Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis
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Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis

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The role of the fibrinolytic system in the development of deep vein thrombosis (DVT) is unclear. We determined the plasma fibrinolytic potential of patients enrolled in the Leiden Thrombophilia Study (LETS), a population-based case-control study on risk factors for DVT. Plasma fibrinolytic potential was determined in 421 patients and 469 control subjects by means of a tissue factor–induced and tissue-type plasminogen activator (tPA)–induced clot lysis assay. Using clot lysis times above the 70th, 80th, 90th, 95th, and 99th percentiles of the values found in control subjects as cut-off levels, we found a dose-dependent increase in risk for DVT in patients with hypofibrinolysis (odds ratios of 1.4, 1.6, 1.9, 2.1, and 2.2, respectively). This indicates a 2-fold increased risk of DVT in subjects with clot lysis times above the 90th percentile. The risk increase was not affected by age or sex (adjusted odds ratio for 90th percentile, 2.0), and after correction for all possible confounders (age, sex, and levels of procoagulant proteins shown to associate with clot lysis times in the control population), the risk estimate was marginally reduced (odds ratio, 1.6 for 90th percentile). Taken together, these results indicate that plasma hypofibrinolysis constitutes a risk factor for venous thrombosis, with a doubling of the risk at clot lysis times that are present in 10% of the population. (Blood. 2005;105:1102-1105)

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Evidence for a role of fibrinolysis, the dissolution of a fibrin clot, in the etiology of venous thrombosis is scarce. Genetic evidence has been limited to reports on patients with hereditary plasminogen deficiency, although the relationship between heterozygous plasminogen deficiency and thrombosis has been questioned (for a review, see Brandt). Moreover, homozygous plasminogen deficiency is not associated with deep vein thrombosis (DVT), but rather presents as ligneous conjunctivitis caused by vascular deposition of fibrin within the eye. Elevated levels of plasminogen activator inhibitor type 1 (PAI-1), and the PAI-1 4G/5G polymorphism have been suggested as a risk factor for venous thrombosis, but data are highly conflicting and unconvincing (for a review, see Francis). Baseline plasma levels of tissue-type plasminogen activator (tPA) did not predict a first episode of venous thrombosis nor did they predict the risk of recurrence. Elevated plasma levels of thrombin activatable fibrinolysis inhibitor (TAFI), however, were a mild risk factor for the development of venous thrombosis. Next to levels of proteins involved in fibrinolysis, levels of markers of fibrinolytic activity were studied in relation to the development of venous thrombosis. Levels of tPA/PAI-1 complex and plasmin-antiplasmin (PAP) complex were not associated with an increased risk for venous thrombosis.

Studies on a relationship between fibrinolysis and venous thrombosis have focused on plasma levels of proteins or protease-inhibitor complexes. Global tests of fibrinolytic activity such as the euglobulin clot lysis time (ECLT) and the dilute whole-blood clot lysis time (DWBCLT) have been used to study the association of thrombosis and hypofibrinolysis, but these studies gave inconclusive results (summarized by Prins and Hirsh). Moreover, the outcome of these tests do not reflect a true global fibrinolytic potential, because these tests are either performed with plasma fractions (ECLT) or in the presence of citrate (DWBCLT), which excludes the interplay between coagulation and fibrinolysis through TAFI and factor XIII.

We have developed a plasma-based, tissue factor-induced clot lysis assay, in which a fibrin clot is lysed by exogenously added tPA. Although this assay was initially developed to study TAFI-related processes, we have shown that the clot lysis time (CLT) in this assay is also influenced by levels of other proteins involved in fibrinolysis and thrombin generation (which is required for TAFI activation), including plasminogen, α2-antiplasmin, PAI-1, and antithrombin. The outcome of this assay therefore reflects an overall plasma fibrinolytic potential. This test has been used to study fibrinolysis in a number of pathologies. We demonstrated a hyperfibrinolytic state in hemophilia, factor XI deficiency, and after use of heparin and heparin-like anticoagulants as a consequence of reduced thrombin generation resulting in defective TAFI activation. In patients with liver disease, plasma fibrinolytic potential was similar to that in controls, despite severely reduced TAFI levels, which could be explained by a concomitant decrease in profibrinolytic proteins. During oral contraceptive use, overall plasma fibrinolytic potential did not change, but factor XI–independent CLTs were prolonged.

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Here, we assessed the contribution of the fibrinolytic system to the
development of venous thrombosis. For this, our CLT assay,
which measures global plasma fibrinolytic potential, taking the
interplay between coagulation and fibrinolysis (eg, through TAFI)
into account, was used. This study was part of the Leiden
Thrombophilia Study (LETS), a large population-based case-
control study designed to estimate the contributions of genetic and
acquired factors to the risk of venous thrombosis.

