered (2 sided alpha=5%, power=80%) to determine whether anticoagulants reduce the risk of VTE in these patients.

Conclusions

Statins and systemic glucocorticoids are commonly prescribed drugs that interfere with the risk of VTE in different ways. Systemic glucocorticoid use increases the relative risk of first VTE by more than three-fold and *confers* an additional 5% *absolute risk of* recurrent VTE per year. These data underscore that prior use of systemic glucocorticoids should be considered when identifying provoking factors of VTE and that treatment strategies to prevent glucocorticoid-associated VTE are needed, especially in patients with higher risk for VTE. Since there is no evidence on which is the best treatment strategy to prevent glucocorticoid-associated VTE, and given the high risk of incident and recurrent VTE associated with oral glucocorticoids, clinical trials are warranted to examine whether prophylactic anticoagulation is beneficial for patients starting an oral glucocorticoid treatment.

On the other hand, rosuvastatin use may reduce the risk of first VTE by 40%. Although the mechanisms behind this association are not fully elucidated, this thesis has shown that rosuvastatin is capable of decreasing the thrombin generation potential by 10% in patients with a prior VTE. The finding that rosuvastatin modulates the coagulation

profile supports the hypothesis that rosuvastatin may be capable of reducing the risk of recurrent VTE, while not increasing the risk of bleeding. However, clinical evidence on the effect of statins on recurrent VTE risk is lacking and the role of statin use in recurrent VTE prophylaxis needs careful evaluation/confirmation in well-defined trials.

In conclusion, this thesis has shown that both statins and systemic glucocorticoids are capable of affecting the risk of VTE, improving the knowledge on the influence of this two commonly prescribed drugs on VTE pathophysiology. These findings have the potential to further refine the assessment of VTE risk since they highlight that the use of these drugs should be considered when evaluating the risk of VTE. Finally, this thesis provides insight into new therapeutic approaches since the results underscore that treatment strategies on VTE prevention in patients already taken statins, which may be sufficient for VTE prevention, are lacking. Treatment strategies to prevent glucocorticoid-associated VTE are also needed.

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Samenvatting en Discussie

SAMENVATTING EN ALGEMENE DISCUSSIE

Het doel van dit proefschrift was om te onderzoeken of vaak voorgeschreven geneesmiddelen, als statines en glucocorticoïden, geassocieerd zijn met concentraties van stollingsfactoren en veneuze trombo-embolie (VTE). In dit hoofdstuk worden de belangrijkste uitkomsten uit dit proefschrift besproken alsook de mogelijke klinische implicaties.

STATINES MODULEREN HYPERCOAGULABILITEIT EN TROMBOSE RISICO

Hoofdstuk 2 betreft een samenvatting van de literatuur over het mogelijke effect van statines op hemostase en VTE risico. Meerdere observationele studies alsook klinische trials hebben aangetoond dat statinegebruikers een lager risico hebben op het krijgen van VTE. In de JUPITER trial, die tot dusverre de enige statine trial is waarbij VTE één van de te onderzoeken uitkomstmaten was, hadden rosuvastatine gebruikers een 40% lager risico op het krijgen van het krijgen van VTE [1]. Observationele studies laten een 14-54% lager risico zien op het krijgen van VTE bij statine gebruikers, en een meta-analyse van gerandomiseerde klinische trials (RCT) rapporteerde een 11-25% lager risico op het krijgen van VTE bij statinegebruikers [2-4]. Verder liet een meta-analyse van observationele studies zien dat statine gebruik geassocieerd is met een 27% lager risico op het krijgen van recidief VTE [5].

