Synergistic Effects of Hypofibrinolysis and Genetic and Acquired Risk Factors on the Risk of a First Venous Thrombosis

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Abbreviations: BMI, body mass index; CI, confidence interval; CLT, clot lysis time; DVT, deep vein thrombosis; MEGA, Multiple Environmental and Genetic Assessment (of risk factors for venous thrombosis); OR, odds ratio; PAI-1, plasminogen activator inhibitor-1; PE, pulmonary embolism; TAFI, thrombin activatable fibrinolysis inhibitor; t-PA, tissue-type plasminogen activator

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ABSTRACT

Background

Previously, we demonstrated that hypofibrinolysis, a decreased capacity to dissolve a blood clot as measured with an overall clot lysis assay, increases the risk of venous thrombosis. Here, we investigated the combined effect of hypofibrinolysis with established risk factors associated with hypercoagulability.

Methods and Findings

Fibrinolytic potential was determined with a plasma-based clot lysis assay in 2,090 patients with venous thrombosis and 2,564 control participants between 18 and 70 y of age enrolled in the Multiple Environmental and Genetic Assessment (MEGA) of risk factors for venous thrombosis study, a population-based case-control study on venous thrombosis. Participants completed a standardized questionnaire on acquired risk factors.

Hypofibrinolysis alone, i.e., clot lysis time (CLT) in the fourth quartile (longest CLT) (in absence of the other risk factor of interest) increased thrombosis risk about 2-fold relative to individuals with CLT in the first quartile (shortest CLT). Oral contraceptive use in women with CLT in the first quartile gave an odds ratio (OR) of 2.6 (95% confidence interval [CI] 1.6 to 4.0), while women with hypofibrinolysis who used oral contraceptives had an over 20-fold increased risk of venous thrombosis (OR 21.8, 95% CI 10.2 to 46.7). For immobilization alone the OR was 4.3 (95% CI 3.2 to 5.8) and immobilization with hypofibrinolysis increased the risk 10.3-fold (95% CI 7.7 to 13.8). Factor V Leiden alone increased the risk 3.5-fold (95% CI 2.3 to 5.5), and hypofibrinolysis in factor V Leiden carriers gave an OR of 8.1 (95% CI 5.3 to 12.3). The combination of hypofibrinolysis and the prothrombin 20210A mutation did not synergistically increase the risk. All ORs and 95% CIs presented are relative to individuals with CLT in the first quartile and without the other risk factor of interest.

Conclusions

The combination of hypofibrinolysis with oral contraceptive use, immobilization, or factor V Leiden results in a risk of venous thrombosis that exceeds the sum of the individual risks.

The Editors' Summary of this article follows the references.

Introduction

A hypercoagulable state, an increased capacity to form thrombin, is known to be associated with an increased risk of venous thrombosis [1]. However, the role of the fibrinolytic system in the development of venous thrombosis has not yet been extensively investigated.

We have shown that reduced fibrinolytic potential, as measured by a plasma-based clot lysis assay, increases the risk of a first deep vein thrombosis (DVT) [2]. An almost 2-fold increased risk of DVT was found in individuals with clot lysis times (CLTs) above the 90th percentile of the values found in control participants compared with individuals with CLTs below this cut-off point.

Venous thrombosis is a multicausal disease, and several genetic and acquired risk factors associated with venous thrombosis have already been described [1]. The occurrence of multiple risk factors within a single patient may be associated with a risk of thrombosis that exceeds the sum of the individual risks. Such an increase is, for example, found in women with the factor V Leiden mutation who also use oral contraceptives. While carriers of the factor V Leiden mutation have a 5-fold increased risk of venous thrombosis, and oral contraceptive use increases the risk about 3-fold, the combination of these risk factors leads to a 20- to 30-fold increased risk (reviewed in [3]). A synergistic effect is also found for the combination of the factor V Leiden mutation and malignancy or air travel [4,5]. We hypothesized that the combination of hypofibrinolysis and hypercoagulability would synergistically enhance the risk of venous thrombosis. This hypothesis was investigated in the Multiple Environmental and Genetic Assessment (MEGA) of risk factors for venous thrombosis study, which is a large population-based case-control study on venous thrombosis. First, we studied hypofibrinolysis as a risk factor for thrombosis, and investigated the joint effect of hypofibrinolysis and several established risk factors for venous thrombosis. Second, we studied the influence of several genetic and acquired factors on the outcome of the clot lysis assay.

Methods

Study Design

The design of the MEGA study has been described previously [4]. Between March 1999 and May 2002, consecutive patients aged 18 to 70 y, with a first DVT of the leg or a first pulmonary embolism (PE), were identified at six anticoagulation clinics in The Netherlands. In these clinics, the anticoagulant therapy of all patients in a well-defined geographical area is monitored, which gave us the opportunity to identify consecutive and unselected patients with venous thrombosis.

Partners of participating patients were invited to take part as control participants. From January 2002 to September 2004, an additional control group was selected from the same area by random digit dialing using the Mitofsky-Waksberg method [6] and was frequency matched to the patients for age and sex. This matching was on group level in which random controls were selected in numbers proportional to the number of patients within strata of sex and 5-y age groups.

Patients or control participants with severe psychiatric problems or those who could not speak Dutch were excluded.

