Lower concentrations of thrombin-antithrombin complex (TAT) correlate to higher recanalisation rates among ischaemic stroke patients treated with t-PA

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Summary
An elevated concentration of the thrombin-antithrombin complex (TAT) has been associated with high mortality rates and poor outcome in ischaemic stroke patients treated with tissue plasminogen activator (t-PA). Moreover, antithrombin drugs have been tested in combination with t-PA in the acute phase of ischaemic stroke to increase treatment efficacy. We aimed to study whether poor outcome associated with TAT among ischaemic stroke patients treated with t-PA could be due to the effects of this complex on recanalisation rates of the middle cerebral artery (MCA) and on haemorrhagic transformation. The TAT levels of 89 patients having a proximal MCA occlusion were measured by ELISA, and the patients were then treated with t-PA. Complete recanalisation was diagnosed by transcranial Doppler (TCD) at 1, 2 and 6 hours post-t-PA infusion and haemorrhagic transformation was identified by computed tomography (CT). Lower levels of TAT were associated with better recanalisation rates at all time-points (1 hour: OR = 24.8 95% CI 1.4-434.8, p = 0.028; 2 hours: OR = 6.3 95% CI 1.5-27, p = 0.014; 6 hours: OR = 6.4 95% CI 1.5-26.5, p = 0.011) after adjustment for stroke risk factors. However, no correlation was found between TAT concentration and haemorrhagic transformation. The elevated mortality rates previously observed in patients with high levels of TAT might have been due to revascularisation resistance. Low levels of TAT are not associated with an increase in haemorrhagic complications after t-PA, indicating that the combination of thrombin blockers and t-PA could be a safe and effective treatment for ischaemic stroke in the future.

Keywords
t-PA, thrombolysis, thrombin, Argatroban, plasmin

Introduction
The benefits of fibrinolytic (thrombolytic) therapy with tissue plasminogen activator (t-PA) for ischaemic stroke patients (1) have been partially limited due to a lack of blood flow restoration, in many occluded patients, and to the risk of symptomatic haemorrhagic transformation, in approximately 1.7 to 7% of cases (2). Low rates of recanalisation are associated with greater cerebral ischaemic lesions, and thus, with poor neurological outcome (3, 4). Therefore, major efforts are being made to identify biological or genetic biomarkers that could predict the response of an individual patient to fibrinolytic treatment (5–11).

The serine protease thrombin is an essential component of the coagulation cascade, executing multiple functions such as cleavage of fibrinogen to fibrin and activation of platelets, which are implicated in physiological and pathological clotting (12). Furthermore, thrombin activates Factor XIII, which stabilises fibrin and clots, and it activates Protein C, which slows down clot formation, thereby regulating coagulation by a different pathway. Several coagulation factors that affect thrombin activity (e.g. FXI and FXII) have been associated with ischaemic stroke (13–15).

Nevertheless, the relationship between thrombin levels and ischaemic strokes is not clear: an elevated level of thrombin
could be either a risk factor for or a consequence of these events. Endogenous fibrinolysis and coagulation might modulate physiological responses to t-PA. Indeed, the proteins plasminogen activator inhibitor-1 (PAI-1) and thrombin activatable fibrinolysis inhibitor (TAFI), both inhibitors of fibrinolysis, influence recanalisation rates (6, 16). Thus, we hypothesised that other regulation factors of the coagulation-fibrinolytic pathways, such as thrombin, might also be associated with recanalisation rates.

Tanne et al. measured levels of thrombin-antithrombin (TAT) complex in ischaemic stroke patients at 24 hours post-t-PA treatment and, based on the results, were able to predict mortality at 3 months (17). However, the biological mechanism behind this correlation remains unknown. In these patients low recanalisation rates have also been associated with mortality at 3 months post-treatment (18).

In this study we aimed to explore firstly whether pre-treatment plasma levels of TAT in stroke patients treated with t-PA correlated to low recanalisation rates, and secondly, if such a correlation were to be found, whether it could explain the relationship between TAT levels and mortality reported for this patient group.

