Venous thrombosis risk associated with plasma hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1

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Abstract

Elevated plasma clot lysis time (CLT) increases risk of venous and arterial thrombosis. It is unclear which fibrinolytic factors contribute to thrombosis risk. In 743 healthy controls we investigated determinants of CLT. By comparison with 770 patients with a first venous thrombosis we assessed plasma levels of fibrinolytic proteins as risk factors for thrombosis. Plasminogen activator inhibitor-1 (PAI-1) levels were the main determinants of CLT, followed by plasminogen, thrombin activatable fibrinolysis inhibitor (TAFI), prothrombin, and α2-antiplasmin. Fibrinogen, factor VII, X, and XI contributed minimally. These proteins explained 77% of variation in CLT. Levels of the fibrinolytic factors were associated with thrombosis risk (odds ratios, highest quartile versus lowest, adjusted for age, sex, and BMI: 1.6 for plasminogen, 1.2 for α2-antiplasmin, 1.6 for TAFI, 1.6 for PAI-1, 1.8 for tissue plasminogen activator (t-PA)). Adjusting for acute-phase proteins attenuated the risk associated with elevated plasminogen levels. The risk associated with increased t-PA nearly disappeared after adjusting for acute-phase proteins and endothelial activation. TAFI and PAI-1 remained associated with thrombosis after extensive adjustment. Concluding, CLT reflects levels of all fibrinolytic factors except t-PA. Plasminogen, TAFI, PAI-1, and t-PA are associated with venous thrombosis. However, plasminogen and t-PA levels may reflect underlying risk factors.
Introduction

Decreased fibrinolytic potential as measured with a plasma-based assay has consistently been shown to be a risk factor for venous and arterial thrombosis.\textsuperscript{1-4} Furthermore, we recently showed that the combination of hypofibrinolysis and risk factors associated with hypercoagulation resulted in a substantially higher risk than expected based on the individual risks conferred by these factors.\textsuperscript{1} The clot lysis assay used in these studies determines time to half maximal lysis of a plasma clot initiated with tissue factor. To induce fibrinolysis, tissue Plasminogen Activator (t-PA) is added to the plasma prior to clot formation. The clot lysis time (CLT) calculated from the turbidity profile of the clot formation and clot lysis is thought to represent overall plasma fibrinolytic capacity. In \textit{in vitro} experiments, we previously investigated the effect of changes in levels of a single fibrinolytic factor on CLT. CLT increased with the addition of active plasminogen activator inhibitor-1 (PAI-1) to pooled normal plasma and with the addition of pooled normal plasma to plasma of a patient with severe $\alpha_2$-antiplasmin deficiency. Furthermore, CLT increased when thrombin activatable fibrinolysis inhibitor (TAFI) was added to immuno-depleted normal plasma. When purified plasminogen was added to immuno-depleted plasminogen deficient plasma, CLT decreased.\textsuperscript{5} In these experiments, a linear relation between TAFI and $\alpha_2$-antiplasmin levels and CLT was found, whereas the relation between plasminogen and CLT was biphasic and between PAI-1 and CLT S-shaped. The relative contribution of the plasma concentration of different fibrinolytic factors to CLT or the association between these factors and CLT in the general population is, however, not known. The procoagulant capacity of plasma has been shown to contribute to some extent to CLT. For example, individuals with the prothrombin G20210A mutation have longer CLTs as a result of increased thrombin generation resulting in increased TAFI activation.\textsuperscript{6}
Based on the experiments with purified proteins, we surmised CLT will be explained by a combination of plasma levels of plasminogen, α2-antiplasmin, TAFI, and PAI-1. As a high concentration of t-PA is added to test plasma to induce fibrinolysis, the role of plasma t-PA levels in CLT is probably minor. In addition, we hypothesized that low levels of plasminogen, and high levels of α2-antiplasmin, TAFI, and PAI-1 constitute a risk factor for venous thrombosis and explain the association between CLT and risk of venous thrombosis.

Studies on the association between plasma levels of individual fibrinolytic factors and risk of venous thrombosis are either lacking or gave inconclusive results. Surprisingly, the association between plasma levels of plasminogen and risk of venous thrombosis has not yet been investigated. Studies on plasminogen deficient individuals, however, have not provided any evidence for a causal role of plasminogen in venous thrombosis risk, but were of limited power to detect an effect.⁷,⁸ Studies investigating levels of α2-antiplasmin and the risk of venous thrombosis are also scarce and have failed to show an association, possibly due to low patient numbers.⁹,¹⁰ Increased TAFI levels were more consistently associated with risk of venous thrombosis¹¹-¹³ although not in thrombophilic families.¹⁴

The role of PAI-1 and t-PA in venous thrombosis is controversial. In the Longitudinal Investigation of Thromboembolism Etiology (LITE) study, a large population-based prospective study on venous thrombosis in middle-aged and elderly individuals no association was found between levels of PAI-1 or t-PA/PAI-1 complex and the risk of venous thrombosis.¹⁵ Although several other studies also failed to show an association between t-PA and PAI-1 and the risk of venous thrombosis, others did find an association (extensively reviewed by Prins and Hirsh¹⁶). In this review it was concluded that there is evidence that increased plasma levels of t-PA and PAI-1 are associated with postoperative thrombosis.
The aim of the present study was to investigate the determinants of CLT in the general population. Furthermore, the association between plasma levels of plasminogen, α2-antiplasmin, TAFI, PAI-1, and t-PA and risk of venous thrombosis was studied.