For the MEGA study, 4,131 patients were eligible. Of those patients, 194 died soon after the venous thrombosis. Of the remaining 3,937 patients, 51 were in the end stage of a disease and 629 were unable or refused to participate, leaving 3,257 patients in the study who filled in a questionnaire (83%). Of the total of 3,257 patients of the MEGA study, 2,360 had an eligible partner. One partner died soon after the request for participation and, of the remaining 2,359 partners, 1,908 participated (81%). Of the nonparticipating partners, 15 were in the end-stage of a disease and 436 refused, were unable to participate, or could not be located. Of the 4,350 eligible random digit dialing control participants, four died before they were able to participate, 15 were in the end stage of a disease, and 1,331 refused to participate or could not be located, leaving 3,000 participants in the study (69%).

All participants signed a written informed consent form. Approval for this study was obtained from the Medical Ethics Committee of the Leiden University Medical Center, Leiden, The Netherlands.

Validation of Thrombosis Diagnosis

Written informed consent to obtain medical records was given by 95% of the patients. Information regarding the diagnostic procedure was obtained via hospital records and general practitioners. For 10% of these patients the information regarding the diagnostic procedure could not be obtained. A PE was considered "definite" when diagnosed with a high-probability ventilation-perfusion scan (VQ scan), positive spiral computed tomography (CT), or angiogram. A PE was considered "probable" when diagnosed with a low- or intermediate-probability VQ scan, inconclusive spiral CT, or angiogram. A deep venous thrombosis was considered definite when a (Doppler) ultrasound showed the presence of a thrombus in the deep veins. When no information regarding the diagnostic procedure was available or when a patient was registered at the anticoagulation clinic with a different or additional diagnosis to the one that had been objectively confirmed, the diagnosis by which the patient was registered at the anticoagulation clinic was added. A registration of a PE was considered probable while a DVT registration was considered definite. Only those patients were included in the study when their diagnosis was definite or probable. Of those patients in whom diagnostic information was available, only 2% was excluded as they did not have a venous thrombotic event according to the medical records.

Data Collection and Current Analysis

All participants were asked to complete a standardized questionnaire on acquired risk factors for venous thrombosis, such as surgery, plaster cast, confinement to bed, injury, oral contraceptive use, hormonal replacement therapy, pregnancy, and malignancy. Body mass index (BMI in kg/m^2) was calculated from self-reported weight and height. All items in the questionnaire referred to the period before the index date. The date of diagnosis of thrombosis as reported by the participant was used as the index date for the patients. For the control participants, the date they filled in the questionnaire was used as index date. Idiopathic venous thrombosis was defined as venous thrombosis in patients who had never had malignancies and without surgery, injury, plaster cast, or confinement to bed in the year prior to the

thrombosis or oral contraceptive use or hormone replacement therapy at the time of the event.

When participants were unable to fill in the questionnaire, questions were asked by telephone, using a standardized mini-questionnaire. Three months after discontinuation of the anticoagulant therapy, a blood sample was taken from patients and participating partners in the anticoagulation clinic. In patients who received prolonged anticoagulant therapy (>1 y), blood was drawn 1 y after the event. The random control participants were invited to the clinic for a blood draw after returning their questionnaire. All participants were interviewed regarding current anticoagulant use.

In the current analyses only patients who had their blood drawn were included ($n = 2,420$). Users of oral anticoagulants at time of blood draw were excluded ($n = 290$) as well as 38 patients who, according to the medical records did not have venous thrombosis, leaving 2,092 patients available for this study.

Of these 2,092 patients, 1,328 partner controls participated in the MEGA study. Blood was drawn of 1,138 partner controls and seven were excluded because they used anticoagulants, leaving 1,131 partner controls available for this study. Of the 3,000 random control participants, 1,460 had their blood drawn and 1,440 were not using oral anticoagulants.

Blood Collection and Laboratory Analysis

Blood samples were drawn into vacuum tubes containing 0.106 M trisodium citrate. Plasma was obtained by centrifugation at $2,000g$ for 10 min at room temperature and stored in aliquots at -80 °C. Samples that were not previously thawed were used for this study. Lysis of a tissue factor–induced clot by exogenous tissue-type plasminogen activator (t-PA) was studied by monitoring changes in turbidity during clot formation and subsequent lysis as described previously [2]. In short, 50 µl plasma was pipetted in a 96-well microtiter plate. Subsequently, 50 µl of a mixture containing phospholipid vesicles (40% L-α-dioleoylphosphatidylcholine, 20% L-α-dioleoylphosphatidylserine, and 40% L-α-dioleoylphosphatidylethanolamine, final concentration 10 µM), t-PA (final concentration 56 ng/ml), tissue factor (final dilution 1:1000), and $CaCl_2$ (final concentration 17 mM), diluted in HEPES buffer (25 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 137 mM NaCl, 3.5 mM KCl, 3 mM $CaCl_2$, 0.1% bovine serum albumin, pH 7.4), was added using a multichannel pipette. After thorough mixing, the plate was incubated at 37 °C in a Spectramax 340 kinetic microplate reader (Molecular Devices Corporation), and the optical density at 405 nm was monitored every 20 s, resulting in a clot-lysis turbidity profile. The CLT was derived from this clot-lysis profile and defined as the time (minutes) from the midpoint of the clear to maximum turbid transition, representing clot formation, to the midpoint of the maximum turbid to clear transition, representing the lysis of the clot. The intra-assay coefficient of variation was 5.5% ($n = 99$) and the inter-assay coefficient of variation was 6.6% ($n = 90$).

