



Full Length Article

Associations between complement pathways activity, mannose-binding lectin, and odds of unprovoked venous thromboembolism[☆]

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ABSTRACT

Introduction: Deep vein thrombosis (DVT) originates in the valvular sinuses of large veins in a local milieu characterized by stasis and severe hypoxia. This may induce complement- and coagulation activation, which potentially increases the risk of venous thromboembolism (VTE). The aim of the present study was to investigate whether the activity of the complement pathways, the level of mannose-binding lectin (MBL) and tissue-factor (TF) induced thrombin generation were associated with risk of unprovoked VTE.

Methods: A case-control study was performed in patients with unprovoked VTE (n = 24) and age- and sex-matched healthy controls (n = 24). Serum complement pathway activity was measured by the total complement screen assay (Wieslab®). MBL was quantified by ELISA. Plasma TF-induced thrombin generation was measured using the CAT-assay.

Results: Activity in the highest quintile of the classical pathway was associated with increased odds of unprovoked VTE (OR 4.5, 95% CI; 0.8–24.7). Moreover, MBL deficiency (≤ 100 ng/ml) was associated with unprovoked VTE (OR 3.5, 95% CI; 0.8–15.3). VTE patients had shortened TF-induced lag-time (4.8 ± 0.6 min vs. 5.8 ± 2.1 min, $p < 0.001$) and a higher endogenous thrombin potential (ETP) (1383 ± 267 nM·h vs. 1265 ± 247 nM·h, $p = 0.07$) than controls. No association between the classical complement pathway activity or MBL deficiency, and parameters of TF-induced thrombin generation was observed.

Conclusion: Our findings suggest that high activity of the classical complement pathway, and MBL deficiency, might be associated with an increased odds of unprovoked VTE, independent of activation of TF-induced coagulation.

1. Introduction

Venous thromboembolism (VTE) is a common disease with a complex, multicausal etiology accompanied by serious short- and long-term complications, including death [1]. Virchow's triad, which includes vessel wall damage/dysfunction, changes in blood flow/stasis, and hypercoagulability, represents the key elements in the pathogenesis of thrombosis [2]. Autopsy- [3] and imaging [4] studies suggest that formation of venous thrombi originate in the valvular sinuses of large

veins. The milieu in the valvular sinuses is characterized by severe hypoxia and stasis [5], conditions known to induce cellular immune responses [6]. Although the incidence of arterial cardiovascular diseases, e.g. myocardial infarction and stroke, have declined by 25–40% during the last two decades [7], the incidence of VTE remains stable or has even increased during the same time period [8]. In order to reduce the incidence of VTE, it is necessary to discover disease mechanisms that could be targets for future prevention and treatment.

The complement system is an important part of the innate immune

Abbreviations: AP, alternative pathway; BMI, body mass index; CAT, calibrated automated thrombogram; CP, classical pathway; DVT, deep vein thrombosis; ETP, endogenous thrombin potential; LP, lectin pathway; MBL, mannose binding lectin; PE, pulmonary embolism; SLE, systemic lupus erythematosus; TF, tissue factor; VTE, venous thromboembolism

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system [9]. Studies suggesting that complement is activated by hypoxic cells and tissues [10], make it reasonable to assume that complement is also activated in the hypoxic milieu in the valvular sinuses. Results from observational and animal studies suggest that the complement system is involved in the early steps in the pathogenesis of VTE. In a large population-based cohort study, subjects with plasma complement C3 concentration in the highest tertile had 31% higher risk of VTE compared to those in lowest tertile [11]. C3-deficient mice had lower incidence of venous thrombosis and reduced thrombus size compared to wild-type mice in a tissue factor (TF)-dependent model of flow-restriction induced venous thrombosis [12]. Further, in a cross-sectional study of patients with systemic lupus erythematosus (SLE), patients with a previous history of VTE showed increased deposition of complement factors C1q, C4 and C3 on platelets [13].

TF-induced thrombin formation in plasma *ex vivo*, i.e. the calibrated automated thrombogram (CAT), is proved useful in assessing thrombosis risk [14, 15]. The CAT displays thrombin activity over time in clotting plasma and allows for assessment of the initiation phase (lag time) and propagation phase (endogenous thrombin potential-ETP) of coagulation. Parameters of the thrombogram (e.g. lag time and ETP) are associated with risk of incident [16] and recurrent VTE [17].