Methods

Study population
Eighty-nine patients with an acute ischaemic stroke admitted at the emergency department of a University Hospital were prospectively studied. Our target group consisted of patients who had had an acute ischaemic stroke admitted within the first 3 hours after symptoms onset. Consecutive patients with a non-lacunar stroke involving the vascular territory of the middle cerebral artery (MCA) were evaluated. Patients who had a documented proximal MCA occlusion on transcranial Doppler (TCD) and received t-PA in a standard 0.9mg/kg dose (10% bolus, 90% continuous infusion during 1 hour) were included in the study.

Clinical and transcranial Doppler protocol
A detailed history of vascular risk factors was obtained from each patient. To identify potential mechanisms of cerebral infarction, a set of diagnostic tests was performed including electrocardiogram, chest radiography, carotid ultrasonography, complete blood count and leukocyte differential and blood biochemistry in all patients; when indicated some patients also underwent special coagulation tests, transthoracic ecocardiography and Holter monitoring. With this information, and the neuroimaging data, previously defined etiologic subgroups were determined (19).

Clinical examination was performed on admission and at 1 and 2 hours post-t-PA administration and again at 12, 24 and 48 hours after symptoms onset. Stroke severity as well as improvement, stability or neurological worsening were assessed by using the National Institutes of Health Stroke Scale (NIHSS) (20). Improvement, stability or neurological worsening were measured at 48 hours post symptoms onset and mortality was measured at three months.

A standard TCD examination was performed by experienced neurologists in the emergency room on admission, before t-PA administration, using 1-channel 2MHz equipment (TCD 100M, Spencer Technologies, Seattle, Washington, USA). A standard set of diagnostic criteria was applied to diagnose arterial occlusion. Proximal MCA occlusions were defined as the absence of flow or the presence of minimal flow signal throughout the MCA at an insonation depth between 45 to 65mm, accompanied by flow diversion in the ipsilateral anterior cerebral artery and posterior cerebral artery, according to the Thrombolysis in Brain Ischemia (TIBI) gradient system (21). To assess recanalisation, follow up recordings were performed at 1 hour after t-PA administration and again at 2 hours and 6 hours. After the site of MCA occlusion was identified, continuous monitoring of the residual flow signals was performed with a Marc 600 head frame (Spencer Technologies) or DWL metal head frame to maintain tight transducer fixation and a constant angle of insonation. Changes on TCD for each patient were determined by a rater using direct visual control of monitoring display.

Complete recanalisation on TCD was diagnosed if the end-diastolic flow velocity improved to normal or elevated values (normal or stenotic signals) in previously demonstrated absent or minimal flow (22).

Intravenous heparin was not administered during the study period. This study was approved by the Ethics Committee of the hospital and all patients or their relatives gave informed consent.

Neuroimaging protocol: CT and MRI
On admission, all patients underwent a Computed Tomography (CT) or Magnetic Resonance (MRI) within the first 3 hours of stroke onset as previously described (23). A CT scan was repeated after 24-48 hours or earlier when rapid neurological deterioration occurred. The presence and type of haemorrhagic transformation were defined according to previously published criteria (24).

To determine thrombus area, MRI examinations were performed at 48 hours post symptoms onset with 1.5T whole-body imagers (Magnetom Vision Plus or Symphony Siemens Medical Systems, Erla) with 24mT/m gradient strength, 300µsec rise time, and an echo-planar–capable receiver equipped with a gradient override. The following images were obtained: a) transverse T2-weighted susceptibility-based echo-planar gradient-echo images (0.8/29 [repetition time msec/echo time msec], one signal acquired, total acquisition time of 2.8 seconds) or b) transverse DW echo-planar spin-echo images (4,000/100, two signals acquired, total acquisition time of 56 seconds). The susceptibility sign on MRI images was defined as presence of hypointensity within the ICA or MCA, in which the diameter of the hypointense signal within the vessel exceeded the contralateral vessel diameter. The magnetic susceptibility produces a non-uniform magnetic field and fast dephasing of spins. This phenomenon is more important in T2 weighted images. The greater (length) and smaller (thickness) diameters of the hyposignal in axial plane were measured (millimeters) (25–28). Thrombus area was calculated as an ellipse: length X thickness X π.