Subjects and methods

Study design, study population, and data collection

For this study patients and control subjects of the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study were used. The design of the MEGA-study has been described extensively.1 Between March 1999 and May 2002, consecutive unselected patients aged 18 to 70 years, with a first deep vein thrombosis of the leg or a first pulmonary embolism were identified at six anticoagulation clinics in the Netherlands. Information on the diagnostic procedure was obtained from hospital records and general practitioners. A deep venous thrombosis was confirmed with Doppler ultrasonography. A pulmonary embolism was confirmed by a ventilation perfusion lung scan, spiral computed tomography, or angiogram. From January 2002 to September 2004, control subjects were selected from the same geographical area as the patients by random digit dialing (RDD) using the Mitofsky-Waksberg method17 and were frequency matched for sex and age to the patients. This matching was performed on group level in which random controls were selected in numbers proportional to the number of patients within strata of sex and 5-year age groups. Patients or control subjects with severe psychiatric problems or those who could not speak Dutch were excluded. Participation rate was 83% among patients and 69% among control subjects. All participants signed an informed consent form in accordance with the Declaration of Helsinki. Approval for this study was obtained from the Medical Ethics Committee of the Leiden University Medical Center, Leiden, the Netherlands.
All participants were asked to complete a standardized questionnaire on acquired risk factors for venous thrombosis. Body mass index (BMI in kg/m²) was calculated from self-reported weight and height. All items in the questionnaire referred to the period before the index date, which was for the patients the date of diagnosis of thrombosis and for the controls the date they filled in the questionnaire. 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, patients were invited to the anticoagulation clinic for a blood sample. Of patients who received prolonged anticoagulant therapy (>1 year), blood was drawn under treatment with vitamin K antagonists. The control subjects were invited to the clinic for a blood draw after returning their questionnaire. All participants were interviewed regarding present anticoagulant use.

For the current study we analyzed all patients included in the MEGA-study who were presented at the anticoagulation clinic between March 1st 2001 and May 31st 2002 (n=770) and all RDD controls invited for participation from January 1st 2002 until December 31st 2003 (n=743) who provided a blood sample, which can be considered random samples of all patients and RDD controls of whom blood samples were available.

As validation population to study determinants of clot lysis time in, the control group of the Study of Myocardial Infarctions Leiden (SMILE) was used, which included 630 men between 18 and 70 years of age. Of these participants, measurements of all fibrinolytic variables were available. These men were without a history of myocardial infarction, without renal disease or severe (neuro)psychiatric problems, with a life expectancy of more than a year, and who had not taken any oral anticoagulants in the 6-month period prior to participation in the study.4

Blood collection and laboratory analysis
Blood samples were primarily drawn in the morning (median 9.40 h, 95% before 11.00 h), without a systematic difference between patients and control subjects. Time between thrombosis and blood draw ranged from 95 days to 877 days with a median of 299 days and 74% of all patients provided a blood sample between 6 and 12 months after the thrombosis. Blood samples were drawn into vacuum tubes containing 0.106 M trisodium citrate. Plasma was obtained by centrifugation at 2000 g for 10 minutes at room temperature and stored in aliquots at -80°C. 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.\(^5\) In short, 50 µl plasma was pipetted in a 96-well microtitre plate. Subsequently, a 50 µl mixture containing phospholipid vesicles, t-PA (final concentration 56 ng/mL), tissue factor, and CaCl\(_2\) diluted in HEPES was added using a multichannel pipette. In a kinetic microplate the optical density at 405 nm was monitored every 20 seconds, resulting in a clot-lysis turbidity profile. The CLT was derived from this clot-lysis profile and defined as the time 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. \(\alpha_2\)-Antiplasmin and plasminogen activity were measured using chromogenic assays (STA Stachrom \(\alpha_2\)-antiplasmin and STA Stachrom plasminogen from Diagnostica Stago, Asnières, France) and were performed on a STA-R coagulation analyzer using a commercial calibration standard (Diagnostica Stago, Asnières, France). PAI-1 antigen levels were measured with a Technozym PAI-1 enzyme-linked immunosorbent assay (ELISA) reagent kit (Kordia, Biopool, the Netherlands). Plasma TAFI activity levels were determined with a chromogenic assay (Pefakit TAFI, Pentapharm LTD, Basel, Switzerland) by converting TAFI into its active form using a reagent containing thrombin-thrombomodulin and subsequently measuring the carboxypeptidase activity. Measurements were run on a BCS coagulation analyser (Dade Behring Inc., Marburg,
Germany). Antigen levels of t-PA were assessed by ELISA using a commercially available mouse anti-t-PA antibody (Nuclilab BV, Ede, The Netherlands) as capture, and a biotin-labelled rabbit anti human t-PA antibody (Nuclilab BV, Ede, The Netherlands) as detecting antibody. Bound detecting antibody was visualised using horseradish peroxidase-labelled streptavidin, followed by Tetramethylbenzidine (TMB) staining. A calibration curve was constructed using purified t-PA (Nuclilab BV, Ede, The Netherlands). The inter-assay coefficients of variation were 6.6% for CLT, 1.6% for plasminogen, 4.6% for \( \alpha_2 \)-antiplasmin, 5.8% for TAFI, 7.2% for PAI-1 and 8.1% for t-PA.

Measurements of antithrombin and protein C levels were performed with a chromogenic assay and prothrombin (factor II) activity, factor VII activity, factor VIII activity, factor X activity, and factor XI activity 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 according to methods of Clauss.\(^{18}\) Von Willebrand factor (VWF) antigen was measured with the immunoturbidimetric method, using the STA Liatest kit, following the instructions of the manufacturer (Diagnostica Stago). Total homocysteine, total cysteine and methionine (as sum methionine + methioninesulfoxide) were measured using liquid chromatography-mass spectrometry.

The abovementioned parameters were all expressed in percentages relative to pooled normal plasma, except t-PA and PAI-1 levels that were expressed in ng/mL, fibrinogen levels that were expressed in g/L, and the sulphur amino acids that were expressed in \( \mu \)mol/L.