The complement system can be initiated by three pathways depending on stimuli: classical, alternative and lectin [18]. These can be evaluated based on specific activation stimuli ending with the terminal pathway as a common readout [19]. Even though there is growing evidence for a role of the complement system in the pathogenesis of VTE, it is not known which pathways of the system that are involved, and to what extent the activity of the different complement pathways are associated with known risk factors of VTE, such as parameters of the thrombogram. Complement deficiencies, as detected by abolished activity in one or several of the pathways, are extremely rare, except for the lectin pathway mannose binding lectin (MBL) molecule. Mannose-binding lectin (MBL) is a C-type lectin that plays an important role in innate immunity. MBL binds to repetitive carbohydrate patterns, such as mannose, on foreign pathogens and altered host cells, to promote opsonophagocytosis and activation of the lectin pathway of the complement system [18, 20]. Around 5–20% of the population is classified as MBL-deficient according to a cut-off level of < 100 ng/ml. However, this cut-off level is disputed due to high rates of haplotype variation [21–23]. In addition to the general activity of the three complement pathways, we therefore also measured the levels of MBL.

In the present study, we aimed to investigate whether activity of the various complement pathways and levels of MBL were (i) associated with VTE risk and (ii) correlated with TF-induced thrombin generation in plasma. To address these questions, we performed a case-control study in patients with a previous history of unprovoked VTE and population-based controls.

2. Material and methods

2.1. Study population

Patients with unprovoked VTE were recruited from the fourth survey of the Tromsø Study (conducted in 1994–95), a single-center prospective, population-based study, with repeated health surveys of inhabitants of Tromsø, Norway [24]. A VTE case in this registry had all of the following criteria fulfilled; (1) objectively confirmed by diagnostic procedures, (2) the medical record indicated that a physician had made a diagnosis of DVT and/or PE, (3) signs and symptoms consistent with DVT or PE were present, and (4) therapy with anticoagulants (heparin, warfarin, or a similar agent), thrombolytics, or vascular surgery was initiated. Unprovoked VTE was defined as complete absence of provoking factors at the time of diagnosis. Provoking factors were defined as recent surgery or trauma (within 8 weeks before the event), an acute medical condition (myocardial infarction, ischemic stroke, major infectious disease), cancer, marked immobilization (bed rest >

3 days, confinement to wheelchair, long-distance travel > 4 h within the last 14 days), pregnancy or puerperium, estrogen supplementation, or another likely provoking factor specifically described by a physician in the medical record (e.g., intravascular catheter).

VTE cases were recruited from a general population health survey (the fourth Tromsø Study) and invited to participate in an additional screening visit. VTE cases were eligible for the current study if they were between 20 and 80 years of age, had an unprovoked VTE event without recurrence 1 to 6 years before inclusion (i.e. blood sampling), had discontinued anticoagulant treatment at least 3 months before the blood samples were collected, and did not have any other medical conditions (e.g. cancer and auto-inflammatory diseases). For each VTE case, one healthy person matched for age and sex was recruited from the fourth Tromsø Study and underwent the same screening visit and blood sampling as the VTE patients [25].

Complete medical history, physical examination, and blood samples were obtained for VTE cases and controls. Details on the occurrence of cardiovascular events, including previous or current transient ischemic attacks, stroke, angina pectoris, and MI, and recurrent venous thrombosis, diabetes mellitus, and other concurrent diseases was obtained with a self-administrated questionnaire that also included dietary habits, physical exercise, and alcohol consumption. Height and weight were measured with the participants in light clothing without shoes. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of height in meters (kg/m²).

Measurements of protein C, protein S and antithrombin were determined by commercially available assays obtained from Diagnostica Stago® (Parsippany, New Jersey, USA) and performed according to the manufactures instructions. Screening for factor V Leiden and factor II G20210A mutations was carried out on genomic DNA as previously described [26]. Measurements of white blood cells, platelets and hemoglobin were performed with the ABX Micros 60 cell counter (HORIBA ABX SAS, Kyoto, Japan).

2.2. Blood collection and storage

Blood was drawn from an antecubital vein in the morning at 7:45 a.m. after 12 hour overnight fasting and 48 hour refrain of exhaustive physical exercise and alcohol consumption. Serum was prepared by clotting whole blood in a glass tube at room temperature for 1 h. Blood for plasma preparation was collected into 4.5 ml vacutainers (Becton Dickinson, Meylan Cedex, France) containing 0.129 M sodium citrate (1 vol anticoagulant and 9 vol whole blood) as anticoagulant. Serum and plasma were prepared by centrifugation at 2000g for 15 min at 22 °C, transferred into cryovials (Greiner laboratechnik, Nürtingen, Germany) in aliquots of 1 ml and stored at –70 °C until further analysis.