Patients, materials, and methods

Patients

The design of the LETS has been extensively described previously.\(^\text{17,18}\) In
short, 474 consecutive patients with an objectively confirmed first episode
of DVT that occurred between January 1988 and December 1992 were
selected from 3 anticoagulation clinics in The Netherlands. Patients were
younger than 70 years and had no known malignancy. Controls were
acquaintances or partners, and they were matched for age and sex.
Blood was collected in tubes containing 0.106 M trisodium citrate.
Plasma was prepared by centrifugation at 2000 g for 10 minutes at room
temperature and stored at \(-70^\circ\mathrm{C}\). Samples were obtained at least 6 months
after the thrombotic event and at least 3 months after discontinuation of oral
anticoagulant treatment. For this study, plasma samples from 469 controls
and 469 patients were available. However, 48 patients were excluded from
the analysis because they were still using oral anticoagulation at the time of
venipuncture. Samples, which were kept at \(-70^\circ\mathrm{C}\) at all times and were not
previously thawed, were used for this study. Approval for this study was
obtained from the Institutional Review Board of the Leiden University
Medical Center. Informed consent was provided according to the Declara-
tion of Helsinki.

Clot lysis assay

Lysis of a tissue factor–induced clot by exogenous tPA was studied by
monitoring changes in turbidity during clot formation and subsequent lysis
essentially as described previously.\(^\text{10}\) Plasma (50 \(\mu\)L) was pipetted into a microtiter plate, after
which 50 \(\mu\)L of a mixture containing tissue factor (diluted Innovin; Dade
Behring, Marburg, Germany; final dilution 1000 times), CaCl\(_2\) (final
concentration 17 mM), tPA (Chromogenix, Mölndal, Sweden; final concen-
tration 30 U/mL; 56 ng/mL), and phospholipid vesicles (consisting of 40%
L-\(\alpha\)-dioleoylphosphatidylcholine, 20\% L-\(\alpha\)-dioleoylphosphatidylserine, and
40\% L-\(\alpha\)-dioleoylphosphatidylethanolamine, all from Sigma, St Louis,
MO), prepared according to Brunner et al\(^{19}\) with minor modifications as
described by van Wijnen et al\(^{20}\); final concentration 10 \(\mu\)M), 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 [BSA], pH 7.4) was added. After thorough mixing,
turbidity at 405 nm was measured in time at 37°C in a Spectramax 340
kinetic microplate reader (Molecular Devices Corporation, Menlo Park,
CA). CLT was defined as the time from the midpoint of the clear to
maximum turbid transition, which characterizes clot formation, to the
midpoint of the maximum turbid to clear transition, which represents clot
lysis. The intra-assay and interassay coefficients of variation were 3.4%
\((n = 16)\) and 4.0\% \((n = 32)\), respectively. All assays were performed
without knowledge of whether the sample was from a patient or a control.

Statistical analysis

Data were analyzed with 2 objectives. First, determinants of CLT were
assessed in the control subjects as reflecting the general population.
Determinants were established by comparing means and using linear
regression. Second, it was investigated whether elevated CLT is a risk factor
for DVT by calculating odds ratios (ORs) and 95\% confidence intervals
(CIs). As cut-off points we used percentiles (70, 80, 90, 95, and 99) of CLTs
measured in the control subjects. We used multivariate modeling by

unconditional logistic regression to adjust for sex, age, and other putative
confounding variables.

Results

In this study, 421 patients and 469 controls were used. The mean age
of the patients and controls was 45 years (range, 14-69 years in
patients; 14-72 years in controls). In the control population 47\%
were male, whereas 43\% of the patients were male.

Determinants of CLTs

Determinants of CLTs in the control population are shown in Table
1. The mean of the CLTs in the 469 control subjects was 61.0
minutes (range, 38.8-135.2 minutes). CLTs were slightly higher in
men than in women (mean difference, 3.3 minutes; 95\% CI, 1.3-5.3). CLTs progressively increased with age and were reduced
by oral contraceptive use, although this effect was small.

To investigate the relationship between levels of clotting factors and
the CLT, univariate regression analysis was performed. Table 2
shows regression coefficients (\(b\)) and their 95\% CIs for all the
tested variables. Although the levels of several coagulation factors
were associated with the CLTs, all associations were weak,
indicating that levels of these factors only to a small extent
determined the CLT in our assay. A weak association between CLT
and the endogeneous thrombin potential was present; however, this
association disappeared after correction for age, indicating that
CLTs were not influenced by the thrombin-generating capacity of
the plasma. No association between CLT and TFPI activity, total
TFPI, factor XIII activity, and factor XIII a-subunit could be
demonstrated.