All laboratory measurements were performed without knowledge of whether the sample was from a patient or a control subject.
Statistical analysis

To study determinants of CLT in the general population, simple and multiple linear regression with CLT as dependent variable were performed in the control group. As the use of vitamin K antagonists influences CLT, these analyses were restricted to subjects not taking oral anticoagulants in whom all investigated parameters (plasminogen, α2-antiplasmin, TAFI, PAI-1, t-PA, prothrombin, factor VII, factor VIII, factor IX, factor X, factor XI, protein S, protein C, antithrombin, fibrinogen, VWF and CLT) were measured (N=733). Nine subjects had missing values for the sulphur containing amino acids (homocysteine, cystein and methionine) and were excluded from the analyses including these three factors. As the distribution of CLT is skewed, CLT was 10log-transformed. t-PA, PAI-1, factor VIII and factor XI, homocysteine and fibrinogen also had skewed distributions and were entered after a 10log-transformation for a better fit of the model. The R² was used as a measure of explained variance. To compare the relative strength of the various determinants within the model, all variables (CLT and other plasma factors) were standardized by calculating Z-scores. The Z-score for an observation of a subject is calculated by subtracting the mean from the observed value and dividing the residual by the standard deviation (SD). Simple or multiple linear regression analysis was performed with the standardized variables. The resulting standardized regression coefficient (β) for a factor indicates the increase in SDs of log-CLT, when that particular factor increases with 1 SD and all other variables in the model are unchanged. The fit of the resulting regression models was examined by plotting the observed CLT versus the predicted CTL and versus the independent variables in the model. If this plot showed a non-linear relationship for any of the independent variables, higher order terms were added to the model.

We estimated the unexplained variance (σ²_{unknown}) by subtracting an estimate of variance due to the measurement error of CLT (σ²_{measurement error}) from the residual variance of
the regression analysis. The variance due to measurement error of CLT was estimated from the inter assay variation. Ninety normal pooled plasma samples were measured on ninety 96-well plates yielding a SD of the 10log-transformed CLT measurement of 0.028.

To study the effect of fibrinolytic factors and CLT on risk of venous thrombosis, levels of plasminogen, α2-antiplasmin, TAFI, PAI-1, t-PA, and CLT were grouped into quartiles based on the distribution among the control subjects. Odds Ratios (ORs) with 95% confidence intervals (CIs) were calculated taking the lowest quartile as the reference group for the OR. Unconditional logistic regression was performed to adjust for age, sex and other potential confounders. In the logistic regression model BMI, levels of factor VIII, fibrinogen, VWF and fibrinolytic factors when used as covariates were included as continuous variables. VWF, fibrinogen, t-PA and PAI-1 were included in the model after 10log-transformation. Entering these factors as categorical variables did not change the results. Participants on oral anticoagulants (7 controls and 92 patients) were excluded in the analyses concerning CLT. SPSS 16.0 (SPSS, Chicago, IL, USA) was used for statistical analyses.

Results

Determinants of clot lysis time

To examine determinants of CLT in the general population, 733 control subjects of the MEGA-study were studied, including 374 men and 359 women with a mean age of 46 years (range 18-70). Mean CLT was 65.3 minutes (median 61.7; range 35.0-204.7).

Linear regression was performed to investigate the association between factors of the coagulation and fibrinolytic system and CLT, using the log-transformed CLT as dependent variable. In Table 1 the standardized regression coefficients (βs) of the analyses including plasminogen, α2-antiplasmin, TAFI, PAI-1, and t-PA are shown. In simple linear regression analyses, all fibrinolytic factors except plasminogen were associated with CLT. The strongest
association was found between PAI-1 and CLT, with a regression coefficient of 0.63 (95%CI 0.58-0.69), indicating that with every SD increase in PAI-1, CLT increases with 0.63 SD (PAI-1 and CLT log-transformed). This model had an R$^2$ of 0.63$^2$=0.40, denoting that with PAI-1 as independent factor, 40% of the variation in CLT was explained. Including all fibrinolytic factors (plasminogen, α2-antiplasmin, TAFI, PAI-1, and t-PA) in a multiple regression model increased the explained variance to 53% (Table 1).

Next, we used a separate model without fibrinolytic variables, but including all coagulation factors and sulphur containing amino acids (prothrombin, factor VII, VIII, IX, X, and XI, protein C, protein S, antithrombin, fibrinogen, VWF, cystein, methionine, and homocysteine) resulting in an R$^2$ of 0.29 (regression coefficients not shown). Combining all coagulation and fibrinolytic factors in one overall multiple model yielded a R$^2$ of 0.58 (regression coefficients not shown). Table 2 (model A) shows a reduced model from a backward selection procedure with a similar R$^2$ (0.58), which included the variables which remained significant at $\alpha=0.10$. PAI-1 remained the strongest determinant of CLT ($\beta = 0.49$ (95%CI 0.43-0.54)).

To obtain an estimation of the accuracy of the model, the predicted log-CLT Z-score was calculated for each study participant, using model A of table 2 and back transformed to the original CLT scale. Figure 1a shows the observed CLT plotted against the predicted CLT. The fit of the model seems adequate for shorter CLT, but the longest CLTs are underestimated using this linear model. The reason for this is the nonlinear or cubic association between PAI-1 and CLT as shown in Figure 1b. Therefore, to better fit the model, a squared and cubic term for PAI-1 were added to the overall model including all coagulation and fibrinolytic factors. Table 2 model B shows the reduced model from a backward selection procedure. CLT predicted with regression coefficients from a multiple regression model including the variables of model B plotted against the observed CLT is shown in Figure 1c.
The $R^2$ of this model increased to 0.69, meaning that the residual variation in log-CLT was 31%. The estimated residual variance was equal to 0.0028. The estimated variance due to measurement error was $0.028^2 = 0.00077$, because our inter assay SD was equal to 0.028. This is equal to 27% of the residual variance. Consequently, 8% (27% of 31%) of the total variation in CLT is due to measurement error and 23% (73% of 31%) of the variation in CLT remains unexplained. Accordingly, 77% of the variation in CLT could be explained.