Hoewel deze bevindingen het gebruik van statine voor VTE preventie lijken aan te moedigen, zijn er een aantal mogelijke valkuilen die een dergelijke conclusie voorbarig maken. Ten eerste kan de JUPITER trial bekritiseerd worden omdat de resultaten voor VTE secundaire onderzoeksresultaten betroffen. Ten tweede waren er slechts 34 deelnemers in de JUPITER trial die een VTE ontwikkelden waardoor een type I fout dit resultaat kan hebben beïnvloed. Ten derde namen de meta-analysen van observationele studies een aantal limitaties van observationeel onderzoek, zoals confounding en survivor bias, niet mee in hun opzet [6, 7]. Als voorbeeld: statine gebruikers kunnen gezonder zijn dan niet statine gebruikers (omdat statine niet wordt voorgeschreven aan mensen met een lage levensverwachting), en daarom, onafhankelijk van statine gebruik een lager VTE risico hebben (‘healthy user effect’) [8]. Ook kan de inclusie van prevalente statine gebruikers (dat zijn degenen die al statine gebruiken voordat de studie begint) leiden tot

het missen van gegevens van mensen die wel statine gebruikten, maar daar ofwel mee gestopt waren, of wel overleden waren alvorens de studie begon (‘survivor bias’). Ook kunnen observationele studies veelal niet corrigeren voor therapietrouw van statine, en kunnen observationele studies dat zeker niet doen als de niet therapietrouwe patiënten gestopt zijn met statine alvorens de studie begon (‘adherence bias’) [6]. Ten vierde, de meta-analyse van RCTs had ongepubliceerde (niet ‘peer reviewed’) data toegevoegd, wat de resultaten mogelijk heeft vertekend. Ten vijfde, de meta-analyse waaruit bleek dat statinetherapie mogelijk het risico op recidief VTE kon verlagen, betrof enkel (kleine) observationele studies en het kan niet uitgesloten worden dat ten gevolge van deze onderzoeksopzet de resultaten vertekend zijn.

Tot dusverre zijn er geen statine trials verricht of op dit moment lopend die recidief trombose als primaire uitkomst hebben. Hoewel betere onderzoeksmethoden nodig zijn om te onderzoeken of statinegebruik het risico op eerste en/of recidief VTE kan verlagen, is de verwachting dat dit de komende jaren niet zal gebeuren omdat dergelijke studies (RCTs) jaren duren en nog niet zijn opgezet. De enige trial die op dit moment patiënten met VTE randomiseert op statine, is de SAVER trial (NCT02679664). Echter, deze trial heeft post-trombotisch syndroom als uitkomstmaat en niet recidief VTE.

Een mogelijke verklaring waarom kostbare trials niet worden uitgevoerd om het effect van statine of (recidief) VTE te onderzoeken is omdat de pathofysiologie van *hoe* statines VTE risico kunnen verlagen niet duidelijk is. Om deze reden werden de effecten van statines op de hemostase samengevat in **Hoofdstuk 2**, waar we zagen dat zowel basale als klinische studies suggereren dat statines plaatjesreactiviteit kunnen beïnvloeden [9-21], de initiatie en amplificatie van de stolling downreguleren [12, 14, 22-32] en de fibrinolyse stimuleren [33-37]. Echter, het mechanisme dat het onderliggende effect van statines op de hemostase doet verklaren is onbekend. De gerapporteerde bevindingen van statine effecten op de hemostase kunnen daarom ook wel worden bekeken worden als de parabel van de blinde reizigers en de olifant. Deze parabel gaat over zes blinde reizigers die op hun levensweg tegen verschillende delen van een olifant aanlopen. Elke blinde reiziger schept vervolgens zijn eigen versie van de realiteit, gebaseerd op zijn eigen beperkte ervaring en perspectief. De moraal van deze parabel is een waarschuwing tegen absolute waarheden op plekken waar scepsis meer op zijn plaats is.

Het meest bekende mechanisme van hoe statines de hemostase kunnen beïnvloeden is via downregulatie van tissue factor (TF) expressie op het endotheel en monocyten door een direct remmend effect op de transcriptieactiviteit van het TF gen [38-42]. Ofschoon dit mechanisme deels de downregulatie van coagulatie kan verklaren, verklaart het niet de remming van plaatjesfunctie en fibrinolyse, wat ook wordt toegeschreven aan statinegebruik. Verder is TF niet geassocieerd met VTE risico [43] en kan om die reden TF antigeen- of activiteitremming door statine niet het effect van statine op vermindering van het VTE risico verklaren. Het zou interessant zijn om te kijken of statine ook globale testen van pro-coagulatie kan beïnvloeden die *wel* geassocieerd zijn met een verhoogd VTE risico.

