Introduction

Human plasma fibrinogen is an important protein in the haemostatic system, in which soluble fibrinogen is converted to insoluble fibrin, leading to clot formation. Fibrinogen is a heterogeneous protein, existing as several natural isoforms. The fibrinogen variant containing γ' is one such isoform, comprising 10 ± 3% of the circulating fibrinogen molecules (1). In the systemic circulation, γ' is mostly present as a heterodimeric protein with the common γA form (γA/γ'), while <1% is present in the γ'/γ form (2). Fibrinogen γ' is a result of alternative mRNA processing, in which 20 amino acids replace the four amino acids at the C-terminal of γA (3, 4). The extension of fibrinogen γ' contains additional binding sites for thrombin (hence the alternative name antithrombin I for γ'-containing fibrinogen molecules (3)) and for the factor XIII B-subunit (6), enhancing binding of factor XIII. The platelet binding site for integrin αIIbβ3 is disrupted, which deducts platelet binding capacity (7).

Recently, we and others reported that an elevated γ' level or γ'/total fibrinogen ratio is associated with cardiovascular disease (1, 8–12), especially in the acute phase of ischaemic stroke. Results of our previous studies suggest that fibrinogen γ' levels as well as the γ'/total fibrinogen ratio alter during an acute phase reaction (1, 8). This may lead to a different clot formation rate and to different characteristics of the fibrin network, and may therefore contribute to the pathology of thrombotic diseases and stroke.

The role of fibrinogen γ' and the γ'/total fibrinogen ratio in the precipitation and course of ischaemic stroke is as yet unclear. To our knowledge, no studies have been published on fibrinogen γ' in relation to outcome of cardiovascular disease, ischaemic stroke in particular.

We aimed to investigate the differences in fibrinogen γ' levels and γ'/total fibrinogen ratio, between patients with ischaemic stroke and control persons. Furthermore, we studied whether there is an association between these levels and short-term outcome in ischaemic stroke.

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Summary

Fibrinogen γ' (γ') is a natural isoform of fibrinogen, and alters the rate of formation and the properties of clots. It could therefore affect outcome after ischaemic stroke. The prognostic significance of γ' fibrinogen levels is, however, still unclear. It was the objective of this study to assess levels of γ' in ischaemic stroke, and its association with short-term outcome. We included 200 ischaemic stroke patients and 156 control persons. Total fibrinogen and γ' levels were measured; outcome at discharge was assessed by means of the modified Rankin Scale score (defined as unfavourable when >2). We compared levels between patients and controls using multiple linear regression analysis, and logistic regression analysis was used to assess the relationship between levels and outcome. All analyses were adjusted for age and sex. Mean γ' levels were significantly higher in patients with ischaemic stroke than in controls (0.37 vs. 0.32 g/l, p<0.001), and patients also had a higher γ'/total fibrinogen ratio (0.102 vs. 0.096, p<0.019). The γ'/total fibrinogen ratio is associated with unfavourable outcome in patients with ischaemic stroke (odds ratio per unit increase of γ'/total fibrinogen ratio 1.27, 95% confidence interval 1.09–1.47). Our study shows that patients with ischaemic stroke have increased levels of fibrinogen γ' and suggests a trend towards an increased γ'/total fibrinogen ratio in ischaemic stroke. Increased fibrinogen γ' relative to total fibrinogen levels are associated with unfavourable outcome in the early phase after stroke.

Keywords

Ischaemic stroke, fibrinogen γ', outcome

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Materials and methods

Study population

All patients and controls participated in the Erasmus Stroke Study (ESS), which is an ongoing prospective registry of all patients with transient ischaemic attack (TIA) or stroke treated at Erasmus MC University Medical Center Rotterdam since December 2005. From all patients, detailed clinical and radiological data, blood samples and DNA are collected. The ESS also includes population-based control persons, mostly friends and spouses of patients, but no family members. All participants in the ESS provided informed consent. The study was approved by the Medical Ethics Committee of the Erasmus MC University Medical Center.