A detailed description of DNA extraction and DNA analysis for the factor V Leiden (G1691A) mutation and the prothrombin (G20210A) mutation in the MEGA study has been published previously [4].

Of two patients (without participating partner), three partners, and four random control participants, no citrated plasma was available or the CLT could not be measured as the plasma was too turbid.

The DNA analyses and the clot lysis assays were performed by staff who had no knowledge of whether the sample was from a patient or a control participant.

Statistical Analysis

We investigated whether elevated CLT is a risk factor for venous thrombosis by calculating odds ratios (ORs), as an approximation of relative risks, and 95% confidence intervals (CIs). CLTs were grouped into quartiles or deciles based on the distribution among all control participants. The lowest quartile or decile was taken as the reference group. ORs adjusted for age (continuous) and sex (categorical) (OR_{adj}) were calculated using multivariate logistic regression. Analyses in subgroups by sex were adjusted only for age. Analyses in subgroups of age were adjusted for sex and for age (continuous) within each stratum of age. Adjustment for age as a dummy variable (five categories) did not change the results compared to models with age as a continuous covariate, and the latter adjustment is presented for all analyses.

A patient and his or her partner control may be more similar than a random pair selected from the population would be, e.g., on (measured and unmeasured) lifestyle factors that could influence fibrinolytic potential. Therefore, we performed conditional logistic regression to adjust for all matching factors in the analyses with partners as control group [5], including 1,128 patient-partner pairs. In the analyses with the random control group, unconditional logistic regression including all patients and random control participants were performed. To obtain a final estimate, results of unmatched and matched analyses were pooled. As both estimates used the same subset of 1,128 patients, the estimates are correlated. Therefore these estimates were pooled using analyses taking this correlation into account [7].

When subgroup analyses were performed on men and women separately, only an unmatched analysis with the random control group was performed, as a matched analysis was not possible because the patient and matched control were nearly always of the opposite sex.

We assessed the joint effect of quartiles of CLT with other risk factors for venous thrombosis by calculating ORs. Participants in the first quartile of CLT and without the other risk factor of interest were used as the reference category, and dummy variables were used for the seven other categories.

To study the association between clotting abnormalities and acquired (risk) factors with CLT in the control group (partners and random controls), mean CLTs of different groups were calculated and confidence intervals were constructed for the difference based on a t-distribution. The association between age and BMI with CLT was assessed using linear regression. Linear regression was also used to determine the association between the time interval between the thrombotic event and blood draw and CLT in the patients. Longer CLTs in those with a small time interval than in those with a large time interval could indicate that increased CLT is rather an acute-phase reaction than a risk factor for venous thrombosis.

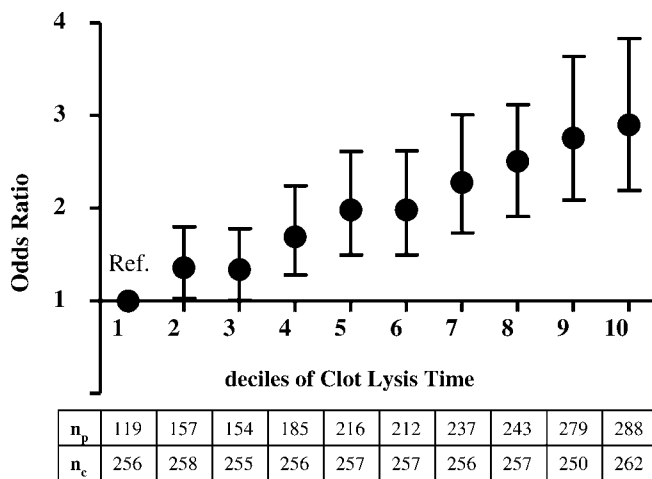Cases and controls with missing data were excluded in the

Figure 1. Risk of Venous Thrombosis for Deciles of Clot Lysis Time

The odds ratios are adjusted for age and sex. Error bars, 95% CI; n_c, number of control participants (all); n_p, number of patients; Ref., reference category.

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analyses. SAS 9.1 (SAS Institute) was used for all statistical analyses.

Results

In this study, 2,090 patients, 1,128 partner controls, and 1,436 random controls were included. Mean age at time of blood draw of patients was 49 y (range 19–71y) and mean age of all control participants was 48 y (range 18–71 y). Of the patient group 926 participants (44%) were men, whereas in the control group 1,226 participants (48%) were men. Of the 2,090 patients, 650 were diagnosed with an isolated PE, 1,245 with an isolated DVT of the leg, and 195 of all the patients were diagnosed with both a PE and a DVT of the leg.