Om deze reden werd in **Hoofdstuk 3** onderzocht of rosuvastatine trombinegeneratie kan beïnvloeden in patiënten met VTE die gerandomiseerd waren in de START trial. De trombinegeneratie test is een globale stollingstest, [44,45] die het risico op eerste VTE en recidief VTE kan voorspellen [49-51]. De START trial is een RCT die als primair onderzoeksdoel heeft om te onderzoeken of het stollingsprofiel verandert door statine bij patiënten die eerder VTE hadden gehad. Nadat patiënten gestopt waren met antistollingstherapie werden zij voor vier weken al dan niet gerandomiseerd op rosuvastatine (20mg/dag). De trombinegeneratie potentiaal werd beoordeeld aan het begin van de studie (baseline) en 4 weken later (end of study). Het primaire eindpunt was het verschil in verandering van endogeen trombine potentiaal (ETP) en trombine generatie piek tussen rosuvastatinegebruikers en niet-statine gebruikers. De analysen werden onder ‘intention to treat’ uitgevoerd en regressiemodellen werden gecorrigeerd voor leeftijd en geslacht. De studie bevatte 245 patiënten, 126 rosuvastatine gebruikers en 199 niet-statine gebruikers. De gemiddelde leeftijd was 58 jaar, 61% was man, 49% had een niet-uitgelokte VTE en 75% had cardiovasculaire (CV) risicofactoren. De ETP nam toe van baseline naar end of study in de niet-statine gebruikers (gemiddeld verschil 97.22nM*min; 95%BI 40.92 tot 153.53) en nam af in rosuvastatine gebruikers (gemiddeld verschil -24.94nM*min; 95%BI -71.81 tot 21.93). Het gemiddeld verschil in ETP verandering tussen beide behandelgroepen was -120.24nM*min (95%BI -192.97 tot -47.51), oftewel een 10.4% reductie in ETP door rosuvastatine. De trombinepiek nam toe in zowel de niet-statine groep (gemiddeld verschil: 20.69nM; 95%BI 9.80 tot 31.58) en de rosuvastatine groep (gemiddeld verschil: 8.41nM; 95%BI -0.86 tot 17.69). Het

gemiddelde verschil in trombine piek tussen beide behandelgroepen was -11.88nM (95%BI -26.11 tot 2.35), oftewel een 5% afname in trombinepiek door rosuvastatine. Andere trombinegeneratie parameters, zoals tijd tot piek, 'lag time'en 'velocity index' veranderden niet substantieel. Subgroep-analysen in relatie tot geslacht en oorzaak van VTE (uitgelokt vs onuitgelokt) lieten soortgelijke resultaten zien als de hoofdanalyse, maar de reductie van ETP was sterker in deelnemers met CV risicofactoren en met een longembolie.

Geconcludeerd werd dat 20mg/dag rosuvastatine het stollingsprofiel verbetert in patiënten met eerdere VTE omdat het de trombinegeneratiepotentiaal verlaagt. Gegeven dat trombinegeneratie geassocieerd is met eerste VTE [16, 28, 29, 31] en het risico op recidief VTE kan voorspellen [25-27, 30], interpreteren wij deze bevinding als zijnde dat statines het potentieel hebben om de kans op VTE te verlagen doordat zij de trombinegeneratie verlagen.

Een andere vraag is of het effect van statines op de hemostase afhankelijk is van de lipiden-veranderende effecten van statine gebruik. *In vitro* studies hebben aangetoond dat statines in staat zijn om TF-expressie en plaatjesactiviteit te onderdrukken via een mechanisme dat niet samenhangt met de lipiden-veranderende effecten van statinegebruik [11, 20, 52]. Echter, of de effecten van statinegebruik op de hemostase volledig via niet-lipiden-veranderende mechanismen lopen is niet aangetoond [16, 53, 54]. Recentelijk zijn apolipoproteïnen (apos) A en B geassocieerd met een afwijkend stollingsprofiel [53]. In **Hoofdstuk 4**, hebben we onderzocht of er een associatie bestond tussen apos C-I,-II,-III and E met coagulatie en VTE risico.