For this case-control study we included 200 patients with ischaemic stroke, and 156 age- and sex matched stroke-free control persons. In all patients, citrated blood was collected and centrifuged at 4,000 rpm for 15 minutes (min). Citrated plasma was stored at −80°C within 2 hours (h) from collection. Blood was drawn eight days (interquartile [IQR] range 3–22 days) from the date of onset, either in the stroke unit or the outpatient clinic.

Total fibrinogen and fibrinogen γ' antigen measurements

We determined total fibrinogen levels according to von Clauss (13) on a fully-automated blood coagulation analyzer (Sysmex CA-1500 system, Siemens Healthcare Diagnostics, Breda, the Netherlands). Normal Reference Plasma was used as reference plasma in this assay (Precision BioLogic, Dartmouth, ON, Canada), the intra-assay coefficient of variation was 3.5%.

Fibrinogen γ’ antigen levels were measured with an enzyme-linked immunosorbent assay as described previously (9), with minor modifications. Briefly, plastic 96-well microtiter plates (Nunc maxisorp, Roskilde, Denmark) were coated with mouse anti-human fibrinogen γ’ (2.G2.H9; Millipore, Billerica, MA, USA) and then incubated overnight at 4°C. Wells were blocked with bovine serum albumin for 1 h at room temperature. Plasma samples were added to each well in duplicate. After 1 h incubation at room temperature, horseradish peroxidase-conjugated rabbit anti-human fibrinogen (DAKO A/S, Glostrup, Denmark) was added to tag the immobilised patient fibrinogen γ’, and incubated for 1 h at room temperature. Colour was developed with Tetra-methylbenzidine substrate solution (BioMérieux, Marcy l’Etiole, France) and was terminated with H2SO4 after 15 min. The plate was read at 450 nm spectrophotometrically. Wells were washed three times between all incubation steps. Pooled normal plasma calibrated against purified human fibrinogen γ’ was used as calibrator (1). The intra-assay variation was less than 10%.

Definitions

Ischaemic stroke was defined as focal neurological deficit of presumed vascular origin, lasting >24 h or leading to death within 24 h, with brain imaging showing no abnormalities or typical signs of brain infarction. Hypertension was defined as the use of antihypertensive drugs before the event, hypercholesterolemia as the use of cholesterol lowering drugs before the event. Diabetes mellitus was defined as the use of oral antidiabetic drugs and/or insulin before the event. All strokes were classified according to TOAST criteria (14) based on all available information.

Functional short-term outcome was assessed by means of the modified Rankin Scale (mRS) (15) score at time of discharge from the stroke unit for hospitalised patients, and from the outpatient clinic for ambulant patients. The mRS is a score for handicap and level of dependency. The scale ranges from 0 (no symptoms) to 6 (dead), with each increase denoting more severe disability. At a score of 2, patients have neurological symptoms affecting their daily life but are still able to live completely independent; at a score of 3 patients cannot live independently due to symptoms. The mRS was dichotomised between scores of 2 and 3, as favourable (≤2, independent living) or unfavourable (>2, dependent or institutionalised living).

Statistical analysis

Differences between baseline characteristics of patients and controls were assessed with Student’s t-test. Differences in fibrinogen levels between patient groups and controls were assessed using Student’s t-test; adjustment for confounders was performed using analysis of variance (ANOVA). Associations between fibrinogen levels and outcome were assessed using multiple logistic regression analysis with odds ratio (OR) with 95% confidence intervals (CI). Analyses were also performed in two subgroups of equal size, based on time from event to blood drawing. This allowed us to study levels of γ’ in the acute and subacute stage after ischaemic stroke. All analyses were adjusted for age and sex. Statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). A probability value <0.05 was considered significant.

Results

Baseline characteristics of patients and controls are shown in Table 1. As expected, prevalence of cardiovascular risk factors (smoking, hypertension and hypercholesterolaemia) was different between patients and controls.

