

# Hemostatic Activation and Outcome After Recombinant Tissue Plasminogen Activator Therapy for Acute Ischemic Stroke

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**Background and Purpose**—Early thrombolytic therapy with intravenous recombinant tissue plasminogen activator (rtPA) improves clinical outcome in acute ischemic stroke (AIS), but impaired endogenous fibrinolysis, thrombin generation, and vascular injury may hamper the efficacy of thrombolysis. We investigated in an exploratory, post hoc analysis the relationship between hemostatic markers and clinical outcomes among patients included in the National Institute of Neurological Disorders and Stroke (NINDS) rtPA Stroke Study.

**Methods**—Tissue plasminogen activator (tPA) antigen, thrombin-antithrombin complex (TAT), soluble thrombomodulin, and fibrinogen levels were measured in patients with AIS included in the NINDS rtPA Stroke Study from plasma samples collected at baseline, at 2 hours after treatment, and after 24 hours.

**Results**—TAT and tPA antigen levels peaked at 2 hours selectively in the rtPA treatment group, whereas fibrinogen levels dropped at 2 hours and remained low after 24 hours ( $P < 0.0001$  for interaction effects between time and treatment). At 24 hours, higher levels of tPA antigen were associated with a lower chance of favorable outcome (odds ratio [OR]=0.34; 95% CI, 0.14 to 0.82) selectively in the rtPA group, and higher levels of TAT (OR=1.72; 95% CI, 1.26 to 2.34) in the entire cohort and of thrombomodulin selectively in the rtPA group (OR=4.45; 95% CI, 1.26 to 15.67) were associated with higher 3-month mortality.

**Conclusions**—Hemostatic activation after AIS appears to be independently associated with clinical outcome in patients treated with rtPA. However, because we have tested for multiple associations, some may have been identified by chance alone and require further confirmatory studies. On the basis of this exploratory analysis, there is a rationale to investigate the safety and efficacy of protocols in which rtPA is complemented by agents that are antithrombotic and enhance fibrinolysis. (*Stroke*. 2006;37:1798-1804.)

**Key Words:** hemostasis ■ stroke, acute ■ thrombolysis

Thromboembolic arterial occlusion is the principal underlying cause of acute ischemic stroke (AIS). Early thrombolytic therapy with intravenous recombinant tissue plasminogen activator (rtPA) and successful reperfusion improve clinical outcome.<sup>1,2</sup> Impaired endogenous fibrinolysis, thrombin generation, and vascular injury may be involved in failure to achieve reperfusion, artery reocclusion, and risk of hemorrhage, which hamper the efficacy of thrombolysis.<sup>2-4</sup> Data on hemostatic markers in AIS and the effect of rtPA therapy are scarce, however, and based on small case series.<sup>5,6</sup>

The usefulness of hemostatic markers in affecting clinical outcomes after thrombolysis has been investigated more extensively in patients with acute myocardial infarction (AMI).<sup>4,7-10</sup> We undertook to investigate in the National Institute of Neurological Disorders and Stroke (NINDS) rtPA

Stroke Trial<sup>1</sup> (1) the extent of altered markers of endogenous fibrinolysis, thrombin generation, and endothelial injury in AIS; and (2) the relationship of these markers to outcomes, including response to intravenous rtPA. We hypothesized that markers of impaired endogenous fibrinolysis, increased thrombin generation, and microvascular injury may be linked to poorer stroke outcome and may blunt the clinical response to rtPA therapy.

## Subjects and Methods

The NINDS rtPA Stroke Trial consisted of 2 sequential placebo-controlled, randomized, double-blind, multicenter clinical trials.<sup>1</sup> Details of the trials' methodology have been published previously. Detailed clinical, historical, and laboratory data were collected on each patient at baseline, and outcome measures were systematically

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**TABLE 1. Associations of tPA Antigen at 3 Time Points With Outcomes of Interest Adjusted for Potential Confounders\***