CLT as a risk factor for DVT

For determination of the contribution of elevated CLTs to the risk
of DVT, cut-off levels of CLTs in the control group were set at the
70th, 80th, 90th, 95th, and 99th percentiles. The ORs for DVT in
subjects with CLTs above these cut-off values, with values below
the cut-off as reference group, progressively increased over the
range of cut-off levels (Table 3). For a cut-off point at the 90th
percentile, 18\% of the individuals with DVT had a CLT above this
cut-off, compared to 10\% (per definition) in the control group. This
implies that CLTs above 74.1 minutes were associated with an
almost 2-fold increased risk of DVT (OR, 1.9; 95\% CI, 1.3-2.9).

After adjustment for sex and age, the OR for the 90th percentile
was 2.0 (95\% CI, 1.3-3.0). After adjustment for all factors that,

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of subjects</th>
<th>CLT, mean min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>198</td>
<td>63.0</td>
</tr>
<tr>
<td>Female</td>
<td>271</td>
<td>59.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Use of oral contraceptives in premenopausal women</th>
<th>No. of subjects</th>
<th>CLT, mean min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>67</td>
<td>53.4</td>
</tr>
<tr>
<td>No</td>
<td>114</td>
<td>56.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age, y</th>
<th>No. of subjects</th>
<th>CLT, mean min</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 or younger</td>
<td>42</td>
<td>53.9</td>
</tr>
<tr>
<td>25-34</td>
<td>55</td>
<td>54.2</td>
</tr>
<tr>
<td>35-44</td>
<td>104</td>
<td>59.1</td>
</tr>
<tr>
<td>45-54</td>
<td>133</td>
<td>62.7</td>
</tr>
<tr>
<td>55 and older</td>
<td>135</td>
<td>66.0</td>
</tr>
</tbody>
</table>
Discussion

This study shows that individuals with reduced fibrinolytic potential, as measured by a plasma-based assay, have an increased risk of developing a first venous thrombosis. The risk of thrombosis increased with increasing plasma CLT. Individuals with a plasma CLT above the 90th percentile of the control subjects have an almost 2-fold increased risk for development of a first DVT. The CLT above the 90th percentile of the control subjects have an increased risk of thrombosis. Based on these data, the population-attributable risk of thrombosis associated with prolonged CLTs reflects abnormalities in the fibrinolytic system. Unfortunately, levels of fibrinolytic proteins, such as plasminogen, α2-antiplasmin, and PAI-1, as well as the PAI-1 4G/5G polymorphism, have not been measured in the LETS population. Consequently, potential denominators of the CLT in terms of proteins known to be important in fibrinolysis could not be elucidated from this study.

Our clot lysis assay is presumably not sensitive for variations in plasma levels of tPA or urokinase, because a fixed amount of exogenous tPA is added in the assay to induce clot lysis. Also, a contributory role of factor XIII in determining CLTs was excluded from the present study. No association between CLT and factor XIII activity or the α-subunit antigen level could be demonstrated. An association between the catalytically inactive factor XIII b-subunit and CLT was present, which may indicate that the b-subunit has an important role in determining CLTs.

In this study we show for the first time a clear association between plasma hypofibrinolysis and the risk of thrombosis. The role of the fibrinolytic system in DVT has been debated. Previous studies have either examined single fibrinolytic parameters (tPA and PAI-1 activity or antigen, PAI-1 4G/5G polymorphism), plasma markers of fibrinolysis (PAP complexes, tPA/PAI-1 complexes), or clot lysis assays in which fibrinolytic potential was only assessed partially, that is, in a plasma fraction, or in the absence of calcium (ELT or DWBCLT, respectively), and were often in small or selected groups of patients. Here, we have used a tissue factor–induced clotting assay, in which fibrinolysis is initiated by exogenous tPA in a large population-based case-control study. The outcome of the assay presumably represents an overall measure for the plasma fibrinolytic potential resulting from a combination of plasma levels of proteins important for clot dissolution. Indeed, we have shown previously that this clot lysis assay is influenced by levels of plasminogen, α2-antiplasmin, PAI-1, and TAFI. In accordance, in this study, in which individuals have been characterized very extensively, no strong associations of proteins involved in clot formation were observed.

Surprisingly, TAFI levels were only weakly associated with CLTs. Previously, it has been demonstrated that addition of purified TAFI to TAFI-depleted plasma dose-dependently prolongs CLT. Moreover, an association between TAFI antigen levels and CLTs were found in plasma samples from 20 healthy volunteers. In this study we show that although an association between TAFI antigen levels and CLTs exists, the contribution of TAFI levels to CLTs is small. In other words, the CLT in a given plasma sample is determined by the total fibrinolytic capacity of the plasma, which is determined by the balance of the levels of all fibrinolytic proteins.