Determination of TAT complex levels
Blood samples were obtained from patients before t-PA infusion (baseline) and stored at –80°C. Plasma samples were obtained using citrate tubes, and TAT levels, which are commonly ac-
Table 1: Demographic data and risk factors for complete recanalisation of ischaemic stroke patients at three different time-points after t-PA infusion. Rec.: complete recanalisation; HT: hypertension; AF: atrial fibrillation; TAT: thrombin-antithrombin complex.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>YES (9)</th>
<th>NON (73)</th>
<th>p-Value</th>
<th>YES (18)</th>
<th>NON (56)</th>
<th>p-Value</th>
<th>YES (22)</th>
<th>NON (43)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men n (%)</td>
<td>8 (88.9)</td>
<td>36 (49.3)</td>
<td>&lt;0.05</td>
<td>13 (72.2)</td>
<td>26 (46.4)</td>
<td>0.065</td>
<td>17 (77.3)</td>
<td>15 (34.9)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Women n (%)</td>
<td>1 (11.1)</td>
<td>37 (50.7)</td>
<td></td>
<td>5 (27.8)</td>
<td>30 (53.6)</td>
<td></td>
<td>5 (22.7)</td>
<td>28 (65.1)</td>
<td></td>
</tr>
<tr>
<td>Current smokers n (%)</td>
<td>2 (25)</td>
<td>16 (23.9)</td>
<td>0.94</td>
<td>2 (11.8)</td>
<td>15 (30)</td>
<td>0.14</td>
<td>4 (20)</td>
<td>9 (23.1)</td>
<td>0.79</td>
</tr>
<tr>
<td>Non-smokers n (%)</td>
<td>6 (75)</td>
<td>51 (76.1)</td>
<td></td>
<td>15 (88.2)</td>
<td>35 (70)</td>
<td></td>
<td>16 (80)</td>
<td>30 (76.9)</td>
<td></td>
</tr>
<tr>
<td>Hypertension n (%)</td>
<td>2 (22.2)</td>
<td>41 (56.2)</td>
<td>0.054</td>
<td>6 (33.2)</td>
<td>33 (58.9)</td>
<td>0.1</td>
<td>8 (36.4)</td>
<td>25 (58.1)</td>
<td>0.12</td>
</tr>
<tr>
<td>Non-Hypertension n (%)</td>
<td>7 (77.8)</td>
<td>32 (43.8)</td>
<td></td>
<td>12 (66.7)</td>
<td>23 (41.1)</td>
<td></td>
<td>14 (63.6)</td>
<td>18 (41.9)</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus n (%)</td>
<td>2 (22.2)</td>
<td>13 (17.8)</td>
<td>0.74</td>
<td>3 (16.7)</td>
<td>11 (19.6)</td>
<td>0.78</td>
<td>3 (13.6)</td>
<td>8 (18.6)</td>
<td>0.74</td>
</tr>
<tr>
<td>Non-Diabetes mellitus n (%)</td>
<td>7 (77.8)</td>
<td>60 (82.2)</td>
<td></td>
<td>15 (83.3)</td>
<td>45 (80.4)</td>
<td></td>
<td>19 (86.4)</td>
<td>35 (81.4)</td>
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</tr>
<tr>
<td>AF n (%)</td>
<td>4 (44.0)</td>
<td>30 (41.1)</td>
<td>0.84</td>
<td>9 (50)</td>
<td>23 (39.3)</td>
<td>0.58</td>
<td>10 (45.5)</td>
<td>18 (41.9)</td>
<td>0.8</td>
</tr>
<tr>
<td>Non-AF n (%)</td>
<td>5 (55.6)</td>
<td>43 (58.9)</td>
<td></td>
<td>9 (50)</td>
<td>34 (60.7)</td>
<td></td>
<td>12 (54.5)</td>
<td>25 (58.1)</td>
<td></td>
</tr>
<tr>
<td>Dyslipemia n (%)</td>
<td>3 (33.3)</td>
<td>28 (38.9)</td>
<td>0.75</td>
<td>8 (44.4)</td>
<td>19 (34.5)</td>
<td>0.58</td>
<td>10 (45.5)</td>
<td>15 (35.7)</td>
<td>0.59</td>
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<tr>
<td>Non-Dyslipemia n (%)</td>
<td>6 (66.7)</td>
<td>44 (61.1)</td>
<td></td>
<td>10 (55.6)</td>
<td>36 (65.5)</td>
<td></td>
<td>12 (54.5)</td>
<td>27 (64.3)</td>
<td></td>
</tr>
<tr>
<td>Previous stroke n (%)</td>
<td>2 (22.2)</td>
<td>17 (23.9)</td>
<td>0.99</td>
<td>4 (22.2)</td>
<td>12 (22.2)</td>
<td>0.82</td>
<td>4 (18.2)</td>
<td>8 (19)</td>
<td>0.98</td>
</tr>
<tr>
<td>Non-Previous stroke n (%)</td>
<td>1 (11.1)</td>
<td>11 (15.1)</td>
<td></td>
<td>1 (5.6)</td>
<td>9 (16.1)</td>
<td>0.43</td>
<td>2 (9)</td>
<td>6 (14)</td>
<td>0.71</td>
</tr>
<tr>
<td>Etiology: n (%)</td>
<td>8 (88.9)</td>
<td>62 (84.9)</td>
<td>0.75</td>
<td>1 (5.6)</td>
<td>47 (83.9)</td>
<td>0.43</td>
<td>2 (9)</td>
<td>6 (14)</td>
<td>0.71</td>
</tr>
</tbody>
</table>