	Baseline (n=395)		2 Hours After Treatment (n=403)		24 Hours After Stroke Onset (n=359)	
	IT <i>P</i>	Individual Effect <i>P</i> , OR (95% CI)	IT <i>P</i>	Individual Effect <i>P</i> , OR (95% CI)	IT <i>P</i>	Individual Effect <i>P</i> , OR (95% CI)
Favorable outcome at 24 h	0.35	0.40, 0.86 (0.61–1.22)	0.65	0.70, 0.94 (0.67–1.31)		
Favorable outcome at 3 mo	0.18	0.05, 0.71 (0.50–1.00)	0.60	0.80, 0.96 (0.67–1.36)	0.09	tPA: 0.02, 0.34 (0.14–0.82); placebo: 0.42, 1.32 (0.67–2.56)
ICH (tPA only) symptomatic all		0.56, 0.74 (0.28–2.01); 0.69, 0.86 (0.41–1.82)		0.12, 4.15 (0.70–24.7); 0.67, 1.19 (0.54–2.64)		
Died by 90 d	0.31	0.21, 1.34 (0.84–2.12)	0.17	0.10, 1.52 (0.92–2.51)	0.99	0.54, 1.25 (0.60–2.61)
Deterioration by 24 h	0.23	0.96, 1.01 (0.63–1.63)	0.74	0.10, 1.52 (0.92–2.51)		
Deterioration by 7–10 d	0.04	tPA: 0.43, 0.75 (0.37–1.52); placebo: 0.02, 2.11 (1.12–3.99)	0.22	0.62, 0.80 (0.34–1.90)	0.40	0.58, 1.37 (0.45–4.16)
All DFI	0.97	0.36, 1.25 (0.78–2.02)	0.94	0.88, 0.97 (0.59–1.57)	0.14	0.95, 1.02 (0.48–2.17)
DFI within 24 h	0.21	0.34, 1.30 (0.75–2.25)	0.75	0.96, 0.98 (0.49–1.96)		
CT volume at 3 mo	0.11	0.68	0.58	0.04	0.37	0.02

IT indicates interaction with treatment; DFI, deterioration following improvement.

\*Adjusted for all biomarkers for potential confounders listed in Appendix and for baseline marker level. The ORs correspond to per-unit change of the log-transformed data. Where an interaction was detected, the ORs are presented for rtPA group and placebo group separately.

collected at 2 hours, 24 hours, 7 to 10 days, and 3 months after stroke. Records of adverse events such as death and intracerebral hemorrhage were collected as the event occurred. Definitions of clinical outcomes of interest were similar to those used in prior analyses from this trial.<sup>1,11,12</sup>

Venous blood samples at baseline, 2 hours, and 24 hours were collected in citrated tubes. Plasma levels of fibrinogen and fibrinogen degradation products (FDP) were determined by a central laboratory (HFHSC, Detroit, Mich) as part of the NINDS rtPA Stroke Trial protocol. The remaining plasma was frozen and stored at  $-70^{\circ}\text{C}$ . The stored plasma samples from the 3 time points were used to determine by enzyme immunoassays the levels of the following hemostatic markers: TAT (Dade Behring), tissue plasminogen activator (tPA) antigen (American Bioproducts), and thrombomodulin (Diagnostic Stago). These laboratory tests were performed at the Veterans Affairs Medical Center, Baltimore, Md.

We combined, categorized, or ranked data before linking the patient data with the hemostatic marker concentrations. The coordinating center staff merged the laboratory data and clinical information from the original database files, and thereafter the identifying information was removed from the merged file.