Apos C-I, C-II, C-III en E zijn geassocieerd met het risico op arteriële cardiovasculaire aandoeningen, maar of deze apos ook prothrombotische eigenschappen hebben, en daarmee het risico op VTE verhogen, is onbekend. Om protrombotische effecten van deze apos te onderzoeken werden 127 VTE patiënten en 299 controles geselecteerd uit de MEGA studie. Apos waarden werden bepaald met massaspectrometrie (LC/MS/MS). In controles waren stijgende waarden van apolipoproteïnen geassocieerd met toegenomen waarden van vitamine K afhankelijke stollingsfactoren, factor XI en clot lysis tijd. Daarnaast waren hogere apos C-III en E geassocieerd met toegenomen factor VIII en von Willebrand factor. C-reactief proteïne was niet geassocieerd met apolipoproteine. De

leeftijd en geslacht gecorrigeerde odds ratio voor VTE van apos E, C-III, CII en CI (per SD toename) waren respectievelijk 1.21 (95% BI, 0.98-1.49), 1.19 (0.99-1.44), 1.24 (0.95-1.61) en 1.06 (95% BI, 0.87-1.30). Deze odds ratios werden niet beïnvloed door correcties voor statinegebruik, oestrogeengebruik, BMI, alcoholgebruik, en diabetes. Geconcludeerd werd dat apos C-I, C-II, C-III en E geassocieerd zijn met meerdere stollingsfactoren. Echter, of deze apos ook geassocieerd zijn met een toegenomen VTE risico is onzeker gezien de analyse in ons onderzoek gebaseerd was op kleine aantallen. De bevindingen zijn echter consistent met uitkomsten uit andere populatiestudies waar apo A en B geassocieerd waren met een mild verhoogd risico op VTE [53]. Toekomstige klinische studies en trials dienen uit te wijzen of de effecten van statine op stollingsfactoren al dan niet lopen via apolipoproteïnen.

ROL VAN GLUCOCORTICOIDEN MET BETREKKING TOT TROMBOSE RISICO: SPELERS OF TOESCHOUWERS?

Systemische glucocorticoïden zijn hormonale steroïden die voorgeschreven worden om opvlammingen van inflammatoire ziekten te remmen [58-62]. Deze middelen zijn effectief, maar hebben ook een aantal bijwerkingen. Experimentele studies hebben aangetoond dat glucocorticoïdgebruik leidt tot stijging van stollingsfactoren, en mogelijk ook het risico op VTE doet verhogen [63-65]. Echter, verschillende confounders zouden het toegenomen VTE risico in deze relatie kunnen doen verklaren, zoals opvlammingen van inflammatoire ziekten [59, 66, 67], co-morbiditeit [58-62,68] en toxiciteit van concomitant medicijngebruik [69]. Het effect van onderliggende ziekte is een probleem omdat onderliggende ziekten als astma, reumatoïde artritis, maligniteit en SLE vaak worden behandeld met glucocorticoïden, terwijl al deze ziekten ook het risico op VTE 3-9-voudig verhogen [59-62, 66, 67, 70, 71]. Wat ook problematisch is, is dat de suppressie van inflammatie door glucocorticoïden het ontstaansproces van VTE kan remmen (‘confounding by indication’) [68,72, 73]. Om deze redenen hebben wij in **Hoofdstuk 5** onderzocht of het risico op eerste VTE en recidief VTE was geassocieerd met gebruik van orale glucocorticoïden, waarbij we (voor het risico op eerste VTE) potentiele confounders als onderliggende ziekte elimineerden met behulp van de ‘self-controlled case-series’ (SCCS) methode. Het effect van oraal glucocorticoïdgebruik op recidief VTE werd onderzocht in een cohortdesign. Patiënten met VTE uit de MEGA-studie werden gekoppeld aan het register van Stichting Farmaceutische Kengetallen (SFK). De