Figure 2 illustrates the effect of the combination of levels of fibrinolytic factors (plasminogen, $\alpha_2$-antiplasmin, TAFI, and PAI-1) on CLT in control subjects. Each fibrinolytic factor was divided in three groups (low, intermediate, and high level, using the mean + or $-\frac{2}{3}\ast SD$ as arbitrary cut-off points), resulting in 81 categories. Mean clot lysis time for each category was calculated. The mean CLTs were classified as low (below $-\frac{1}{3}\ast SD$), intermediate ($-\frac{1}{3}\ast SD$ to $\frac{1}{3}\ast SD$), high ($\frac{1}{3}\ast SD$ to 2*SD) and very high (>2*SD). Groups of similar mean CLT are indicated with the same color, with the more intense colors representing higher mean CLT. Thus, confirming data from the analyses in tables 1 and 2, the figure shows a clear increase in CLT with increasing plasma levels of PAI-1, TAFI, and $\alpha_2$-antiplasmin and decreasing levels of plasminogen. Remarkable is the individual in the upper right corner of the figure with low levels of plasminogen and high levels of $\alpha_2$-antiplasmin, TAFI, and PAI-1 with a CLT 3.5*SD above mean CLT.

To validate the model, we performed an additional regression analysis in the control group of the Study of Myocardial Infarctions LEiden (SMILE) (n=630) using the variables of the model of Table 1. Prothrombin levels were not measured in SMILE and we were thus not able to use the model as presented in table 2. Regression coefficients in this analysis were similar to those found in MEGA: -0.01 for plasminogen, 0.23 for $\alpha_2$-antiplasmin, 0.17 for TAFI, and 0.53 for PAI-1. The $R^2$ of this model was 0.39.
Fibrinolytic proteins and risk of venous thrombosis

Next, the association between plasma levels of the individual fibrinolytic components and risk of venous thrombosis was investigated. In these analyses, 770 patients and 743 control subjects were included. Mean age at time of blood draw of patients was 49 year (range 19-71) and mean age of control participants was 46 year (range 18–70). In the control group 379 participants (51%) were men and in the patient group 347 participants (45%) were men. Of all patients 215 (28%) were diagnosed with an isolated pulmonary embolism, 475 (62%) with an isolated deep vein thrombosis of the leg, and 80 (10%) with both a pulmonary embolism and a deep vein thrombosis of the leg.

Increased levels of each of the individual fibrinolytic proteins were associated with an increased risk of venous thrombosis after adjustment for age and sex (Table 3). As levels of fibrinolytic factors increase with increasing BMI, risks were further adjusted for BMI. After adjusting for BMI, the ORs were 1.6 (95%CI 1.2-2.2) for plasminogen, 1.6 (95%CI 1.2-2.1) for TAFI, 1.6 (95%CI 1.1-2.1) for PAI-1, and 1.8 (95%CI 1.3-2.6) for t-PA for the highest quartile compared with the lowest. α2-Antiplasmin was no longer associated with risk of venous thrombosis (OR 1.2; 95%CI 0.9-1.7).

Previously it has been shown that plasminogen, PAI-1, and t-PA are markers of inflammation. Indeed the risks of thrombosis somewhat decreased after adjustment for markers of inflammation. The risk of venous thrombosis, adjusted for age, sex, BMI, and levels of the acute-phase proteins fibrinogen and factor VIII, decreased to 1.3 (95%CI 0.9-1.8) for the highest quartile of plasminogen. After adjusting for the same factors the risk for the highest quartile of PAI-1 was still 1.7-fold increased (95%CI 1.2-2.3) and for the highest levels of t-PA, the adjusted OR decreased to 1.5 (95%CI 1.0-2.1), all compared with the first quartile.
t-PA can also be seen as a marker of endothelial activation,\textsuperscript{22} so we further adjusted for plasma levels of VWF. The risk of venous thrombosis for individuals with the highest levels of t-PA reduced to 1.3 (95% 0.9-1.9) after adjusting for age, sex, BMI, fibrinogen, factor VIII and VWF. Similar adjustments in the analyses of the other fibrinolytic factors did not considerably change the results. Adjusting for age, sex, fibrinogen, factor VIII, and VWF resulted in ORs for the 4\textsuperscript{th} quartile of 1.4 (95%CI 0.9-2.0) for plasminogen, 1.0 (95%CI 0.7-1.5) for $\alpha$2-antiplasmin, 1.4 (95%CI 1.0-2.0) for TAFI, and 1.6 (95%CI 1.1-2.2) for PAI-1. Finally we adjusted the risks for each fibrinolytic variable additionally for the other fibrinolytic factors. The risk for the 4\textsuperscript{th} quartile of plasminogen adjusted for age, sex, BMI, factor VIII, fibrinogen, VWF, and for plasma levels of $\alpha$2-antiplasmin, PAI-1, t-PA and TAFI was 1.3 (95%CI 0.9-2.0). Similar analysis for the other fibrinolytic factors gave an OR of 1.0 (95%CI 0.7-1.4) for $\alpha$2-antiplasmin, 1.4 (95%CI 1.0-2.0) for TAFI, 1.5 (95%CI 1.0-2.1) for PAI-1, and 1.1 (95%CI 0.7-1.6) for t-PA. Hence PAI-1 and TAFI were still associated with venous thrombosis in these models.