Hypofibrinolysis as a Risk Factor for Venous Thrombosis

The association between CLT and the risk of venous thrombosis is shown in Figure 1. Using deciles of CLT based on the values found in all control participants, we found a clear dose-response relation between CLT and the risk of venous thrombosis. The OR_{adj} for individuals in the tenth decile of CLT compared with individuals in the first decile was 2.9 (95% CI 2.2 to 3.8). In Table 1 the risk of venous thrombosis is presented in quartiles. Individuals with hypofibrinolysis, i.e., CLTs in the highest quartile, had an increased relative risk of venous thrombosis of 2.4 (95% CI

2.0 to 2.8) compared with individuals in the lowest quartile of CLT. Adjustment for age and sex did not change this result (OR_{adj} 2.4, 95% CI 2.0 to 2.8). Using only the random control group yielded an OR_{adj} of 2.5 (95% CI 2.1 to 3.1), and the matched analysis with only partner controls resulted in a similar OR_{adj} of 2.0 (95% CI 1.5 to 2.6) for those with hypofibrinolysis.

Hypofibrinolysis was associated with a 3.2-fold increased risk of a simultaneous DVT of the leg and a PE (OR_{adj} 3.2, 95% CI 1.9 to 5.3) (Table 2). Hypofibrinolysis conferred a 2.1-fold increased risk of an isolated PE (OR_{adj} 2.1, 95% CI 1.6 to 2.8). For an isolated DVT of the leg an OR_{adj} of 2.6 was found (95% CI 2.1 to 3.2).

The relative risk of venous thrombosis in individuals with hypofibrinolysis was most pronounced in women below age 50 y (OR_{adj} 3.2, 95% CI 2.2 to 4.6) (Table 3). As blood of these women was drawn after the event and most patients but not the control participants had ceased oral contraceptive use before time of blood draw, this difference could have influenced the risk found in younger women. For that reason we also calculated the risk in women below age 50 y who did not use oral contraceptives at time of the index date and still did not use oral contraceptives at time of blood draw. In those women, an OR_{adj} of 2.3 (95% CI 1.3 to 4.1) was found. In women over age 50 y, the OR_{adj} was 2.8 (95% CI 1.7 to 4.6). The relative risk in men below age 50 y with hypofibrinolysis was slightly lower (OR_{adj} 2.2, 95% CI 1.4 to 3.5) than in men over age 50 y (OR_{adj} 2.8, 95% CI 1.8 to 4.3).

The risk of idiopathic thrombosis in those with hypofibrinolysis was approximately 3-fold increased (OR_{adj} 3.1, 95% CI 2.2 to 4.4).

Hypofibrinolysis in Combination with Other Established Risk Factors

Table 4 shows the risk of venous thrombosis for individuals with hypofibrinolysis (i.e., the highest quartile of CLT) in combination with established risk factors. The reference group is the group with the lowest CLTs (i.e., in the first quartile) and in absence of the other risk factor. To study the joint effect of hypofibrinolysis and oral contraceptive use on venous thrombosis, only women below the age of 50 y were included. Women with the lowest CLTs who were taking oral contraceptives had a 2.6-fold increased risk of venous thrombosis (OR_{adj} 2.6, 95% CI 1.6 to 4.0) compared with women with the lowest CLTs who did not take oral contraceptives. In those with the highest CLTs who did not take oral contraceptives, an OR_{adj} of 1.9 (95% CI 1.1 to 3.3) was found

Table 1. Relative Risk of Venous Thrombosis According to Quartiles of Clot Lysis Time

	CLT Quartile 1 (Reference)	CLT Quartile 2	CLT Quartile 3	CLT Quartile 4
Lysis time at cut-off (min)	—	57.9	65.6	75.9
Cases (*n*)	340	491	558	701
Controls (all, *n*)	641	641	641	641
OR (95% CI)	1	1.5 (1.3 to 1.8)	1.8 (1.5 to 2.2)	2.4 (2.0 to 2.8)
OR_{adj} (95% CI)	1	1.5 (1.3 to 1.8)	1.8 (1.5 to 2.2)	2.4 (2.0 to 2.8)

OR, crude odds ratio; OR_{adj}, odds ratio adjusted for age and sex.

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Table 2. Relative Risk of Venous Thrombosis According to Quartiles of Clot Lysis Time in Subgroups of Venous Thrombosis

Diagnosis		CLT Quartile 1 (Reference)	CLT Quartile 2	CLT Quartile 3	CLT Quartile 4
DVT of the leg	Cases (n)	200	285	340	420
	Controls (all, n)	542	545	504	514
	OR_adj (95% CI)	1	1.5 (1.2 to 1.8)	2.1 (1.6 to 2.6)	2.6 (2.1 to 3.2)
DVT of the leg + PE	Cases (n)	24	50	53	68
	Controls (all, n)	428	407	339	358
	OR_adj (95% CI)	1	2.1 (1.2 to 3.4)	2.6 (1.6 to 4.4)	3.2 (1.9 to 5.3)
PE	Cases (n)	116	156	165	213
	Controls (all, n)	481	467	426	425
	OR_adj (95% CI)	1	1.4 (1.1 to 1.8)	1.6 (1.2 to 2.1)	2.1 (1.6 to 2.8)

OR$_{adj}$, odds ratio adjusted for age and sex.
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compared with the reference group. The combination of hypofibrinolysis and oral contraceptive use gave a 22-fold increased risk (OR$_{adj}$ 21.8, 95% CI 10.2 to 46.7).