koppeling werd gemaakt op basis van een combinatie van leeftijd, geslacht, 4 karakter postcode en vitamine K antagonist gebruik in de maand rondom datum van VTE. Voorschriften van orale glucocorticoïden werden daarna gekoppeld op individueel niveau aan deze patiënten uit de MEGA studie. Het relatief risico op eerste VTE werd berekend met de SCCS methode. De associatie tussen oraal glucocorticoïd gebruik en recidief VTE werd geschat met behulp van Cox regressie. In totaal werden 2547 patiënten gekoppeld aan het SFK register. Van hen ontvingen 363 patiënten ten minste één medicijnvoorschrift voor orale glucocorticoïden. Het risico op eerste VTE was 3.5-voudig verhoogd over de totale follow-up periode (incidence rate ratio [IRR] 3.5, 95% BI, 2.6-4.8). De IRR voor eerste VTE was 2.5 (95% BI, 1.1– 5.7) in de week voordat de behandeling startte, 5.3 (95% BI, 2.9 – 9.5) in de eerste 7 dagen van behandeling, 3.7 (95% BI, 2.6 – 5.2) tot aan 6 maanden van behandeling en 1.6 (95% BI, 0.8 – 3.1) na 6 maanden behandeling met orale glucocorticoïden. Een dosis-response effect tussen oraal glucocorticoïd gebruik en VTE risico werd gezien, waarbij de IRR van oraal glucocorticoïd gebruik en VTE risico 3.4 (95% BI, 2.3 – 5.0) was met cumulatieve glucocorticoïd doseringen beneden 300 mg per 30 dagen tot 4.9 (95% BI, 1.7 – 14.0) met cumulatieve doseringen boven de 2000mg per 30 dagen. De IRR voor DVT (3.9; 95% BI, 2.9 – 9.5) was vergelijkbaar met de IRR voor PE (3.1; 95% BI, 2.0 – 4.9) en iets lager voor de IRR voor niet-uitgelokte VTE (2.4; 95% BI, 1.3 – 4.7) dan voor uitgelokte VTE (4.2; 95% BI, 2.9 – 6.0). De incidenties voor recidief VTE waren verhoogd in patiënten met niet-uitgelokte eerste VTE en in patiënten die een eerste VTE onder glucocorticoïdgebruik hadden ontwikkeld. Het risico op recidief VTE was 2.7-voudig verhoogd (95% BI, 1.6 – 4.8) gedurende behandelperioden met glucocorticoïden. Geconcludeerd werd dat patiënten die glucocorticoïden gebruiken een >3-voudig verhoogd risico op eerste VTE hebben en een 2-voudig verhoogd risico voor recidief VTE hebben. Verklarende factoren als onderliggende ziekte bij patiënten die glucocorticoïden gebruiken konden deze bevindingen maar deels verklaren. Een klinische implicatie van deze bevindingen is dat tromboseprofylaxe strategieën op hun plaats lijken te zijn in patiënten die worden behandeld met glucocorticoïden. Klinische trials zijn nodig om deze bevindingen te bevestigen.

Conclusies

Statines en systemische glucocorticoïden zijn vaak voorgeschreven geneesmiddelen en kunnen het risico op VTE op verschillende manieren beïnvloeden. Systemisch

glucocorticoïd gebruik verhoogt het risico op eerste VTE meer dan 3-voudig en leidt tot een 5% hoger *absoluut risico* op het krijgen van een recidief VTE. Deze data onderstrepen dat tijdelijk gebruik van glucocorticoïden ten tijde van trombose dient te worden gezien als uitlokkende factor van VTE. Ook benadrukken deze data dat behandelstrategieën nodig zijn om het risico van VTE te verlagen ten tijde van glucocorticoïd gebruik. Deze behandelstrategieën dienen te worden onderzocht in klinische trials.