To investigate whether CLT increases the risk of venous thrombosis also independently of the fibrinolytic factors, risk of venous thrombosis for quartiles of CLT was estimated after adjustment for age and sex, and fibrinolytic factors. Age and sex adjusted ORs were 1.5 (95%CI 1.1-2.2) for individuals in the 2\textsuperscript{nd} quartile of CLT, 2.7 (95%CI 1.9-3.7) for the 3\textsuperscript{rd} and 3.4 (95%CI 2.5-4.9) for the 4\textsuperscript{th} quartile, all compared with the lowest. After further adjustment for plasma levels of PAI-1 the risk of venous thrombosis remained 1.5-fold (95%CI 1.1-2.2), 2.5-fold (95%CI 1.8-3.6), and 3.0-fold (95%CI 2.1-4.5) increased for the 2\textsuperscript{nd}, 3\textsuperscript{rd}, and 4\textsuperscript{th} quartile respectively. Entering squared and cubic terms for PAI-1 into the model did not change these results (data not shown). Further adjustment for levels of plasminogen, $\alpha$2-antiplasmin, TAFI, t-PA, and prothrombin gave ORs of 1.4 (95%CI 1.0-2.0) for the 2\textsuperscript{nd}, 2.6 (95%CI 1.8-3.8) for the 3\textsuperscript{rd} and 3.2 (95%CI 2.0-5.2) for the 4\textsuperscript{th} quartile of CLT.
Discussion

Increased clot lysis time as measured with an overall plasma-based assay is associated with an increased risk of venous and arterial thrombosis.\(^1\)\(^-\)\(^4\)\(^,\)\(^23\) However, the factors influencing CLT in the general population were unknown, which prompted us to look for determinants of CLT in a large group of healthy individuals derived from the MEGA-study. In this study we could explain 77% of the variation in CLT. Plasma levels of PAI-1 explained the majority of the variance in CLT, followed by TAFI levels, which is in line with the observation from the present study that elevated levels of PAI-1 and TAFI are independent risk factors for venous thrombosis. Plasma levels of \(\alpha\)2-antiplasmin and plasminogen were associated with CLT to similar extent as TAFI. \(\alpha\)2-Antiplasmin was not associated with venous thrombosis. Surprisingly, plasminogen was positively associated with venous thrombosis. Levels of t-PA did not determine CLT but were associated with risk of venous thrombosis. Plasminogen and t-PA, however, may be just markers of other risk factors such as inflammation and endothelial activation. Besides fibrinolytic factors, prothrombin is an important determinant of CLT. Fibrinogen, factor VII, X, and XI contributed to the variation in CLT to a lesser extent.

Determinants of clot lysis time

Our results are in agreement with previous \textit{in vitro} experiments in which CLT increased with increasing levels of \(\alpha\)2-antiplasmin, TAFI, and PAI-1, and with decreasing levels of plasminogen.\(^3\) Moreover, we could replicate the associations found in the MEGA-study to a large extent in the control group of the SMILE, an independent study population. In our CLT assay t-PA is added to initiate fibrinolysis, mimicking the \textit{in vivo} release of endothelial t-PA after stimulation.\(^22\) Consequently, plasma levels of t-PA do not influence the CLT. Elevated prothrombin levels increase CLT, probably via increased TAFI activation,
which is in line with studies showing that individuals with the prothrombin 20210A mutation had longer CLTs than those without the mutation, and with studies showing that the lysis time of prothrombin-enriched plasma clots was prolonged proportionally to the amount of added prothrombin in a TAFI-dependent manner.\textsuperscript{1,6} Finally, levels of fibrinogen or the sulphur containing amino acids which also have been found to be associated with stiffer or denser clots that are more resistant to fibrinolysis\textsuperscript{24} were minimally or not associated with CLT.

With a statistical model including all factors associated with CLT, we could explain 77\% of the variation in CLT. The 23\% unexplained variation may be due to factors not measured in our study, but known to influence fibrinolysis, or by yet unknown factors. Possible candidates are coagulation factor XIII and Tissue Factor Pathway Inhibitor (TFPI), which were, although not strongly, previously shown to be associated with CLT using an age-adjusted model in the control group of the LEiden Thrombophilia Study (LETS).\textsuperscript{2} Other proteins known to influence fibrinolysis are for instance lipoprotein(a),\textsuperscript{25} which competes with plasminogen for binding to fibrinogen, vitronectin,\textsuperscript{26} which binds to PAI-1 and stabilizes it, histidine-rich glycoprotein which binds to plasminogen and modulates plasminogen bioavailability for plasmin generation,\textsuperscript{27} and the plasmin inhibitors $\alpha_2$-macroglobulin, $\alpha_1$-antitrypsin, and C1 inhibitor.\textsuperscript{28} We are currently examining possible genetic factors influencing CLT through Quantitative Trait Loci (QTL) analysis in an extended thrombophilic pedigree.\textsuperscript{23} Part of the unexplained variation may be the result of lack of fit of the model as the linear regression model assumes a linear relation between the proteins and CLT which may not always be completely accurate. Additionally, only plasma antigen or activity levels of the fibrinolytic factors are included. Consequently the functionality or stability of the factors and the interplay between the factors in the coagulation and fibrinolytic cascade are not taken into account. Plasma levels of plasminogen and $\alpha_2$-antiplasmin are presumably not the limiting factors in fibrinolysis as they circulate at high concentrations in
healthy subjects. The amount of plasmin formed and subsequent lysis of the clot may therefore depend more on regulating steps prior to final activation of plasminogen than on the total amount of plasminogen or $\alpha_2$-antiplasmin present in plasma.\textsuperscript{29}

Although CLT could largely be explained by the levels of the fibrinolytic factors and prothrombin, the association between CLT and venous thrombosis remained after adjusting for the fibrinolytic factors measured. This suggests that indeed CLT is not fully explained by these factors, and that additional proteins are involved. Alternatively, a complex interplay between fibrinolytic factors and prothrombin, not fully accounted for by the statistical models is responsible for the association between elevated CLT and venous thrombosis.