The risk of venous thrombosis in individuals with a combination of hypofibrinolysis and immobilization (defined as either plaster cast, confinement to bed for more than 4 d, or surgery) was also assessed (Table 4). The OR$_{adj}$ for individuals with hypofibrinolysis and no immobilization was 2.4 (95% CI 1.9 to 2.9), and for individuals with CLT in the lowest quartile who were immobilized it was 4.3 (95% CI 3.2 to 5.8). Those who were immobilized and who had hypofibrinolysis had a 10-fold increased risk of venous thrombosis compared with individuals without these risk factors (OR$_{adj}$ 10.3; 95% CI 7.7 to 13.8). When plaster cast, confinement to bed, and surgery were analyzed separately in combination with hypofibrinolysis, the same trends were found as when these three risk factors were combined into one variable (unpublished data).

In individuals with hypofibrinolysis without the factor V Leiden mutation an OR$_{adj}$ of 2.4 (95% CI 2.0 to 2.9) was found (Table 4). Carriers of the factor V Leiden mutation with the lowest CLTs had a risk of venous thrombosis that was 3.5-fold (95% CI 2.3 to 5.5) increased. The risk of venous thrombosis

increased 8.1-fold (95% CI 5.3 to 12.3) for individuals with hypofibrinolysis and the factor V Leiden mutation. Similar analyses for the prothrombin 20210A mutation gave an OR$_{adj}$ of 2.3 (95% CI 2.0 to 2.8) for hypofibrinolysis only, 2.4 (95% CI 0.7 to 8.1) for the prothrombin 20210A mutation only, and 4.4 (95% CI 2.5 to 7.6) for the combination. When patients with pulmonary emboli were excluded, the same trend in risks was found, but somewhat higher ORs were found for the risk of factor V Leiden alone and the combination of factor V Leiden with hypofibrinolysis (unpublished data).

In each of the above analyses of combinations of risk factors, the risks of venous thrombosis in the 2nd and 3rd quartile of CLT (in the presence or absence of the other risk factor) showed a consistent trend. With each increasing quartile of CLT, the risk of venous thrombosis also increased (unpublished data).

Determinants of Clot Lysis Time

All control participants were used to study genetic and acquired factors as determinants of CLTs (Table 5). Mean CLT in the control group was 69.8 min (5th to 95th percentile, 49.4 to 105.1). CLT increased with age (3.1 min

Table 3. Relative Risk of Venous Thrombosis According to Quartiles of Clot Lysis Time

Group		CLT Quartile 1 (Reference)	CLT Quartile 2	CLT Quartile 3	CLT Quartile 4
Women <50 y	Cases (n)	175	219	172	152
	Random controls (n)	186	131	85	51
	OR_adj (95% CI)	1	1.8 (1.3 to 2.4)	2.2 (1.5 to 3.0)	3.2 (2.2 to 4.6)
Women <50 y not taking oral contraceptives[a]	Cases (n)	47	49	50	43
	Random controls (n)	92	74	56	41
	OR_adj (95% CI)	1	1.4 (0.8 to 2.3)	1.9 (1.1 to 3.2)	2.3 (1.3 to 4.1)
Women ≥50 y	Cases (n)	35	85	126	200
	Random controls (n)	56	72	79	117
	OR_adj (95% CI)	1	1.9 (1.1 to 3.2)	2.6 (1.6 to 4.4)	2.8 (1.7 to 4.6)
Men <50 y	Cases (n)	53	65	98	113
	Random controls (n)	91	90	65	80
	OR_adj (95% CI)	1	1.2 (0.7 to 1.9)	2.4 (1.5 to 3.9)	2.2 (1.4 to 3.5)
Men ≥50 y	Cases (n)	77	122	162	236
	Random controls (n)	72	96	85	80
	OR_adj (95% CI)	1	1.2 (0.8 to 1.8)	1.8 (1.2 to 2.7)	2.8 (1.8 to 4.3)

[a]At index date and at time of blood draw.
OR$_{adj}$, odds ratio adjusted for age.
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Table 4. Hypofibrinolysis, Presence of Established Genetic and Acquired Risk Factors, and Relative Risk of Venous Thrombosis

Risk Factor	Present?	Quartile of CLT	Cases (n)	Controls (n)[a]	OR$_{adj}$ (95% CI)
Oral contraceptive use at index date[b,c]	No	1	50	93	1 (reference)
		4	44	42	1.9 (1.1 to 3.3)
	Yes	1	125	93	2.6 (1.6 to 4.0)
		4	107	9	21.8 (10.2 to 46.7)
Immobilization[c,d]	No	1	194	550	1 (reference)
		4	415	550	2.4 (1.9 to 2.9)
	Yes	1	142	88	4.3 (3.2 to 5.8)
		4	282	85	10.3 (7.7 to 13.8)
Factor V Leiden	No	1	288	605	1 (reference.)
		4	590	608	2.4 (2.0 to 2.9)
	Yes	1	52	36	3.5 (2.3 to 5.5)
		4	111	33	8.1 (5.3 to 12.3)
Prothrombin 20210A	No	1	332	636	1 (reference)
		4	661	620	2.3 (2.0 to 2.8)
	Yes	1	8	5	2.4 (0.7 to 8.1)
		4	40	21	4.4 (2.5 to 7.6)