### Statistical Methods

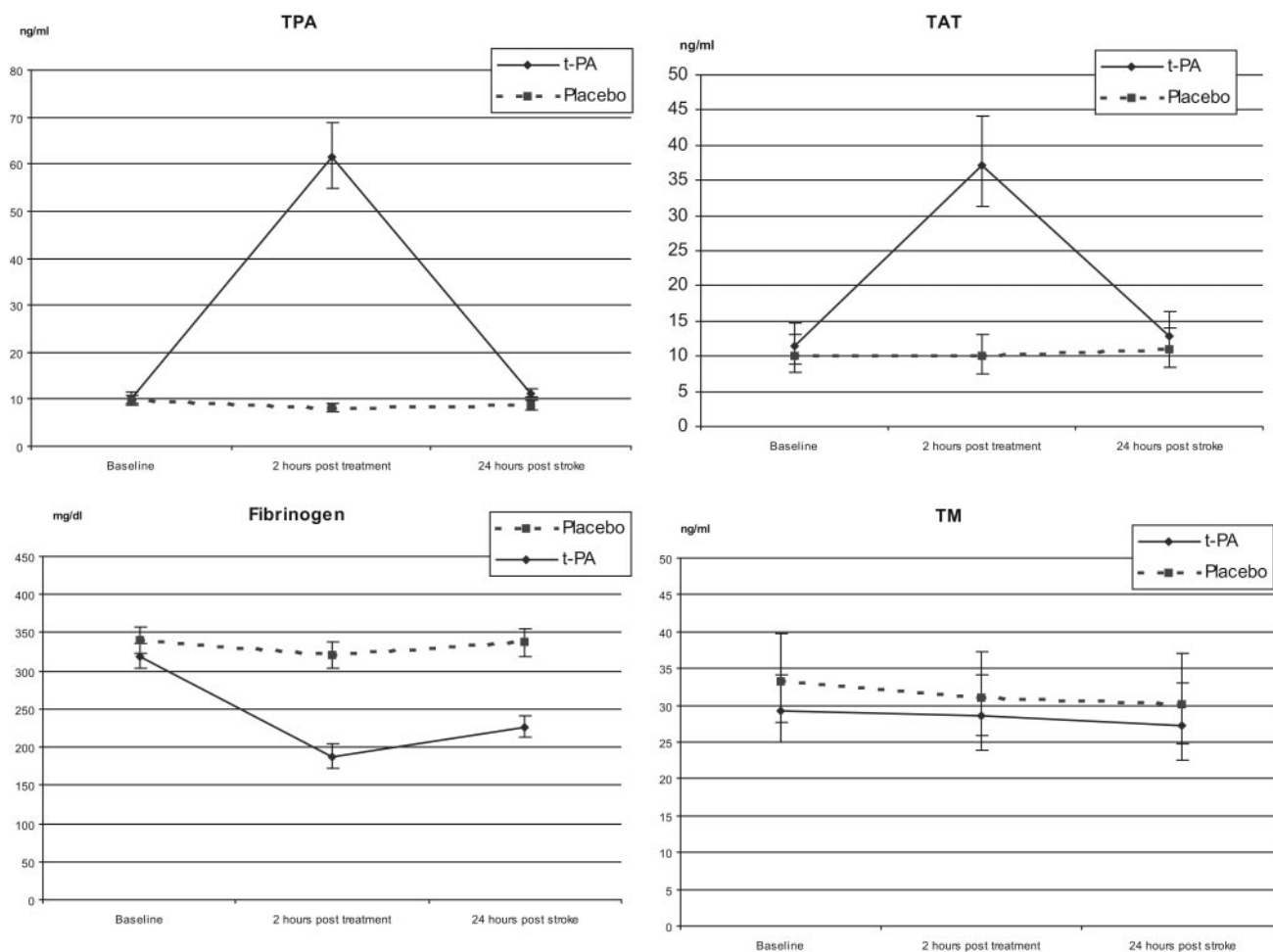
To study the relationship of each marker to the clinical outcomes of interest, we identified potential confounders from among the covariates previously shown to be associated with 1 or more outcomes in previous NINDS rtPA Stroke Trial publications and those variables shown to be unbalanced between the rtPA and placebo groups in the subset with samples available for this study (see Appendix). We included at each time point all patients for whom blood samples were tested at that time point but excluded patients for whom the outcome

**TABLE 2. Associations of TAT at 3 Time Points With Outcomes of Interest Adjusted for Potential Confounders\***

	Baseline (n=394)		2 Hours After Treatment (n=402)		24 Hours After Stroke Onset (n=361)	
	IT <i>P</i>	Individual Effect <i>P</i> , OR (95% CI)	IT <i>P</i>	Individual Effect <i>P</i> , OR (95% CI)	IT <i>P</i>	Individual Effect <i>P</i> , OR (95% CI)
Favorable outcome at 24 h	0.12	0.75, 0.98 (0.84–1.14)	0.19	0.29, 0.90 (0.75–1.09)		
Favorable outcome at 3 mo	0.48	0.58, 1.04 (0.90–1.22)	0.19	0.20, 0.89 (0.75–1.06)	0.17	0.21, 0.89 (0.75–1.06)
ICH (tPA only) symptomatic all		0.49, 1.18 (0.74–1.87); 0.20, 1.29 (0.88–1.88)		0.82, 1.13 (0.40–3.18); 0.28, 1.60 (0.69–3.75)		
Died by 90 d	0.99	0.97, 1.00 (0.81–1.23)	0.53	0.096, 1.26 (0.96–1.66)	0.32	0.0006, 1.72 (1.26–2.34)
Deterioration by 24 h	0.096	tPA: 0.29, 1.20 (0.86–1.66); placebo: 0.21, 0.83 (0.63–1.11)	0.91	0.64, 0.88 (0.53–1.48)		
Deterioration by 7–10 d	0.47	0.69, 0.96 (0.78–1.18)	0.64	0.68, 0.90 (0.53–1.52)	0.12	0.27, 1.28 (0.82–2.00)
All DFI	0.42	0.81, 1.03 (0.82–1.29)	0.76	0.61, 1.09 (0.79–1.48)	0.49	0.49, 0.85 (0.52–1.37)
DFI within 24 h	0.99	0.13, 1.23 (0.94–1.61)	0.87	0.12, 1.41 (0.91–2.17)		
CT volume at 3 mo	0.088	tPA: 0.18; placebo: 0.53	0.36	0.49	0.48	0.095