\textit{Fibrinolytic proteins and risk of venous thrombosis}

To our knowledge this is the first large study on levels of plasminogen and $\alpha_2$-antiplasmin and risk of venous thrombosis. Although plasminogen levels were negatively associated with CLT, unexpectedly plasminogen was positively associated with risk of venous thrombosis. Plasma levels of plasminogen are strongly linked to plasma levels of other coagulation and fibrinolytic factors (see supplemental table), which may mask a protective effect of elevated plasminogen levels on venous thrombosis. The role of plasminogen in thrombosis risk, however, has always been unclear. Although plasmin is thought to be the key enzyme responsible for fibrin degradation, plasminogen deficient subjects do not appear to suffer from thrombotic events but primarily from ligneous conjunctivitis, a rare form of chronic conjunctivitis characterized by the development of firm fibrin-rich lesions mainly on the tarsal conjunctivae (reviewed by Brandt\textsuperscript{30}). In contrast, population-based studies have found increased plasminogen levels to be associated with an increased risk of arterial thrombosis.\textsuperscript{19,20} One explanation for this association is that plasminogen levels are increased by inflammatory processes.\textsuperscript{21} We have previously demonstrated that the positive association
between plasma levels of plasminogen and myocardial infarction disappears after adjustment for markers of inflammation. (M.E.M., C.J.M, Ph.G.G, F.R.R., and T.L., manuscript submitted January 2010) In the current study, adjustment for the acute-phase proteins fibrinogen and factor VIII attenuated the association between plasminogen and venous thrombosis, indeed suggesting that plasminogen is a marker of inflammation. However, plasminogen could play a role in venous thrombosis through alternative pathways. Plasmin has other substrates besides fibrin, such as protease-activated receptor-1 (PAR-1), the extracellular matrix, TFPI, and factor V, and plasmin could induce endothelial damage, all potentially important in thrombosis risk.31-35

Although α2-antiplasmin was positively associated with CLT, no association was found between α2-antiplasmin and risk of venous thrombosis after adjusting for BMI. This is in agreement with two small studies in which levels of α2-antiplasmin were not associated with postoperative thrombosis.9,10 As stated previously, α2-antiplasmin normally circulates at high levels and may therefore not be a limiting factor.

TAFI defines the molecular connection between the coagulation and fibrinolytic cascades.36,37 We find TAFI levels to be associated with an increased risk of venous thrombosis. This is in accordance with previous studies investigating the association between TAFI levels and first thrombosis, either in a general population11 or in factor V Leiden carriers,12 and with a study on TAFI and recurrent venous thrombosis.13 In these studies, TAFI levels above the 90th percentile of the control group were associated with a 2 to 4-fold increased risk of venous thrombosis.

The role of t-PA and PAI-1 in venous thrombosis is controversial. Although several studies have found a positive relation with venous thrombosis others have not. In an extensive review it was concluded that PAI-1 and t-PA may be important in venous thrombosis especially in individuals undergoing surgery.16 In present study, elevated levels of PAI-1 and
t-PA were associated with venous thrombosis. Adjustment for acute-phase proteins and VWF attenuated the increased risk found in individuals with high t-PA levels. As most of the t-PA antigen in plasma is bound to PAI-1 and therefore inactive it is plausible that t-PA is just a marker of underlying processes, such as inflammation and endothelial activation. This observation is in agreement with two studies investigating the association between t-PA and arterial thrombosis. Increased PAI-1 levels were still associated with venous thrombosis, even after extensive adjustment, which suggests that hypofibrinolysis caused by elevated PAI-1 levels indeed increases thrombosis risk.

It should be noted that the adjustments made for plasma proteins in all analyses presented must be considered with caution. Adjustments are justified for factors that are risk factors for venous thrombosis and that influence the fibrinolytic factor of interest but are not influenced by the fibrinolytic factor themselves. As coagulation factors and fibrinolytic factors tend to cluster and correlate and possibly share genetic regulation, causal inference and justification of the adjustments is difficult.

In conclusion, variation in clot lysis time could be explained for 77%. PAI-1 was the principal determinant of CLT, followed by plasminogen, TAFI, prothrombin, and $\alpha_2$-antiplasmin. Increased plasma levels of plasminogen, TAFI, PAI-1, and t-PA were associated with an increased risk of venous thrombosis, although plasminogen and t-PA may be markers of other risk factors for venous thrombosis.

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MD) who made the recruitment of patients possible. The interviewers (J.C.M. van den Berg, B. Berbee, S. van der Leden, M. Roosen, and E.C. Willems of Brilman) performed the blood draws. We also thank I. de Jonge, MSc, R. Roelofsen, MSc, M. Streevelaar, L.M.J. Timmers, MSc, and J.J. Schreijer for their administrative support and data management. The fellows I.D. Bezemer, PhD, J.W. Blom, MD, A. van Hylckama Vlieg, PhD, E.R. Pomp, PhD, L.W. Tick, MD, and K.J. van Stralen, PhD took part in every step of the data collection. M.A. Weine, L.M. Leverink, E.J. Hoenderdos, W.F. Kopatz, S. Moschatsis, J. Adelmeijer, C.J.M. van Dijk, R. van Eck, J. van der Meijden, P.J. Noordijk, and T. Visser performed the laboratory measurements. We express our gratitude to all individuals who participated in the MEGA study. This research was supported by the European Hematology Association (2005/04), the Netherlands Heart Foundation (NHS 98.113 and 2005B060), the Dutch Cancer Foundation (RUL 99/1992) and the Netherlands Organisation for Scientific Research (912-03-033|2003). The funding organizations did not play a role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript.