Daarnaast kan rosuvastatine mogelijk het risico op eerste VTE met 40% reduceren. Hoewel het mechanisme achter deze bevinding niet volledig duidelijk is, heeft dit proefschrift laten zien dat rosuvastatine in staat is om de trombinegeneratiepotentiaal met 10% te verminderen bij patiënten die eerder een VTE hebben gehad. Dat rosuvastatine het stollingsprofiel kan moduleren terwijl rosuvastatine niet geassocieerd is met een verhoogd bloedingsprofiel, maakt langdurige behandeling van VTE met rosuvastatine een aantrekkelijke keus. Echter, of statines het risico op recidief VTE kunnen verlagen is niet onderzocht in dit proefschrift en dient elders te worden onderzocht met behulp van klinische trials.

In conclusie, dit proefschrift heeft aangetoond dat zowel statines als systemische glucocorticoïden het risico op VTE kunnen beïnvloeden. Deze bevindingen kunnen klinisch relevant zijn gezien de bevindingen impliceren dat beide geneesmiddelen in overweging moeten worden genomen bij het beoordelen van VTE risico. Daarnaast onderstrepen deze data dat behandelstrategieën nodig zijn met betrekking tot het voorkomen van VTE met statines als dat ook behandelstrategieën nodig om VTE te voorkomen bij patiënten die glucocorticoïden gebruiken.

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Fernanda

Curriculum Vitae

Fernanda Loureiro de Andrade Orsi was born on December 9, 1975, in São Paulo, Brazil. She started in the Brazilian private education system in 1977. In 1992, she graduated from high school. In 1993, Fernanda commenced her medical studies at the University of Campinas (UNICAMP) School of Medical Sciences, and graduated in 1998. Her contact with scientific research started in the first years of medical school, when she was involved in a student project to study the prevalence of familial thrombophilia in patients with sickle cell disease in the Discipline of Hematology. The study was published in 1998 and was her first published manuscript. From 1999 to 2003, she attended the Medical Residency in Internal Medicine and in Hematology at UNICAMP. Fernanda restarted her research career in 2003 when she started a Master of Science in Bone Marrow Transplantation. In 2008, she started her PhD under the supervision of Prof. Dr. Joyce Annichino-Bizzacchi, at UNICAMP, studying coagulation disorders associated with hemorrhagic fevers. Part of her PhD research was performed at the Children´s Hospital of Philadelphia and supervised by Prof. Dr. Long Zheng. The PhD finished in 2013 and in 2014 Fernanda was admitted, through public tender, as Assistant Professor of the Department of Clinical Pathology of UNICAMP. During her research career, Fernanda had the opportunity to study laboratory and clinical aspects of thromboembolic and hemorrhagic diseases. She was awarded with various Educational, Travel and Reach the World grants from the American Society of Hematology and from the International Society of Thrombosis and Hemostasis. Fernanda also received research grants from São Paulo Research Foundation (FAPESP) and the Brazilian National Council for Scientific and Technological Development (CNPq) to develop studies in the field of antiphospholipid syndrome and thrombosis. Because of her growing interest in studying the epidemiology of hemostasis and venous thrombosis, she started an internship at the Department of Clinical Epidemiology in the Leiden University Medical Center in July 2017 and had the opportunity to start on the PhD Program of the Department of Clinical Epidemiology under the supervision of Prof. Suzanne Cannegieter and Dr. Willem Lijfering. The internship had financial support from UNICAMP and FAPESP. In 2018, Fernanda returned to Brazil where she has been following a career of Professor of Medicine at the School of Medical Sciences of UNICAMP. Since 2019, Fernanda is the representative of Brazil in the Latin American Cooperative Group of Hemostasis and Thrombosis (CLAHT).

List of Publications

1. Orsi FA, Lijfering WM, Van der Laarse A, Ruhaak LR, Rosendaal FR, Cannegieter SC, Cobbaert C. Association of apolipoproteins C-I, C-II, C-III and E with coagulation markers and venous thromboembolism risk. Clinical Epidemiology. 2019;11:625-33.

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