Levels of total fibrinogen and fibrinogen γ’ were significantly higher in patients compared with controls, after adjustment for age and sex (Table 2A). Levels of the γ’/total fibrinogen ratio were similar in patients and controls.
The median time from onset of symptoms to blood drawing was 7.5 days. Two subgroups of patients were formed, by dividing patients in two equal groups based on time from onset of symptoms to blood drawing, the acute phase and the subacute phase. Differences in γ/total fibrinogen ratio between patients and controls were most pronounced in the group with blood drawing in the acute phase of stroke (Table 2B). In this group γ/total fibrinogen ratio was significantly increased, in line with our previous results (8). In the group of patients in the subacute phase of ischaemic stroke, the γ ratio was not different from controls.

Outcome was unfavourable (modified Rankin Scale score >2) in 13.5% of patients with ischaemic stroke. These patients could no longer live independently due to neurological symptoms, had severe disabilities, or died.

Levels of fibrinogen, fibrinogen γ and γ/total fibrinogen ratio with respect to favourable or unfavourable outcome are shown in Table 3. After adjustment for confounders, γ and total fibrinogen were associated with unfavourable outcome. Patients with unfavourable outcome had significantly lower levels of total fibrinogen and γ/total fibrinogen ratio than patients with favourable outcome, after adjustment for confounders. The Table 3 shows the results of Table 2B.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>200</td>
<td>156</td>
</tr>
<tr>
<td>Age (years), mean (SD)</td>
<td>62 (13)</td>
<td>59 (12)</td>
</tr>
<tr>
<td>Female, %</td>
<td>45</td>
<td>51</td>
</tr>
<tr>
<td>Current smoking, %</td>
<td>36</td>
<td>24</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>55</td>
<td>29</td>
</tr>
<tr>
<td>Hypercholesterolaemia, %</td>
<td>34</td>
<td>18</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>Body mass index, mean (SD)</td>
<td>26.0 (4.2)</td>
<td>26.7 (4.5)</td>
</tr>
<tr>
<td>TOAST classification N(%)</td>
<td>Large artery atherosclerosis</td>
<td>39 (20)</td>
</tr>
<tr>
<td>Cardioembolism</td>
<td>21 (11)</td>
<td></td>
</tr>
<tr>
<td>Small vessel occlusion</td>
<td>57 (29)</td>
<td></td>
</tr>
<tr>
<td>Other etiology</td>
<td>12 (6)</td>
<td></td>
</tr>
<tr>
<td>Undetermined etiology</td>
<td>71 (36)</td>
<td></td>
</tr>
<tr>
<td>Time from onset of symptoms to blood drawing (days), median (IQR)</td>
<td>7.5 (3–22)</td>
<td></td>
</tr>
<tr>
<td>Modified Rankin Scale at discharge, median (IQR)</td>
<td>1 (1–2)</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Characteristics of the study population.

Table 2: Levels of total fibrinogen, fibrinogen γ and γ/total fibrinogen ratio in patients and controls (A) and in subgroups of patients and controls (B). Subgroups: patients were divided in two equal groups of 100 patients based on time from event to blood drawing.

A

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Patients</th>
<th>Model 1, p-value*</th>
<th>Model 2, p-value†</th>
<th>Model 3, p-value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fibrinogen (g/l)</td>
<td>3.37 (0.72)</td>
<td>3.69 (0.96)</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fibrinogen γ (g/l)</td>
<td>0.32 (0.13)</td>
<td>0.37 (0.15)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>γ/total fibrinogen ratio</td>
<td>0.096 (0.028)</td>
<td>0.102 (0.036)</td>
<td>0.19</td>
<td>0.30</td>
<td>0.495</td>
</tr>
</tbody>
</table>

Presented are means (SD). *Model 1: adjustment for age and sex. †Model 2: adjustment for age, sex and time to blood drawing. ‡Model 3: adjustment for age, sex, time to blood drawing, current smoking, hypertension and hypercholesterolaemia.