Contributions: M.E.M. designed the present study, analyzed and interpreted the data, drafted the manuscript; T.L. designed the present study, interpreted the data, critically reviewed the analyses and participated in writing the manuscript. P.G.d.G., J.C.M.M. and S.l.C. interpreted the data and critically reviewed the analyses and the manuscript; C.J.M.D. designed the overall study, performed the data collection, interpreted the data, and critically reviewed the analyses and the manuscript; F.R.R. designed the overall study, interpreted the data, and critically reviewed the analyses and the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
Reference List

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fibrinolysis and identification as histidine-rich glycoprotein. *J Biol Chem.*


Table 1 Mean change in clot lysis time* with 1 standard deviation increase in fibrinolytic factor.

<table>
<thead>
<tr>
<th></th>
<th>Simple models†</th>
<th>Multiple Model‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β (95% CI)</td>
<td>R²</td>
</tr>
<tr>
<td>plasminogen</td>
<td>0.04 (-0.03-0.12)</td>
<td>0</td>
</tr>
<tr>
<td>α2-antiplasmin</td>
<td>0.26 (0.19-0.33)</td>
<td>0.07</td>
</tr>
<tr>
<td>TAFI</td>
<td>0.40 (0.33-0.47)</td>
<td>0.16</td>
</tr>
<tr>
<td>PAI-1*</td>
<td>0.63 (0.58-0.69)</td>
<td>0.40</td>
</tr>
<tr>
<td>t-PA*</td>
<td>0.39 (0.32-0.45)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

TAFI thrombin activatable fibrinolysis inhibitor; PAI-1 plasminogen activator inhibitor 1; t-PA tissue plasminogen activator

* Clot lysis time, PAI-1 and t-PA were 10log-transformed.

† five different models. In each model clot lysis time was the dependent variable and only one of the fibrinolytic factors as independent variable.

‡ Clot lysis time as dependent variable and all five fibrinolytic factors simultaneously as independent variables in the model.
Table 2 Multiple linear regression on clot lysis time* without (model A) and with a squared and cubic term (model B) for PAI-1.

<table>
<thead>
<tr>
<th></th>
<th>Model A</th>
<th></th>
<th></th>
<th>Model B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$ (95% CI)</td>
<td>$R^2$</td>
<td>$\beta$ (95% CI)</td>
<td>$R^2$</td>
<td></td>
</tr>
<tr>
<td>prothrombin</td>
<td>0.15 (0.08-0.22)</td>
<td>0.58</td>
<td>0.16 (0.11-0.22)</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>factor VII</td>
<td>0.05 (-0.09-0.11)</td>
<td></td>
<td>0.07 (0.02-0.12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>factor IX</td>
<td>0.06 (-0.01-0.12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>factor X</td>
<td>0.07 (-0.00-0.13)</td>
<td></td>
<td>0.06 (0.00-0.12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>factor XI*</td>
<td></td>
<td></td>
<td>0.05 (0.01-0.10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein C</td>
<td>0.07 (0.00-0.13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>antithrombin</td>
<td>-0.05 (-0.11-0.00)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fibrinogen</td>
<td>0.08 (0.02-0.14)</td>
<td></td>
<td>0.10 (0.05-0.15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>plasminogen</td>
<td>-0.26 (-0.33--0.20)</td>
<td></td>
<td>-0.29 (-0.34--0.23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha$2-antiplasmin</td>
<td>0.14 (0.09-0.20)</td>
<td></td>
<td>0.16 (0.11-0.21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAFI</td>
<td>0.21 (0.15-0.26)</td>
<td></td>
<td>0.23 (0.18-0.28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1*</td>
<td>0.49 (0.44-0.54)</td>
<td></td>
<td>0.28 (0.22-0.35)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1 (squared)*</td>
<td></td>
<td></td>
<td>0.27 (0.22-0.32)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1 (cubic)*</td>
<td></td>
<td></td>
<td>0.22 (0.14-0.29)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TAFI thrombin activatable fibrinolysis inhibitor; PAI-1 plasminogen activator inhibitor 1

* Clot lysis time, factor XI, and PAI-1 were log-transformed.
**Table 3** Fibrinolytic factors and risk of venous thrombosis.

<table>
<thead>
<tr>
<th>Quartile of fibrinolytic factor</th>
<th>1 (reference)</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasminogen (cut off in %)</strong>*</td>
<td>88</td>
<td>96</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td># patients</td>
<td>131</td>
<td>180</td>
<td>209</td>
<td>250</td>
</tr>
<tr>
<td># control subjects</td>
<td>188</td>
<td>187</td>
<td>185</td>
<td>182</td>
</tr>
<tr>
<td>OR age &amp; sex</td>
<td>1</td>
<td>1.3 (1.0-1.8)</td>
<td>1.5 (1.1-2.0)</td>
<td>1.8 (1.4-2.5)</td>
</tr>
<tr>
<td>OR age, sex, BMI</td>
<td>1</td>
<td>1.2 (0.9-1.7)</td>
<td>1.3 (1.0-1.8)</td>
<td>1.6 (1.2-2.2)</td>
</tr>
<tr>
<td><strong>α2-antiplasmin (cut off in %)</strong>*</td>
<td>95</td>
<td>102</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td># patients</td>
<td>175</td>
<td>179</td>
<td>205</td>
<td>211</td>
</tr>
<tr>
<td># control subjects</td>
<td>197</td>
<td>197</td>
<td>183</td>
<td>165</td>
</tr>
<tr>
<td>OR age &amp; sex</td>
<td>1</td>
<td>1.0 (0.8-1.3)</td>
<td>1.2 (0.9-1.7)</td>
<td>1.4 (1.0-1.9)</td>
</tr>
<tr>
<td>OR age, sex &amp; BMI</td>
<td>1</td>
<td>1.0 (0.7-1.3)</td>
<td>1.1 (0.8-1.5)</td>
<td>1.2 (0.9-1.7)</td>
</tr>
<tr>
<td><strong>TAFI (cut off in %)</strong>*</td>
<td>106</td>
<td>117</td>
<td>127</td>
<td></td>
</tr>
<tr>
<td># patients</td>
<td>143</td>
<td>195</td>
<td>166</td>
<td>266</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>121 (93-155) %</td>
</tr>
</tbody>
</table>