Descriptive and frequency statistical analyses were performed and comparisons were made using the SPSS statistics package, version 12.0. Statistical significance for intergroup differences was assessed by the χ² test or Fisher’s exact test for categorical variables. When indicated, Mann-Whitney U and Kruskal-Wallis tests were used. Spearman correlation was used to determine if TAT levels were related to thrombus area. To calculate the sensitivity and specificity for TAT values and predict complete recanalisation, a receiver operating characteristic (ROC) curve was configured. A logistic regression analysis was performed to determine factors that could be considered independent predictors of MCA recanalisation. We took into account recanalisation rates at different time points after t-PA infusion. Univariate analysis was performed using the variables previously associated with recanalisation in other studies. The statistically significant variables were included in the multivariable analysis. Other risk factors for stroke were also included in the logistic regression.

Sample size estimations were determined using Ene software. A p-value < 0.05 was considered statistically significant.

Results

We included in the study 85 patients (45.9% women) with an acute ischaemic stroke involving the MCA territory. Mean age was 72.4 years (ranging from 45 to 91 years). A total of 52.9% of patients were hypertensive, 38.6% were dyslipaemic and 17.6% had a history of diabetes mellitus. Median NIHSS score of the series on admission was 18 (range 4 to 28). Successful complete recanalisation occurred in 11% (n=9) of patients at 1 hour, 24.3% (n=18) at 2 hours and 33.8% (n=22) at 6 hours post-tPA administration. Main baseline characteristics of patients regarding the presence of an effective complete recanalisation following t-PA infusion are shown in Table 1. Among them only sex, presence of hypertension at baseline or TAT levels were associated with revascularisation rates.

TAT intra-assay variability was 4.24% and the inter-assay difference was 1.9%. No association was found between TAT and presence of hypertension at baseline or TAT levels were associated with recanalisation.
Figure 1: Correlation between pre-treatment plasma thrombin-antithrombin complex (TAT) levels (µg/L) and complete recanalisation rates at different time-points after t-PA infusion. Rec.: complete recanalisation. Patients with < 24µg/L baseline TAT presented better revascularisation rates by the end of t-PA infusion: 19.5% (n=8), as compared with those with > 24µg/L TAT: 2.4% (n=1), OR=8 95% CI 1–61.1, p=0.013. Recanalisation at 2h (< 24µg/L TAT: 36.1% [n=13], > 24µg/L TAT: 13.2% [n=5], OR=2.7 95% CI 1.1–6.9, p=0.03). Recanalisation 6 hours after thrombolysis (< 24µg/L TAT: 50% [n=16], > 24µg/L TAT: 18.2% [n=6], OR=2.7 95% CI 1.2–6.1, p=0.009).