B

<table>
<thead>
<tr>
<th></th>
<th>0–7 days</th>
<th>≥8 days</th>
<th>0–7 days vs. controls, p-value*</th>
<th>≥8 days vs. controls, p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fibrinogen (g/l)</td>
<td>3.76 (1.11)</td>
<td>3.62 (0.78)</td>
<td>&lt;0.001</td>
<td>0.033</td>
</tr>
<tr>
<td>Fibrinogen γ (g/l)</td>
<td>0.40 (0.17)</td>
<td>0.34 (0.12)</td>
<td>&lt;0.001</td>
<td>0.194</td>
</tr>
<tr>
<td>γ/total fibrinogen ratio</td>
<td>0.110 (0.040)</td>
<td>0.095 (0.029)</td>
<td>0.015</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Presented are means (SD). *p value after adjustment for age and sex.
Table 3: Levels of total fibrinogen, fibrinogen $\gamma'$ and $\gamma'/\text{total fibrinogen ratio}$ in patients with favourable or unfavourable outcome.

<table>
<thead>
<tr>
<th></th>
<th>Favourable outcome (modified Rankin Scale ≤2)</th>
<th>Unfavourable outcome (modified Rankin Scale &gt;2)</th>
<th>Model 1*, p-value</th>
<th>Model 2†, p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>173</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total fibrinogen, g/l (SD)</td>
<td>3.64 (0.89)</td>
<td>3.97 (1.34)</td>
<td>0.112</td>
<td>0.013</td>
</tr>
<tr>
<td>Fibrinogen $\gamma'$, g/l (SD)</td>
<td>0.36 (0.14)</td>
<td>0.47 (0.18)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$\gamma'/\text{total fibrinogen ratio}$, SD</td>
<td>0.098 (0.029)</td>
<td>0.129 (0.059)</td>
<td>&lt;0.001</td>
<td>0.006</td>
</tr>
</tbody>
</table>

*Model 1: adjusted for age and sex. †Model 2: adjusted for age, sex, time to blood drawing.

Table 4: Associations between total fibrinogen levels, fibrinogen $\gamma'$ levels and $\gamma'/\text{total fibrinogen ratio}$ in patients with unfavourable outcome.

<table>
<thead>
<tr>
<th></th>
<th>Unfavourable outcome, N (%)</th>
<th>Model 1*</th>
<th>Model 2†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fibrinogen (g/l)</td>
<td>1.35 (0.90–2.01)</td>
<td>1.31 (0.87–1.96)</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen $\gamma'$ (dg/l)</td>
<td>1.52 (1.18–1.96)</td>
<td>1.48 (1.15–1.91)</td>
<td></td>
</tr>
<tr>
<td>$\gamma'/\text{total fibrinogen ratio}$, %</td>
<td>1.28 (1.10–1.48)</td>
<td>1.27 (1.09–1.47)</td>
<td></td>
</tr>
</tbody>
</table>

* Model 1: adjusted for age and sex. † Model 2: adjusted for age, sex, and time to blood drawing.

nogen levels and the $\gamma'/\text{total fibrinogen ratio}$ were significantly higher in patients with unfavourable outcome, compared with patients with a favourable outcome.

Increased fibrinogen $\gamma'$ levels and $\gamma'/\text{total fibrinogen ratio}$ were significantly associated with unfavourable outcome (OR per unit increase $\gamma'$ 1.48, 95%CI 1.15–1.91 and OR per unit increase $\gamma'/\text{total fibrinogen ratio}$ 1.27, 95%CI 1.09–1.47; both adjusted for age, sex and time to blood drawing; Table 4). The $\gamma'/\text{total fibrinogen ratio}$ adjusts fibrinogen $\gamma'$ levels for total fibrinogen levels, the prognostic effect of fibrinogen $\gamma'$ on outcome was thus independent of total fibrinogen level. Effects were similar in subgroups based on time to blood drawing, showing that the $\gamma'/\text{total fibrinogen ratio}$ was associated with unfavourable outcome in the first week as well as later in the course of ischaemic stroke (OR 1.25, 95%CI 1.05–1.49 and OR 1.20, 95%CI 0.84–1.70, respectively).

Discussion

Our study shows that levels of total fibrinogen, fibrinogen $\gamma'$ and the $\gamma'/\text{total fibrinogen ratio}$ are increased in the acute phase of ischaemic stroke. Furthermore, this is the first study to show that increased relative levels of fibrinogen $\gamma'$ are significantly associated with unfavourable outcome. The $\gamma'/\text{total fibrinogen ratio}$ was associated with unfavourable outcome both in the acute and subacute phase after ischaemic stroke.