2.3. Calibrated Automated Thrombinoscope

Thrombin generation was assessed using a CAT and was performed as described by Hemker et al. [14] and according to the manufacturer's instructions (Thrombinoscope BV, Maastricht, the Netherlands). Thrombin generation was measured in a Fluoroscan Ascent Fluorometer (Thermolabsystems OY, Vantaa, Finland) equipped with a dispenser. Fluorescence intensity was detected at wavelengths of 355 nm (excitation filter) and 460 nm (emission filter). Briefly, 80 µl of the plasma samples were dispensed into the wells of round bottom 96-well microtiter plates (Immulon, Lab Consult, Lillestrøm, Norway). Twenty µl of a mixture containing TF (Innovin, Bode Behring) and phospholipids (PL) (Cephaline, from rabbit brain) was added to the plasma samples to obtain a final concentration of 5 pM and 4 µM, respectively. For each experiment, a fresh mixture of 2.5 mM fluorogenic substrate (Z-Gly-Gly-Arg-AMC from Bachem, Bubendorf, Switzerland) and 0.1 M CaCl₂ was prepared using buffer containing 20 mM Hepes (Sigma Aldrich, St Louis, USA) and 60 mg/ml BSA (A-7030, Sigma

Aldrich) with pH 7.35. The calibrator with thrombin activity of 600 nM was obtained from Thrombinoscope BV (Maastricht, The Netherlands). The thrombin calibrator corrects for donor-to-donor differences in color of plasma and inner filter effect [27]. The computer software calculated lag time (min), the time to peak (min), the peak of thrombin generation (nM) and the area under the thrombin generation curve (nM*min) or endogenous thrombin potential (ETP).

2.4. Assessment of complement pathway activity in human serum samples

The activity of the classical, alternative and lectin pathways of the complement system was assessed by a commercially available assay (Wielisa COMPL300 Total Complement Functional Screen kit from Wieslab AB, Lund, Sweden) and conducted according to the instructions provided in the manual. In brief, strips of wells for classical pathway (CP) evaluation were pre-coated with IgM, strips for alternative pathway (AP) determination were coated with LPS, and lectin pathway (LP) strips were coated with mannan. Sera were diluted in specific buffers (1/101 for the CP and LP assays, and 1/18 for the AP assay), and were incubated for 1 h at 37 °C. After washing the strips, alkaline phosphatase-conjugated antihuman C5b-9 was added before incubation at room temperature for 30 min. Additional washing was performed, substrate was added, and the wells were incubated for 30 min. Finally, absorbance values were read at 405 nm.

In each assay, standard positive and negative control serum provided in the kit were reconstituted with distilled water. The positive serum was a pool of five sera from healthy individuals, and the negative control consisted of heat-inactivated sera (56 °C for 20 min). Complement activity was calculated using the following formula:

$$\text{Activity} = \frac{\text{mean A405 (sample)} - \text{mean A405 (negative control)}}{\text{mean A405 (standard serum)} - \text{mean A405 (negative control)}} \times 100\%$$

Samples as well as standard serum and negative control serum were tested in duplicates at a fixed dilution. All complement activity values are provided as % of activity in pooled normal serum. The assay is designed as a screening assay to detect deficiencies in the various complement pathways. It is not suitable to detect *in vivo* activation, but reflects the total activity potential that could be activated within each complement pathway *in vitro* [19].

2.5. Measurement of serum mannose binding lectin (MBL)

Measurement of serum concentrations of MBL was performed using an MBL ELISA kit (BIOPORTO Diagnostics A/S, Hellerup, Denmark) according to the manufacturer's instructions.

2.6. Statistics

The Pearson's correlation coefficient was used to test for correlation between variables. Differences between groups with regard to complement pathway activity and parameters of the thrombogram were tested with Student's *t*-test for independent samples. Logistic regression models were used to determine odds ratio (OR) per 1 standard deviation (SD) increase and to determine OR of VTE in extreme categories (highest and lowest quintiles/quartiles) of the complement pathways activity and thrombogram parameters, respectively, compared to all other categories. Statistical analyses were performed using SPSS for Windows, version 22.0 (SPSS Inc. Chicago, IL, USA) and GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA, USA). *p*-Values < 0.05 were considered statistically significant. The measurements of complement pathway activities were visually investigated for normal distribution, and found satisfactory. To analyze the correlation between high activity of the three complement pathways and odds of unprovoked VTE, the pathway activities was divided into quintiles.