No association between the thrombin-generating capacity of the plasma (endogenous thrombin potential [ETP]) and CLT could be demonstrated, which is in contrast with earlier observations in which the extent of TAFI activation was linked to the amount of thrombin generated after clot formation via the factor XI feedback loop. Also, both in the present study and in literature, CLTs were associated with prothrombin plasma levels, which were shown to be accompanied by an increased generation of activated TAFI. Because it was previously demonstrated that ETP values are strongly linked to prothrombin plasma levels, it is surprising that no association between ETP and CLTs times was found now.

The risk for DVT associated with plasma hypofibrinolysis did not disappear after adjustment for all variables shown to influence CLTs in the control population. This again is an indication that the risk of DVT associated with prolonged CLTs reflects abnormalities in the fibrinolytic system. Unfortunately, levels of fibrinolytic proteins, such as plasminogen, α2-antiplasmin, and PAI-1, as well as the PAI-1 4G/5G polymorphism, have not been measured in the LETS population. Consequently, potential denominators of the CLT in terms of proteins known to be important in fibrinolysis could not be elucidated from this study.

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| Table 2. Association of CLTs with levels of coagulation factors and the ETP |
|-----------------|-----------------|-----------------|-----------------|
| Fibrinogen, g/L | 3.251 (1.758-4.744) | 1.963 (0.540-3.387) |
| Factor II, %    | 0.160 (0.092-0.227) | 0.163 (0.102-0.225) |
| Factor VII, %   | 0.121 (0.076-0.166) | 0.090 (0.050-0.135) |
| Factor VIII, %  | 0.004 (0.002-0.034) | 0.016 (0.004-0.013) |
| Factor IX, %    | 0.058 (0.049-0.111) | 0.033 (0.017-0.083) |
| Factor X, %     | 0.064 (0.001-0.129) | 0.105 (0.044-0.166) |
| Factor V, %     | 0.070 (0.041-0.100) | 0.038 (0.010-0.068) |
| Factor XI, %    | 0.091 (0.041-0.141) | 0.074 (0.028-0.122) |
| TAFI, %         | 0.228 (0.149-0.306) | 0.188 (0.115-0.261) |
| Factor XIII, b-subunit, % | 0.182 (0.109-0.255) | 0.145 (0.074-0.214) |
| Free TFPI, ng/mL | 0.953 (0.715-1.192) | 0.762 (0.519-1.004) |
| Antithrombin III, % | 0.050 (0.047-0.146) | 0.107 (0.017-0.196) |
| C-reactive protein, μg/mL | 0.095 (0.107-0.287) | 0.076 (0.110-0.263) |
| ETP, mM l̊a · min | –0.001 (–0.002-0.000) | 0.000 (–0.001-0.000) |

The regression coefficient b shows the increase in clot lysis time (in minutes) per unit increase in the variable studied. Also shown are regression coefficients after correction for age. Levels of coagulation factors are expressed as percentage of the 90th percentile of the controls), high levels of factor IX22/ETP, nM IIa as indicated.

Table 3. Risk of venous thrombosis according to CLTs

<table>
<thead>
<tr>
<th>Cut-off, percentile</th>
<th>Lysis time at cut-off, min</th>
<th>No. of patients</th>
<th>No. of controls</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>65.4</td>
<td>158</td>
<td>141</td>
<td>1.4 (1.1-1.8)</td>
</tr>
<tr>
<td>80</td>
<td>68.6</td>
<td>119</td>
<td>94</td>
<td>1.6 (1.2-2.1)</td>
</tr>
<tr>
<td>90</td>
<td>74.1</td>
<td>75</td>
<td>47</td>
<td>1.9 (1.3-2.9)</td>
</tr>
<tr>
<td>95</td>
<td>79.3</td>
<td>42</td>
<td>23</td>
<td>2.1 (1.3-3.6)</td>
</tr>
<tr>
<td>99</td>
<td>97.8</td>
<td>8</td>
<td>4</td>
<td>2.2 (0.7-7.5)</td>
</tr>
</tbody>
</table>

Cut-off levels of CLTs in the control group were set at the 70th, 80th, 90th, 95th, and 99th percentiles, and ORs for the development of DVT were calculated.
until now incompletely understood role in modulation of fibrinolysis. Thus, the outcome of our assay represents a tPA- and factor XIII-independent measure of the plasma fibrinolytic potential.

Our findings that hypofibrinolysis plays a role in the development of a first venous thrombosis is supported by a recent publication in which an asymptomatic venous thrombus was detected in 1% of a healthy population (n = 1213). The fact that small, nonsymptomatic thrombi occur this frequently may explain why a defective fibrinolytic system, that is, the incapacity to remove these asymptomatic thrombi, is associated with a higher risk for thrombosis.

In conclusion, hypofibrinolysis, as measured by a tissue factor– and tPA-induced plasma-based clot lysis assay is a risk factor for the development of a first DVT. Whether this hypofibrinolytic state is determined by genetic or acquired factors, or a combination of these, and which proteins are involved is at present unknown.

Acknowledgments

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