*median (5-95th percentile)
<table>
<thead>
<tr>
<th># control subjects</th>
<th>199</th>
<th>180</th>
<th>179</th>
<th>184</th>
<th>117 (92-147) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>OR&lt;sub&gt;age &amp; sex&lt;/sub&gt;</td>
<td>1</td>
<td>1.4 (1.1-1.9)</td>
<td>1.2 (0.9-1.7)</td>
<td>1.8 (1.3-2.4)</td>
<td></td>
</tr>
<tr>
<td>OR&lt;sub&gt;age, sex &amp; BMI&lt;/sub&gt;</td>
<td>1</td>
<td>1.3 (1.0-1.8)</td>
<td>1.1 (0.8-1.6)</td>
<td>1.6 (1.2-2.1)</td>
<td></td>
</tr>
</tbody>
</table>

### PAI-1 (cut off in ng/mL)*

<table>
<thead>
<tr>
<th># patients</th>
<th>148</th>
<th>169</th>
<th>120</th>
<th>332</th>
<th>146 (40-552) ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td># control subjects</td>
<td>185</td>
<td>186</td>
<td>186</td>
<td>185</td>
<td>113 (36-401) ng/mL</td>
</tr>
<tr>
<td>OR&lt;sub&gt;age &amp; sex&lt;/sub&gt;</td>
<td>1</td>
<td>1.1 (0.8-1.5)</td>
<td>0.8 (0.6-1.1)</td>
<td>2.2 (1.6-2.9)</td>
<td></td>
</tr>
<tr>
<td>OR&lt;sub&gt;age, sex &amp; BMI&lt;/sub&gt;</td>
<td>1</td>
<td>1.1 (0.8-1.4)</td>
<td>0.7 (0.5-0.9)</td>
<td>1.6 (1.1-2.1)</td>
<td></td>
</tr>
</tbody>
</table>

### t-PA (cut off in ng/mL)*

<table>
<thead>
<tr>
<th># patients</th>
<th>134</th>
<th>135</th>
<th>209</th>
<th>292</th>
<th>7.0 (3.8-12.0) ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td># control subjects</td>
<td>185</td>
<td>186</td>
<td>186</td>
<td>185</td>
<td>6.1 (3.4-10.9) ng/mL</td>
</tr>
<tr>
<td>OR&lt;sub&gt;age &amp; sex&lt;/sub&gt;</td>
<td>1</td>
<td>1.0 (0.8-1.4)</td>
<td>1.6 (1.2-2.2)</td>
<td>2.3 (1.7-3.3)</td>
<td></td>
</tr>
<tr>
<td>OR&lt;sub&gt;age, sex &amp; BMI&lt;/sub&gt;</td>
<td>1</td>
<td>0.9 (0.7-1.3)</td>
<td>1.3 (0.9-1.8)</td>
<td>1.8 (1.3-2.6)</td>
<td></td>
</tr>
</tbody>
</table>

TAFI thrombin activatable fibrinolysis inhibitor; PAI-1 plasminogen activator inhibitor 1; t-PA tissue plasminogen activator; and BMI body mass index.
* PAI-1 levels were not measured in one patient; TAFI, plasminogen, α2-antiplasmin, t-PA, and PAI-1 levels were not measured in one control subject.
a. Predicted CLT (model A) vs. Observed CLT. $R^2 = 0.58$

b. Log transformed PAI-1 vs. Log transformed CLT. Cubic regression line, squared regression line, linear regression line.

c. Predicted CLT (model B) vs. Observed CLT. $R^2 = 0.69$
**Figure 1a** Observed clot lysis time (CLT) plotted against the predicted CLT using regression coefficients derived from a linear regression model including levels of prothrombin, factor VII, factor IX, factor X, protein C, antithrombin, fibrinogen, plasminogen, α2-antiplasmin, TAFI and PAI-1 (Table 2; model A). **1b** Association between PAI-1 and CLT. **1c** Observed CLT plotted against predicted CLT using regression coefficients derived from a linear regression including levels of prothrombin, factor VII, factor X, factor XI, fibrinogen, plasminogen, α2-antiplasmin, TAFI, PAI-1 and a squared and cubic term for PAI-1 (Table 2; model B).
CLT and PAI-1 were based on 10log transformed values.

Cut-offs for fibrinolytic factors (μ=population mean; SD=standard deviation): low: <μ−2/3SD; intermediate: μ−2/3SD to μ+2/3SD; high: >μ+2/3SD. Cut-off for CLT low: <μ−1/3 SD; intermediate: μ−1/3SD to μ+1/3SD; high: μ+1/3SD to μ+2SD; very high: >μ+2SD. Cut-offs for CLT and PAI-1 were based on 10log transformed values.

**Figure 2. CLT for different combinations of levels of fibrinolytic factors.**

Numbers in the squares indicate the number of control subjects included in each category.

CLT clot lysis time, TAFI thrombin activatable fibrinolysis inhibitor, PAI-1 plasminogen activator inhibitor.
Venous thrombosis risk associated with plasma hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1

Mirjam E. Meltzer, Ton Lisman, Philip G. de Groot, Joost C.M. Meijers, Saskia le Cessie, Carine J.M. Doggen and Frits R. Rosendaal