Table 1

Characteristics of patients with a previous history of unprovoked venous thromboembolism (VTE) and healthy age- and sex-matched controls recruited from a general population. Values are means \pm 1 standard deviation (SD) or percentages with numbers in brackets.

Variables	VTE patients (n = 24)	Controls (n = 24)	<i>p</i> -Values
Age (years)	54 \pm 16	54 \pm 16	0.96
Female sex	58 (14)	58 (14)	1.00
BMI (kg/m ²)	28.3 \pm 4.4	26.3 \pm 3.9	0.10
Smoking (%)	33 (8)	17 (4)	0.18
Inherited thrombophilia			
FV-Leiden (heteroz.) (%)	13 (3)	4 (1)	0.30
FII G20210A (heteroz.) (%)	4 (1)	0 (0)	0.31
Protein-C (value in %)	108 \pm 22	93 \pm 21	0.02
Protein-S (value in %)	101 \pm 18	94 \pm 19	0.16
Antithrombin (%)	102 \pm 9	104 \pm 8	0.37
Family history of VTE (%)	21 (5)	13 (3)	0.44
Hematological variables			
Hemoglobin (g/dl)	14.4 \pm 1.2	14.3 \pm 1.2	0.91
White blood cells ($\times 10^9/l$)	6.7 \pm 1.9	5.8 \pm 1.5	0.10
Platelets ($\times 10^9/l$)	251 \pm 64	247 \pm 54	0.81

3. Results

We included 24 patients with unprovoked VTE and 24 age- and gender-matched healthy controls recruited from the general population. For cases, the timing between the VTE event and blood sampling is displayed in Supplementary Fig. 1. Baseline characteristics are shown in Table 1. Except for protein C concentration, which was higher in cases than in controls (108 \pm 22% vs. 93 \pm 21%, *p* = 0.02), there were no significant differences between the groups (Table 1).

The activity of complement pathways and the corresponding ORs of unprovoked VTE are displayed in Table 2. The complement activity of both the classical and the alternative pathways was detectable in serum for all study participants, with median pathway activity above 100% in both cases and controls (Table 2). In contrast, a number of both VTE patients (*n* = 10) and healthy controls (*n* = 5) had undetectable lectin pathway activity, consistent with the high frequency of MBL deficiency in the population. With a threshold level of normal lectin pathway activity set at 10% [19], 42% of VTE patients and 21% of controls had undetectable lectin pathway activity. VTE patients with classical pathway activity in the highest quantile had a higher odds of unprovoked VTE compared to subjects in the lower quantiles (OR 4.5, 95% CI 0.8–24.7). The OR of VTE was 3.5 (95% CI 0.8–15.3) for MBL < 100 ng/ml compared to MBL > 100 ng/ml. Moreover, low activity of the lectin pathway (< 10%) was associated with unprovoked VTE (OR 2.7, 95% CI 0.8–9.8). As expected, lectin pathway activity and MBL concentration was highly correlated (*r* = 0.9, *p* < 0.0001). For the alternative pathway, neither total pathway activity nor levels of pathway activity were associated VTE risk. Activity of the classical and alternative pathway was, as expected, significantly correlated (*r* = 0.7, *p* < 0.0001), whereas no correlation was found between the classical pathway and MBL (*r* = -0.13, *p* = 0.4). Finally, there was no difference in the activity of the classical pathway in subjects without and with MBL deficiency (Fig. 1).

Parameters of the TF-induced thrombin generation curve and their OR for unprovoked VTE are shown in Table 3. VTE patients presented an increased ability to generate thrombin, characterized by a shortened lag time and time to peak, and an increased peak thrombin concentration and endogenous thrombin potential (Table 3, Fig. 2). One standard deviation prolongation of lag time (1.74 min) was associated with an OR of 0.4 (95% CI 0.2–0.8), whereas VTE patients with lag time in the upper quartile had a OR of 0.05 (95% CI 0.01–0.44). Lag time and time to peak were strongly correlated (*r* = 0.9 *p* < 0.001) and displayed a similar risk pattern for VTE. One SD increase in ETP (263.4 nM*h) yielded an OR of 1.8 for VTE (95% CI 0.9–3.5), whereas

Table 2

Complement pathway activity and serum levels of mannose binding lectin (MBL) in patients with previous unprovoked venous thromboembolism (VTE) and age- and sex-matched healthy controls with corresponding odds ratios (OR) with 95% confidence intervals (95% CI) of VTE in linear and stratified analyses. Values are means \pm 1 standard deviation or percentages with numbers in parentheses.