Some methodological issues of our study have to be discussed. Strengths of our study are the extent and quality of clinical data and availability of short-term follow-up, which enabled us to study fibrinogen $\gamma'$ in relation to outcome of stroke. Limitations of our study are the relatively small number of patients, and the low rate of unfavourable outcome among our patients. However, this study provided sufficient precision to allow meaningful conclusions about the relationship between fibrinogen $\gamma'$ and outcome.

We observed that the $\gamma'$ levels were increased most in patients who were presented at the hospital soon after the onset of stroke. In general, patients with severe stroke were admitted to the hospital sooner after the onset of complaints than patients with milder stroke. Therefore, we cannot distinguish between the effects of the acute phase reaction that was initiated by the stroke, and the severity of stroke. Still, adjustment in the analysis for the time since onset of complaints has only a minor effect on the associations of $\gamma'$ levels with outcome of stroke. After adjustment for cardiovascular risk factors, levels of fibrinogen $\gamma'$ were still significantly increased in patients, but the $\gamma'/\text{total fibrinogen ratio}$ was similar in patients and controls. Also, when looking at associations with outcome, the absolute $\gamma'$ level had a stronger association with outcome than the $\gamma'/\text{total fibrinogen ratio}$. This may indicate that the absolute level of fibrinogen $\gamma'$ is perhaps more important than the $\gamma'/\text{total fibrinogen ratio}$.

Our results confirm those of our previous studies that showed increased total fibrinogen and fibrinogen $\gamma'$ levels in the acute phase of ischaemic stroke (8). Also, earlier studies have found a relation between higher post-stroke total fibrinogen levels and poor outcome measured on the modified Rankin Scale (16). We found that higher total fibrinogen levels are associated with unfavourable outcome as well, but in addition we found that the association between increased fibrinogen $\gamma'$ levels and unfavourable outcome after ischaemic stroke is independent of total fibrinogen levels.

Fibrinogen $\gamma'$ has antithrombotic properties; one of these is a high affinity binding site for thrombin, which may result in lower lysis rate than clots formed from $\gamma'$-rich fibrin (18, 19). In vitro studies have reported that fibrinogen $\gamma'$-rich clots show a lower clot lysis rate than clots formed from $\gamma A$ fibrinogen (20, 21).

Increased total fibrinogen levels have been shown to increase risk of cardiovascular disease (22–24). Several studies in the convalescent phase of thrombotic diseases show a decreased fibrinogen

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What is known about this topic?
- Fibrinogen γ' has antithrombotic properties and may contribute to the pathology of cardiovascular diseases such as ischaemic stroke.
- Fibrinogen γ' levels increase in the acute phase of ischaemic stroke.

What does this paper add?
- Levels of fibrinogen γ' and the γ'/total fibrinogen ratio are significantly increased in patients with unfavourable outcome.
- Levels of fibrinogen γ' are associated with unfavorable outcome after ischaemic stroke.
- The fibrinogen γ'/total fibrinogen ratio is increased in patients early after stroke, but is normal in a later phase.

γ' level, which is thought to resemble the level before occurrence of an event. When considering fibrinogen γ' as a protein with mostly antithrombotic properties, it is possible that increased baseline fibrinogen levels, combined with a low fibrinogen γ'/total fibrinogen ratio, could predispose to atherothrombotic disease. In the acute phase of thrombotic diseases, the fibrinogen γ' level then increases, as was seen in previous studies (1) as well as our study. Based on findings from previous studies and our study, it is still too early to use fibrinogen γ' assays for diagnostic or prognostic evaluation in acute stroke, for which further studies are needed.

In conclusion, we have found that levels of fibrinogen γ' are increased in the acute phase of ischaemic stroke. Furthermore, increased levels of fibrinogen γ' are associated with unfavourable outcome. Future research should be aimed at unraveling the role of fibrinogen γ' in the pathogenesis and prognosis of cardiovascular diseases such as ischaemic stroke, in which the genetic background of fibrinogen γ' levels may be of particular interest.

Acknowledgement
We would like to thank Reinilde J. Scheffer for her technical support.

References