Table 2: Odds ratio for pre-treatment plasma thrombin-antithrombin complex (TAT) levels after logistic regression and univariate analysis before logistic regression. 24µg/L was the cut-off of TAT. The covariates of the multivariable model were: male sex, stroke risk factors (age, hypertension, smoking, diabetes mellitus), baseline NIHSS and stroke etiology. TAT level was independently associated with complete MCA recanalisation at different time points (Complete recanalisation at 1 hour: OR=24.8 95% CI 1.4–434.8, p=0.028, for TAT < 24µg/L). Similar results were found for complete recanalisation at 2h and 6h (Table 2). In contrast logistic regression with TAT as a continuous variable did not reveal a significant association after logistic regression, although there was a trend detected (OR=0.97 95% CI 0.93–1, p=0.095).

Discussion

In this study, we report that levels of pre-treatment plasma levels of TAT complex in ischaemic stroke patients correlate with the efficacy of t-PA treatment (gauged as complete recanalisation of the occluded artery). Moreover, we found that low TAT complex levels are not associated with the type of haemorrhagic transformation. A previous study of ischaemic stroke patients from the NINDS group found an association between TAT levels (measured 24 hours after t-PA infusion) and mortality at 3 months (18). The authors reported that a high level of TAT is a predictive factor for mortality after fibrinolytic therapy but did not explain the clinical basis of this association. In another study tained from the ROC curves (53% sensitivity, 89% specificity) showed the best association with complete recanalisation (see Supplementary Figure 2 online at www.thrombosis-online.de). Patients with <24µg/L baseline TAT presented better revascularisation rates by the end of t-PA infusion: 19.5% (n=8), as compared with those with > 24µg/L TAT: 2.4% (n=1), OR=8 95% CI 1–61.1, p=0.013. Similar results were found for complete recanalisation at 2h (< 24µg/L TAT: 36.1% [n=13], > 24µg/L TAT: 13.2% [n=5], OR=2.7 95% CI 1.1–6.9, p=0.03) and at 6 hours after thrombolysis (< 24µg/L TAT: 50% [n=16] of these patients present complete recanalisation, >24µg/L TAT: 18.2% (n=6) of these patients present complete recanalisation, OR=2.7 95% CI 1.2–6.1, p=0.009. No association was found between TAT concentration and haemorrhagic transformation (Parenchymal Haematoma type-2 [n=2]: 30.7µg/L, 95% CI 33.9–95.4µg/L, Parenchymal Haematoma type-1 [n=6]: 15.9µg/L, 95% CI 2.81–28.96µg/L, Haemorrhagic Infarct type-2 [n=8]: 44.2µg/L, 95% CI 11.31–77.06µg/L, Haemorrhagic Infarct type-1 [n=9]: 48.4µg/L, 95% CI 23.28–73.56µg/L, Non-haemorrhagic transformation [n=54]: 32.5µg/L, 95% CI 24.77–40.3µg/L, p=0.23).

TAT concentration was not associated with mortality (mortality after t-PA [n=16]: 39.2µg/L, versus no mortality after t-PA [n=69]: 32.8µg/L, p=0.61) nor neurological improvement (improvement [n=47]: 34.6µg/L, stability [n=28]: 31µg/L, worsening [n=9]: 43.5µg/L, p=0.68).

No correlation was found between TAT and area, length or thickness of the thrombus (p=0.76, p=0.65, and p=0.74 respectively) in a subgroup of 19 patients in whom measurement of the thrombus could be obtained.