	Cases (n = 24)	Controls (n = 24)	OR (95% CI)	OR (95% CI) ^b
<i>Classical pathway (%)</i>				
Pathway activity	112 \pm 14	109 \pm 13	1.30 (0.72–2.40) ^a	1.25 (0.68–2.30) ^a
Quintile 1 \leq 103	25.0 (6)	16.7 (4)	1.67 (0.40–6.87)	1.86 (0.40–8.51)
Quintile 5 \geq 122	29.2 (7)	8.3 (2)	4.53 (0.83–24.7)	3.39 (0.58–19.96)
<i>Alternative pathway (%)</i>				
Pathway activity	131 \pm 64	107 \pm 66	1.16 (0.65–2.01) ^a	1.19 (0.45–3.11) ^a
Quintile 1 \leq 71	16.7 (4)	25.0 (6)	0.60 (0.15–2.47)	0.67 (0.13–3.55)
Quintile 5 \geq 197	20.8 (5)	16.7 (4)	1.32 (0.31–5.65)	1.11 (0.22–5.60)
<i>Lectin pathway (%)</i>				
Pathway activity	60 \pm 64	77 \pm 54	0.86 (0.5–1.52) ^a	0.83 (0.45–1.51) ^a
Pathway activity level \leq 10	41.7 (10)	20.8 (5)	2.71 (0.76–9.78)	3.51 (0.89–13.89)
<i>MBL level (ng/ml)</i>				
Total	412 \pm 670	483 \pm 610	0.82 (0.46–1.50) ^a	0.77 (0.40–1.49) ^a
\leq 100	33.3 (8)	12.5(3)	3.50 (0.80–15.34)	4.65 (0.96–22.42)
\leq 400	50.0 (12)	33.3 (8)	2.00 (0.62–6.42)	2.16 (0.64–7.31)

^a OR for VTE per SD increase.

^b Adjusted for age, sex and BMI.

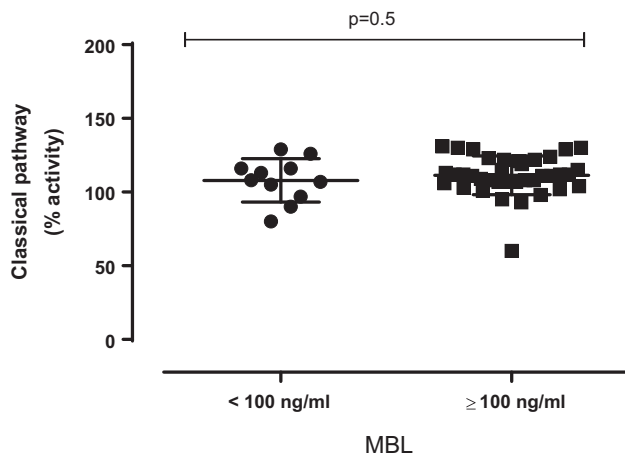


Fig. 1. Dot-plot showing the activity of the classical complement pathway sorted by mannose binding lectin (MBL) deficiency (MBL < 100 ng/ml). Lines are means with standard deviations.

ETP in the upper quartile was associated with an OR of 2.5 (95% CI 0.6–9.8).

Associations between parameters of the thrombogram across categories of complement pathway activities and MBL levels were evaluated for the whole study population (n = 48) (Table 4). There were no association between categories of the classical- and lectin pathways and parameters of the thrombogram. The activity of the alternative pathway was, however, significantly correlated with both peak thrombin generation and ETP ($r = 0.5$, $p < 0.01$ and $r = 0.4$, $p < 0.01$, respectively), and peak thrombin generation and ETP increased significantly across quintiles of the alternative pathway (Table 4).

4. Discussion

In the present case-control study, we investigated the association between activities of complement pathways, thrombin generation and VTE risk. In accordance with previous findings [28], we found that parameters of the thrombogram, lag time in particular, were associated with increased odds of VTE. Cases with unprovoked VTE had higher odds of activity levels within the highest quintile of the classical pathway. Moreover, low lectin pathway activity, as well as MBL deficiency, was associated with increased odds of VTE. There was no

association between activities of the classical- and lectin pathways and parameters of the thrombogram. Our results suggest that the classical pathway of the complement system and MBL deficiencies with low lectin pathway activity might be associated with risk of VTE by mechanisms not mediated by an interplay with TF-induced thrombin generation.