[a]In analyses on hypofibrinolysis and oral contraceptive use, random controls are used. In the other analyses all controls are used.
[b]Analyses in women below the age of 50 y.
[c]Information about oral contraceptive use was unavailable for one patient and information about immobilization was unavailable for eight cases and nine control participants.
[d]Immobilization was defined as plaster cast, confinement to bed for more than 4 d, or surgery.
OR$_{adj}$, odds ratio adjusted for age (in analyses on hypofibrinolysis and oral contraceptive use) or age and sex (in other analyses).
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per decade, 95% CI 2.6 to 3.6). In men a plateau was reached at age 60. Overall, mean CLT in men (70.9 min, 5th to 95th percentile 50.2 to 106.2) was slightly higher than mean CLT in women (68.7 min, 5th to 95th percentile 48.6 to 103.0), although the difference was small (2.2 min, 95% CI 0.7 to 3.7). CLTs substantially increased with increasing BMI. The increase in CLT per 1 kg/m^2 increase in BMI was 2.0 min (95% CI 1.8 to 2.1). When we used conventional classification of BMI as underweight (<20 kg/m^2), normal (20–24 kg/m^2), overweight (BMI 25–29 kg/m^2), and obese (BMI ≥ 30 kg/m^2), mean CLT was 58.0 min (5th to 95th percentile 44.8 to 73.2) in those who were underweight, 63.5 min (5th to 95th percentile 48.0–84.3) in those who had normal BMI, 73.8 min (5th to 95th percentile 52.3 to 109.2) in individuals who were overweight, and 83.9 min (5th to 95th percentile 56.3 to 131.0) in obese individuals. In women not using oral contraceptives, the mean CLT was 5.4 min longer (95% CI 3.0 to 7.7) than in women taking oral contraceptives. Diabetes was associated with a 17% increase in CLT (11.7 min, 95% CI 7.2 to 16.2) compared with individuals without diabetes. In carriers of the prothrombin 20210A mutation, mean CLT was 12% increased (8.1 min, 95% CI 2.3 to 13.8) compared with noncarriers. Carriers of the factor V Leiden mutation had similar CLTs to noncarriers (difference 2.1 min, 95% CI −1.2 to 5.5).

It is known that BMI is associated with plasminogen activator inhibitor-1 (PAI-1) levels [8] and the prothrombin 20210A mutation was found to be associated with increased thrombin-activatable fibrinolysis inhibitor (TAFI) activation in a study that used a clot lysis assay similar to that used in our study [9]. We adjusted the ORs in Table 2 for BMI and the prothrombin 20210A mutation to see whether the increased risk of venous thrombosis in individuals with hypofibrinolysis could (in part) be explained by the increase in these fibrinolytic factors. Adjustment for BMI and the prothrombin 20210A mutation only moderately decreased the association

between hypofibrinolysis and venous thrombosis (ORs [95% CIs] for quartile 2, 3, and 4: 1.4 [1.2 to 1.7], 1.6 [1.3 to 1.9], and 1.8 [1.5 to 2.1]).

Within the patient group, we studied the association between the time interval between the event and the day of blood draw and CLT. The 5th to 95th percentile of this time interval was 188–502 d, with a mean of 318 d (median 301 d). No association was found between the time interval and CLT (0.004 min decrease in CLT/d increase; 95% CI −0.006 to 0.014).

Discussion

In this large population-based case-control study, including over 4,500 individuals, we have shown that decreased fibrinolytic activity, as measured with a plasma-based assay, was associated with an increased risk of a first venous thrombosis. The combination of hypofibrinolysis with immobilization, factor V Leiden, and especially oral contraceptive use, resulted in high relative risks. CLTs were associated with age, BMI, diabetes, oral contraceptive use, and the prothrombin 20210A mutation.

The risk of venous thrombosis for individuals with hypofibrinolysis was 2.4-fold increased compared with those with CLTs in the first quartile. Furthermore, there was a clear dose-response relation between CLT and the risk of venous thrombosis. Our findings support the results of the Leiden Thrombophilia Study (LETS) [2]. In this case-control study, CLTs above the 90th percentile of the levels found in the control group were associated with an almost 2-fold increased risk of venous thrombosis.

Because hypercoagulation is a well-established risk factor for venous thrombosis [1,4], we hypothesized that hypercoagulation and hypofibrinolysis have synergistic effects on venous thrombosis. First, we studied the risk of venous thrombosis in individuals with hypofibrinolysis combined

Table 5. Clot Lysis Times in All Control Participants

Group	Subgroup	Participants (n)	CLT, Mean (5th to 95th Percentile)
Age at blood draw (y), men	All	1,226	70.9 (50.2 to 106.2)
	18–29	96	63.6 (44.5 to 98.8)
	30–39	228	68.1 (49.5 to 102.9)
	40–49	286	72.9 (52.5 to 106.5)
	50–59	345	73.8 (50.5 to 108.3)
	60–71	271	70.2 (50.0 to 110.4)
Age at blood draw (y), women	All	1,338	68.7 (48.6 to 103.0)
	18–29	117	57.7 (44.5 to 73.8)
	30–39	242	61.9 (47.1 to 81.9)
	40–49	331	66.2 (47.9 to 100.3)
	50–59	410	73.9 (52.0 to 114.2)
	60–71	238	75.6 (52.4 to 110.8)
Use of oral contraceptives in women aged <50 y	Yes	266	60.0 (46.2 to 76.3)
	No	424	65.3 (48.2 to 96.3)
Diabetes[a]	Yes	71	81.1 (54.2 to 140.7)
	No	2,389	69.3 (49.3 to 102.5)
BMI (kg/m²)[b]	<20	119	58.0 (44.8 to 73.2)
	20–24	1,117	63.5 (48.0 to 84.3)
	25–29	949	73.8 (52.3 to 109.2)
	≥30	312	83.9 (56.3 to 131.0)
Factor V Leiden mutation	AA or AG	132	71.8 (48.5 to 118.2)
	GG	2,432	69.7 (49.4 to 104.0)
Prothrombin 20210A mutation	AG	43	77.7 (54.5 to 124.9)
	GG	2,521	69.6 (49.4 to 104.9)