IT indicates interaction with treatment; DFI, deterioration following improvement.

\*Adjusted for all biomarkers for potential confounders listed in Appendix and for baseline marker level. The ORs correspond to per-unit change of the log-transformed data. Where an interaction was detected, the ORs are presented for rtPA group and placebo group separately.



**Figure 1.** Observed changes in concentrations of hemostatic markers over time in the rtPA group and placebo group, based on 273 patients with hemostatic markers measured in all 3 time points.

of interest occurred at or before the time of the relevant blood draw. Repeated-measures ANOVA on log-transformed data (tPA, TAT, and thrombomodulin) or raw data (fibrinogen) was used to test for time-by-treatment interactions for each of the hemostatic factors, among patients for whom hemostatic markers were assessed in all 3 time points.

Having identified potential confounders, we then used logistic regression to test for hemostatic marker-by-treatment interactions, adjusting for these potential confounders, for each clinical outcome of interest (listed in Tables 1 and 2). Generalized estimating equations were used to take the within-patient correlations among outcomes into account in estimating the treatment effect, its variance, and the test statistic in a logistic regression. In analyzing the effect of markers on CT lesion volumes at 3 months, a Poisson regression model was used on the cube root transformed CT lesion volume to reduce the skewness and variability.<sup>13</sup> Before conducting the logistic regression analyses, we performed log transformations on the hemostatic marker data that were not normally distributed (tPA, TAT, and thrombomodulin) after adding 1 to each hemostatic marker to gain linearity in the log odds. An interaction between a hemostatic marker and treatment was considered significant for  $P \leq 0.10$ .<sup>14</sup> If a marker-by-treatment interaction was detected, then the effect of the marker was assessed separately for each treatment group; if an interaction was not detected, then the effect of a marker was tested including treatment as a covariate, deleting the interaction term. This was a post hoc exploratory analysis designed to provide directions for future investigation rather than to provide definitive answers. Any findings will require confirmation in other studies. Thus, we performed our analyses without adjustment for multiple comparisons.

## Results

Among the 624 patients in the NINDS rtPA Stroke Trial, in 465 patients plasma samples were available at baseline, after 2 hours, or after 24 hours and were used to study the relationship of each marker to the clinical outcomes of interest. In 281 patients, all 3 plasma samples were available and were used to test for time-by-treatment interactions for each of the hemostatic markers. The only baseline variable that was significantly different between patients with all blood sample and those without was history of cardiac disease other than atrial fibrillation (53% versus 44%;  $P < 0.02$ ). Among the patients with all blood samples measured, 2 baseline variables (ranked weight and aspirin use) were unbalanced ( $P < 0.05$ ) between the rtPA and the placebo treatment groups. The latter imbalance had been observed in the trial as a whole.<sup>1</sup>

Changes in concentrations of hemostatic markers over time in the rtPA group and placebo group are shown in Figure 1. Concentrations of TAT and of tPA antigen were highest 2 hours after treatment in the rtPA group, whereas fibrinogen levels dropped, were lowest at 2 hours, and remained low after 24 hours. Repeated-measures ANOVA showed significant interaction effects between time and treatment on TAT, tPA antigen, and fibrinogen ( $P < 0.0001$ ). Within the rtPA

treatment group, fibrinogen concentrations at 2 hours after treatment were inversely correlated with TAT, tPA antigen, and FDP. The correlations between fibrinogen and tPA and FDP persisted after 24 hours. Thrombomodulin concentrations were not significantly correlated with any of the other hemostatic markers at any of the time points within the rtPA treatment group.