Several studies have shown that parameters of the thrombogram are associated with both first and recurrent VTE [16, 17]. In the Leiden thrombophilia study (LETS) including 360 VTE patients and 404 controls, elevated ETP (> 90% percentile) was associated with a 1.7-fold higher odds of first unprovoked VTE [29]. Similarly, subjects with peak thrombin levels in the highest quartile had a 1.8-fold higher odds of VTE in a nested case-control study from the Longitudinal Investigation of Thromboembolism Etiology (LITE) including 434 cases and 1004 controls [28]. Accordingly, we found that cases with unprovoked VTE had an almost 2-fold higher odds of having ETP levels in the highest quartile. In addition, we found that lag time and time to peak were shorter in patients with unprovoked VTE, suggesting that both the initiation phase (lag time) and propagation phase (ETP) of coagulation are disturbed in subjects who have experienced a VTE event.

MBL deficiency is frequent in humans, ranging up to 20–30% when including those with levels below 400–500 ng/ml [21]. Plasma levels of MBL are independent of sex, remain stable throughout life, and varies mainly due to genotype [30, 31]. Whereas most individuals with low MBL are healthy, deficiency has been reported to be associated with various autoimmune [32, 33] and infectious disorders [34, 35]. Conflicting results have, however, been published regarding the association between MBL deficiency and risk of cardiovascular diseases. In a nested case-control study including 946 cases who experienced a myocardial infarction (MI) or died of cardiovascular diseases (CAD) and 1799 matched controls, men with MBL levels in the highest quartile had a 1.6-fold higher risk of CAD than those in the lowest quartile after adjustment for traditional cardiovascular risk factors. In women, no such relation was observed [36]. In contrast, results from a case-control study, derived from the HUNT study including 370 young (< 62 years) MI patients and 370 age-matched controls, showed that variant haplotypes causing MBL deficiency were associated with a 2-fold higher risk of MI [37]. Several lines of evidence support a relation between MBL deficiency and risk of cardiovascular diseases. First, MBL deficient mice have larger atherosclerotic lesions [38] and MBL deficiency in humans is associated with risk of severe atherosclerosis in young subjects [39]. Second, MBL deficiency is associated with increased and delayed clearance of postprandial lipidemia which is known to augment

Table 3

Parameters of tissue-factor (TF)-induced thrombograms in patients with previous unprovoked venous thromboembolism (VTE) and healthy age- and sex-matched controls with corresponding odds ratios (OR) with 95% confidence intervals (95% CI) of VTE in linear and stratified analyses. Values are means ± 1 standard deviation or percentages with numbers in parentheses.

	Cases (n = 24)	Controls (n = 24)	OR (95% CI)	OR (95% CI) ^b
<i>Lag time (min)</i>				
Total	4.9 + -0.6	6.5 + -2.1	0.19 (0.06–0.68)	0.18 (0.05–0.67) ^a
Quartile 1 ≤ 4.5	29.2 (7)	20.8 (5)	1.56 (0.42–5.86)	1.62 (0.41–6.43)
Quartile 4 ≥ 6.1	4.2 (1)	45.8 (11)	0.05 (0.01–0.44)	0.05 (0.01–0.45)
<i>Time to peak (min)</i>				
Total	8.5 + -1.1	10.9 + -3.5	0.22 (0.06–0.77) ^a	0.21 (0.06–0.75) ^a
Quartile 1 ≤ 8.1	41.7 (10)	20.8 (5)	2.71 (0.76–9.73)	3.18 (0.80–12.71)
Quartile 4 ≥ 9.8	8.3 (2)	41.7 (10)	0.13 (0.02–0.67)	0.10 (0.02–0.58)
<i>Peak (nM)</i>				
Total	195 + -37	161 + -50	2.46 (1.15–5.23) ^a	2.75 (1.10–6.87) ^a
Quartile 1 ≤ 151	12.5 (3)	37.5 (9)	0.24 (0.06–1.03)	0.23 (0.05–1.17)
Quartile 4 ≥ 200	29.2 (7)	20.8 (5)	1.56 (0.42–5.86)	1.41 (0.31–6.30)
<i>ETP (nM*h)</i>				
Total	1402 + -267	1267 + -247	1.81 (0.92–3.56) ^a	1.73 (0.83–3.60) ^a
Quartile 1 ≤ 1159	20.8 (5)	29.2 (7)	0.64 (0.17–2.39)	1.07 (0.23–4.99)
Quartile 4 ≥ 1466	33.3 (8)	16.7 (4)	2.50 (0.64–9.82)	2.57 (0.56–11.68)

^a OR for VTE per SD increase.