[a]Information about diabetes was unavailable for 95 control participants.
[b]Information about BMI was unavailable for 67 control participants.
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with established acquired risk factors. We found a 22-fold increase in risk in women with hypofibrinolysis who were taking oral contraceptives compared with women with neither risk factor. Although the thrombosis risk associated with oral contraceptive use is well known, the mechanisms behind the increased risk of venous thrombosis are still incompletely elucidated [3]. Oral contraceptive use induces complex changes in the hemostatic system, including increased levels of several procoagulant factors, reduction in levels of anticoagulant factors, and activated protein C resistance. In addition, oral contraceptive use results in complex changes in fibrinolytic variables suggestive of increased fibrinolytic capacity. However, the net effect of these changes appears to be prothrombotic. The combination of oral contraceptive use and factor V Leiden was previously shown to result in a thrombosis risk larger than the sum of the individual risks (reviewed in [3]). This effect is presumably due to further exacerbation of thrombin-generating capacity induced by both risk factors. The substantially increased risk associated with hypofibrinolysis and oral contraceptive use possibly relates to a concomitant presence of excessive thrombin-generating capacity and defective clot breakdown.

The combination of immobilization (confinement to bed, surgery, or plaster cast) and hypofibrinolysis resulted in a synergistic effect, possibly via a similar mechanism because immobilization leads to an increase of thrombin formation. The increased risk of venous thrombosis during stasis may be caused by endothelial cell activation and expression of P-selectin, allowing tissue factor–bearing microvesicles to initiate coagulation and thrombosis [10]. Furthermore, increased thrombin generation occurs after surgery, as demonstrated,

for example, by elevated levels of thrombin–antithrombin complexes [11,12]. Although no other studies have investigated the combined risk of immobilization and hypofibrinolysis, in a review it was concluded that there was some evidence for an association between decreased fibrinolytic potential (measured using different assays to the one used in the present study) and postoperative thrombosis [13]. Furthermore, it is found that, among hospitalized patients, the prevalence of asymptomatic thrombi is high, up to about 40% (reviewed in [14]). Although most asymptomatic thrombi resolve without causing clinical consequences, it is plausible that those individuals with a decreased capacity to remove these thrombi have a higher risk of eventually developing a symptomatic venous thrombosis. Similarly, the risk of an unprovoked (idiopathic) thrombosis was also increased in individuals with hypofibrinolysis, which may be explained by defective breakdown of asymptomatic thrombi, as 1% of a healthy population was found to have such an asymptomatic thrombus [15].

Second, we evaluated the effect of hypofibrinolysis in combination with established genetic risk factors, i.e., factor V Leiden or the prothrombin 20210A mutation. The joint presence of factor V Leiden and hypofibrinolysis resulted in a large increase in risk of DVT. Individuals with both factor V Leiden and hypofibrinolysis had an 8.1-fold increased risk, compared with the absence of both risk factors. This joint effect can also be explained by the presence of both an increased procoagulant state and a decreased fibrinolytic potential.

The combination of hypofibrinolysis and the prothrombin 20210A mutation did not lead to a large increase in risk. A difficulty in evaluating the combination of these two risk

factors is the apparent effect of prothrombin 20210A on CLT. In accordance with results previously reported [9], we found that individuals with the prothrombin 20210A mutation had increased CLTs, conceivably related to increased activation of TAFI.

We found shorter CLTs in women using oral contraceptives compared with women not taking oral contraceptives. This is in contrast with the results of a crossover study showing no difference in CLT before and during oral contraceptive use [16]. In that study, t-PA activity, plasminogen, and plasmin-α_2-antiplasmin complexes were increased during oral contraceptive use, and levels of PAI-1 antigen and activity and t-PA antigen were decreased. It was suggested that this increased fibrinolytic activity is counteracted by the increased TAFI levels that were also found. However, a direct comparison of the studies is complicated, because the CLT assays used were slightly different. In the crossover study, the plasma was clotted with thrombin and t-PA, while in our assay tissue factor and t-PA is used.

We found a positive relation between BMI and CLT and increased CLTs in individuals with diabetes. It is plausible that PAI-1 plays an important role in these associations, as it is known that PAI-1 is overexpressed in patients with diabetes and obesity (reviewed in [8]).

In our previous study, we showed that the risk of venous thrombosis in individuals with hypofibrinolysis as measured with our clot lysis assay did not disappear after adjustment for several coagulation factors shown to influence CLTs in the control population [2]. This result suggests that the CLTs are mostly determined by fibrinolytic factors. It is also supported by the association between BMI and CLT and the increased CLTs in individuals with diabetes and those with the prothrombin 20210A mutation. Which fibrinolytic factor or which combination of factors is important for this increased risk is yet to be investigated.