After logistic regression adjusted for male sex, stroke risk factors (age, hypertension, smoking, diabetes mellitus), baseline NIHSS and stroke etiology, TAT level was independently associated with complete MCA recanalisation at different time points (Complete recanalisation at 1 hour: OR=24.8 95% CI 1.4–434.8, p=0.028, for TAT < 24µg/L). Similar results were found for complete recanalisation at 2h and 6h (Table 2). In contrast logistic regression with TAT as a continuous variable did not reveal a significant association after logistic regression, although there was a trend detected (OR=0.97 95% CI 0.93–1, p=0.095).
Thrombin is the main enhancer of the coagulation cascade; accordingly it seems reasonable that patients with low levels of TAT present a higher chance to recanalise than patients with high level of TAT. As such, the high mortality rates of patients with elevated levels of TAT described by Tanne et al. could have been due to resistance to recanalisation. Nevertheless, in our group of patients, there was no statistically significant correlation between pre-fibrinolytic therapy levels of TAT and mortality rates. This result could be explained by two factors. Firstly, we measured TAT before t-PA infusion, whereas Tanne et al. measured TAT at 24 hours post-t-PA. Indeed, thrombin activation and regulation might change after t-PA administration. Moreover, TAT levels could also be associated with mortality, if they were measured after 24 hours of t-PA infusion. Secondly, we studied only 89 patients, whereas Tanne et al. studied 465 patients. Therefore, our sample size is not powerful enough for other endpoints (e.g. mortality). Indeed, considering the levels of TAT that we obtained, and assuming a p-value of 0.05 and a power of 80, the total number of patients needed to find an association between TAT levels and mortality would be 660 (330 patients who died after t-PA + 330 patients who survived after t-PA), as calculated with the Ene software. Given our sample size, it would have been very difficult to observe a statistically significant correlation. However, we did find that patients with levels of TAT > 24µg/L had nearly twice the mortality rate as did those with levels < 24µg/L (23.3% vs. 14.3%, respectively). This suggests that with a larger patient population, we might have been able to observe such an association between baseline TAT levels and mortality rates.

However, despite the small sample size, we did observe significant differences when using recanalisation as a surrogate marker for treatment outcome.

We also did not observe any correlation between TAT levels and the area, length or thickness of the thrombus (as measured on MRI). This could be due to the small number of patients in whom thrombus could be measured (n=19). Alternatively, since the measurements made based on the MRI images are only an approximation of the in vivo dimensions of the thrombi, they might not have been accurate enough to reflect statistical significance.

Our study is limited by the fact that we did not have angiographic data for the patients, which might have provided us with more information on the mechanisms of thrombin activation and its role in t-PA treatment of ischaemic stroke patients. Furthermore, we used an ROC curve to calculate a cutoff value of TAT concentration (24µg/L) for predicting recanalisation, instead of establishing a cut-off before having begun the study, which might have been better in terms of statistical methodology.

The association of TAT with complete recanalisation of the artery in ischaemic stroke patients treated with t-PA is further supported by the fact that thrombin blockers are potentially useful new drugs in the treatment of ischaemic stroke. Thrombin blockers bind selectively to the catalytic site of thrombin, acting as a competitive inhibitor. Argatroban is a thrombin inhibitor that has been shown to reduce thrombus formation and ischaemia in animal models of microvascular thrombosis and of embolic stroke (32, 33). It is currently approved for treatment of acute ischaemic stroke in Japan, but not in Europe or the USA. Administration of Argatroban within 48 hours of stroke has been shown to improve overall patient outcome without increasing intracranial haemorrhage (34, 35). Moreover, LaMonte et al. (36) evaluated the safety of Argatroban in 171 acute stroke patients in the first ever study of this drug in North America, showing that symptomatic intracranial haemorrhage and 90-day mortality did not differ significantly between treated and non-treated patients.

Our observation that lower endogenous levels of TAT correlate with better recanalisation rates is consistent with the findings of LaMonte et al. (36) and suggests that inhibition of thrombin in conjunction with t-PA administration could prove effective for treatment of patients with acute ischaemic stroke. Nevertheless, well-controlled clinical trials are required to prove this hypothesis.

**Study limitations**

In the present study, we found an association of TAT and recanalisation after logistic regression, when analysed as a categorical variable, while no association could be observed when analysed as a continuous variable. However, TAT was associated with recanalisation at a significant level when analysed as continuous before logistic regression (Table 1). Nevertheless the level of significance obtained for TAT was lower for the categorical analysis than for the continuous one. This is probably due to the sample size needed. Indeed, using the Ene software, we calculated that the analysis of TAT as a continuous variable required a sample size of 189 compared to 100 as categorical. We can then speculate that the power to observe an association after logistic regression was too low and thus the results did not reach significance. We believe that a bigger sample size could permit to observe an association of TAT with recanalisation also as a continuous variable; however this point would have to be confirmed by another independent study.

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