^b Adjusted for age, sex and BMI.

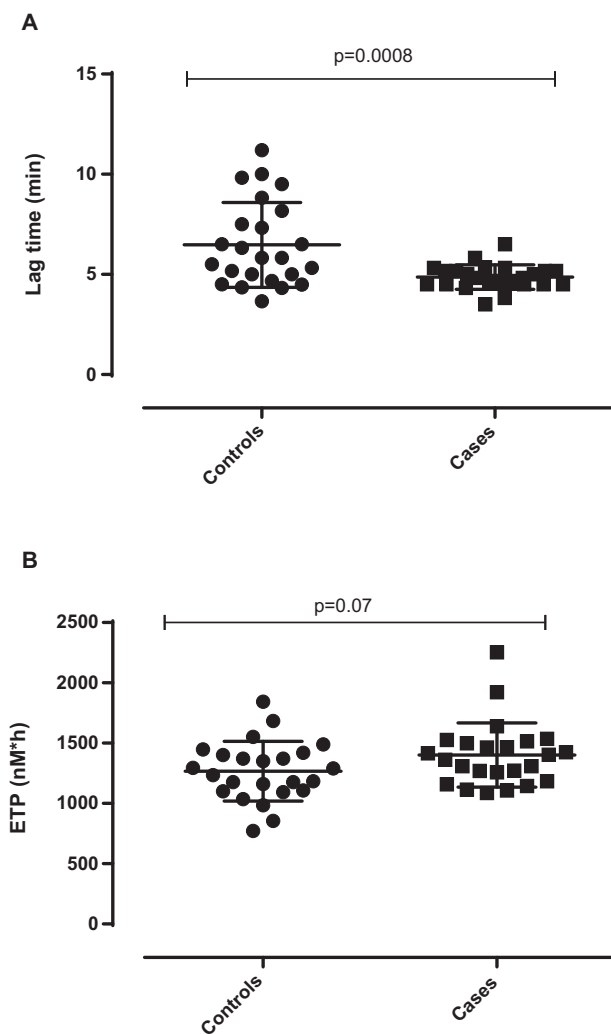


Fig. 2. Dot-plots showing lag time (min) (panel A) and endogenous thrombin potential (ETP) (panel B) of tissue-factor induced thrombograms in VTE cases and controls. Lines are means with standard deviations.

Table 4

Associations between categories of various complement pathway activities and parameters of the TF-induced thrombograms. Values are means ± 1 standard deviation.

	Lag time (min)	Time to peak (min)	Peak (nM)	ETP (nM*h)
<i>Classical pathway (% activity)</i>				
Quintile 5 ≥ 122	5.2 ± 0.7	9.0 ± 1.3	195 ± 29	1416 ± 93
Quintile 1–2 ≤ 108	5.0 ± 2.2	8.7 ± 3.7	173 ± 47	1291 ± 257
p-Value	0.52	0.33	0.05	0.22
<i>Alternative pathway (% activity)</i>				
Quintile 5 ≥ 197	5.3 ± 1.1	9.3 ± 1.9	219 ± 48	1536 ± 341
Quintile 1–2 ≤ 89	4.9 ± 0.9	8.8 ± 1.1	174 ± 27	1252 ± 185
p-Value	0.16	0.095	0.004	0.0004
<i>Lectin pathway (% activity)</i>				
Pathway activity ≥ 10	5.8 ± 1.9	9.8 ± 3.2	183 ± 52	1291 ± 249
Pathway activity ≤ 10	5.4 ± 1.4	9.4 ± 2.1	189 ± 32	1350 ± 298
p-Value	0.43	0.70	0.74	0.66

atherosclerosis formation [40]. Third, MBL deficiency may promote plaque formation by reduced ability to remove apoptotic cells [41] with subsequent increased proatherogenic inflammation.