A drawback of using case-control studies for finding associations between the risk of venous thrombosis and blood parameters is that the blood of the patients is drawn after the thrombotic event. In this way decreased fibrinolysis could be a consequence of the disease rather than a cause. However, when CLT would increase as a result of the event, one would expect such an acute-phase effect to attenuate with time after the thrombotic event. In this study no relation was found between CLT and the number of days between the event and the blood draw.

We conclude that the risk of venous thrombosis is increased in individuals with decreased fibrinolytic potential, as measured by an overall plasma-based assay. The combination of hypofibrinolysis with established genetic and acquired risk factors associated with hypercoagulation has a synergistic effect on the thrombosis risk. This joint effect is observed in individuals with hypofibrinolysis who use oral contraceptives, are immobilized, or are carriers of the factor V Leiden mutation, but not in patients who carry the prothrombin 20210A mutation.

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Editors' Summary

Background. When a blood vessel is injured, proteins in the blood called clotting factors "coagulate" (solidify) the blood at the injury site. The resultant clot (thrombus) plugs the wound and prevents blood loss. When the injury has healed, other proteins dissolve the clot, a process called "fibrinolysis." Sometimes, however, a thrombus develops inside an undamaged blood vessel and partly or completely blocks the blood flow. A clot that occurs in one of the veins (vessels that take the blood to the heart) deep within the body (usually in the leg) is a deep vein thrombosis (DVT). Some DVTs have no symptoms; others cause pain, swelling, and tenderness in one leg. They are usually treated with heparin and warfarin, anticoagulant drugs that stop the clot growing. If left untreated, part of the clot (an embolus) can break off and travel to the lungs, where it can cause a life-threatening condition called a pulmonary embolism (PE).

Why Was This Study Done? Most people are very unlikely to develop venous thrombosis (the collective term for DVT and PE), but anything that makes blood "hypercoagulable" (prone to clotting) increases this risk. Genetic risk factors can be inherited changes in blood clotting proteins (for example, a mutation in a gene coding for one protein, factor V, which is involved in clotting, is known as factor V Leiden—Leiden, The Netherlands, was where it was first described). There are also acquired risk factors such as taking oral contraceptives or being immobilized (for example, during bed rest). These risk factors often act in such a way that the risk of developing venous thrombosis for a person with multiple risk factors is greater than the sum of the individual risks. Another recently identified but little studied risk factor for venous thrombosis is "hypofibrinolysis," a decreased capacity to dissolve blood clots. In this study (part of the "MEGA" study on risk factors for venous thrombosis), the researchers investigate the combined effect of hypofibrinolysis and established risk factors associated with hyper-coagulability on the risk of developing venous thrombosis.

What Did the Researchers Do and Find? The researchers collected blood from more than 2,000 individuals after their first DVT or PE and from a similar number of persons without venous thrombosis (controls). For each blood sample, they measured the time it took to dissolve a clot generated from that blood in a test tube (the clot lysis time or CLT) and determined which participants had the factor V Leiden mutation or a genetic change in the clotting factor prothrombin that also increases blood coagulability. The study participants also completed a questionnaire about acquired risk factors for venous thrombosis. The researchers divided the participants into four equal-sized groups (quartiles) based on their CLT and used the quartile with the lowest CLT as the reference group for their statistical analyses; hypofibrinolysis was defined as a CLT in the highest quartile (the longest times). Participants with hypofibrinolysis alone were twice as likely to develop venous thrombosis as those with a CLT in the lowest quartile (the shortest times). Oral contraceptive use alone increased the risk of venous thrombosis 2.5-fold, whereas the combination of oral contraceptive use and hypofibrinolysis increased the risk 20-fold. The researchers also found synergistic effects on thrombosis risk for hypofibrinolysis combined with immobilization or with the factor V Leiden mutation but not with the prothrombin mutation.

What Do These Findings Mean? These findings confirm that persons with hypofibrinolysis and hence longer CLTs have a greater risk of developing venous thrombosis than those with short CLTs. Because CLTs were measured after venous thrombosis had occurred, hypofibrinolysis could be an effect rather than a cause of this condition. However, this is unlikely because there was no association between how long after the venous thrombosis the blood sample was taken and the measured CLT. These findings also show that the combination of hypofibrinolysis with immobilization, the factor V Leiden mutation, and oral contraceptive use greatly increases the risk of venous thrombosis. This new information about the risk factors for venous thrombosis should help physicians to advise patients about reducing their chances of developing this life-threatening condition.

Additional Information. Please access these Web sites via the online version of this summary at http://dx.doi.org/10.1371/journal.pmed.0050097.

- The MedlinePlus encyclopedia has pages on blood clots, deep vein thrombosis, and pulmonary embolism (in English and Spanish)
- The US National Heart Lung and Blood Institute provides information on deep vein thrombosis, including an animation about how DVT causes pulmonary embolisms
- The UK National Health Service Direct health encyclopedia provides information for patients on deep vein thrombosis (in several languages)
- More information about the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study is available on the Leiden University Medical Center Web site
- Wikipedia has pages on coagulation and on fibrinolysis (note that Wikipedia is a free online encyclopedia that anyone can edit; available in several languages)