Previously, two smaller studies have investigated the association between genotypes associated with MBL deficiency and risk of VTE in patients with systemic lupus erythematosus (SLE). In a cohort of 91 SLE patients followed for a median of 9.1 years, 14 patients developed VTE which was unrelated to the MBL genotype [42]. In a cross-sectional study including 114 SLE patients, the prevalence of VTE was higher in subjects with MBL-deficient genotypes, most probably due to the co-existence with anti-phospholipid syndrome [43]. To the best of our knowledge, no study has investigated the prevalence of MBL deficiency in VTE patients and controls recruited from the general population. In our case-control study, cases had 3.5-fold higher odds of MBL deficiency (≤100 ng/ml) than controls. The MBL levels were strongly correlated with lectin pathway activity, and similarly to MBL levels, low lectin pathway activity showed a trend towards an increased risk of VTE. There was no correlation between low lectin pathway activity levels/ MBL deficiency and high activity in the classical pathway,

indicating that MBL deficiency in itself might be associated with risk of VTE. The underlying mechanisms for the possible association between MBL deficiency and VTE risk is unknown, but may be explained by the fact that individuals with MBL deficiency are predisposed to autoimmune- [33, 44] and infectious disease [35, 45], both of which are associated with risk of VTE [46, 47]. Additionally, the lectin pathway and MBL associated serine protease (MASP-1) has been shown to be a significant contributor to coagulation in a mouse model with occlusive thrombosis [41]. However, due to lack of association between MBL deficiency and parameters of the thrombogram, our findings suggest that, the trend towards increased odds of VTE in individuals with MBL deficiency is not mediated by an effect on TF-induced coagulation activation.

In a case-control study including 69 SLE patients and 69 age- and sex-matched controls, the SLE patients displayed increased deposition of C1q, C3d and C4d on platelets compared to controls patients [13]. Furthermore, the SLE patients with a history of VTE had an increased deposition of C1q, C3, and C4d on platelets compared to SLE patients without VTE, suggesting that activation of the classical pathway of the complement system may play a role in the pathogenesis of VTE among SLE patients. Accordingly, we found an OR of 4.5 for the association between high serum activity of the classical pathway and unprovoked VTE. However, due to the small number of participants in each quintile in our study the results were not statistically significant and should therefore be interpreted with caution. Since thrombin is known to activate C3 of the complement pathway [48], one possible explanation for the association between high complement activity and VTE could be hypercoagulability. However, in our study we found that high activity of the classical complement pathway was not associated with parameters of TF-induced thrombin generation. This indicated that the association between activity of the complement cascade and VTE risk was independent of coagulation parameters. The possible association between high classical pathway activity and increased VTE risk might be explained by the hypoxic state found in the vein, particularly in the deepest recess of the valvular sinus, where the thrombus forms [49, 50]. Hypoxia has been found to activate the complement system [10] and reoxygenation of hypoxic HUVEC cells incubated with 30% serum leads to activation of the classical pathway and thus C3 depositions on cells [51]. In our study, the activity of the classical pathway of the complement system was not associated with parameters of the TF-induced thrombogram, suggesting that the impact of the classical pathway of the complement system is not mediated by a direct effect on coagulation activation.

To the best of our knowledge, no study has investigated the relationship between the activity of the alternative pathway and VTE risk. Although we found an association between the activity of the alternative pathway and parameters of thrombin generation (lag time and ETP), activity of the alternative pathway was not associated with risk of VTE. The alternative pathway is continuously undergoing a low-grade activation and functions as an amplifier of other routes of complement activation [52]. Measurements of activation products of the classical and alternative pathway in blood could shed additional light on the mechanisms of complement activation in VTE.

Strengths of our study include the recruitment of VTE patients from a population-based cohort and age- and sex-matched apparently healthy controls from the same source population. In order to minimize the effect of the acute VTE on the measurements of interest (reverse causation), all samples were collected more than one year after the VTE event. However, the fact that blood samples were collected after unprovoked VTE makes it impossible to affirm the direction of the association between exposure (activities of complement pathways) and outcome (VTE). Another limitation of this study is the low number of participants included with a considerable risk of statistical type 1 and 2 errors. Statistical analysis of such a small sample size has limitations, and the results should be interpreted with caution. Thus, a larger, prospective study should be conducted to validate our findings. Due to the explorative nature of the study,

we did not adjust for multiple testing, which imply that some of the significant associations may occur by chance.

In conclusion, results from our case-control study suggest that low activity of the lectin pathway, reflected by MBL deficiency, and high activity of the classical pathway might be associated with risk of unprovoked VTE. These findings are hypothesis generating and exploratory, and a larger prospective study is warranted to validate our findings.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.thromres.2018.06.019>.

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