### tPA Antigen

Higher 24-hour levels of tPA antigen were associated with infarct volume by CT at 3 months ( $P=0.02$ ) and were associated, particularly in the rtPA treatment group, with lower odds of favorable outcome at 3 months (odds ratio [OR]=0.34; 95% CI, 0.14 to 0.82; Figures 2 and 3). No significant associations were noted with other outcome variables. Among baseline measurements, only weak and inconsistent associations were identified (Table 1).

### Thrombin-Antithrombin Complex

Increasing quartiles of TAT levels were associated with 3-months death rates in the entire cohort of 9.9%, 14.4%, 21.1%, and 28.9%, respectively (Figure 2). Higher TAT levels at 24 hours were independently associated with higher odds of death by 3 months (OR=1.72; 95% CI, 1.26 to 2.34;  $P=0.0006$ ; Figure 3). Among baseline measurements, only weak and inconsistent associations were identified (Table 2).

### Thrombomodulin and Fibrinogen

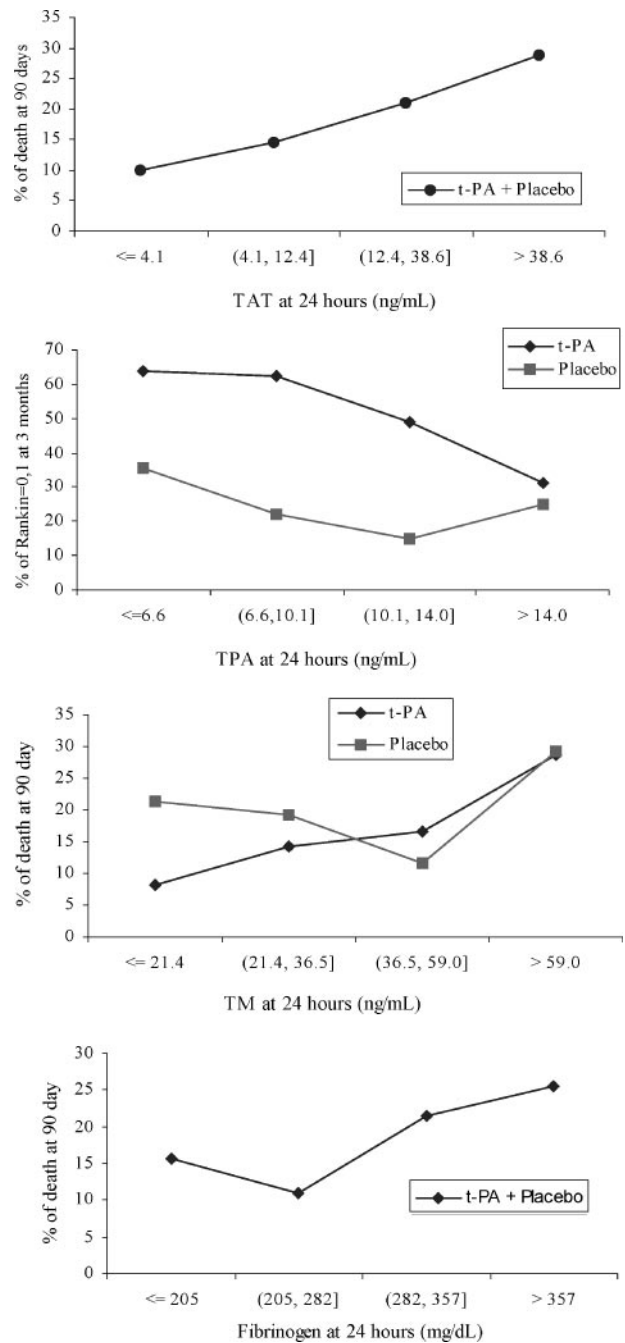
Higher 24-hour levels of soluble thrombomodulin were associated with >4-fold odds of death at 3 months (OR=4.45; 95% CI, 1.26 to 15.67;  $n=361$ ; Figures 2 and 3) particularly in the rtPA group, consistent with a thrombomodulin-by-treatment interaction. Higher level of fibrinogen at baseline was associated within the entire study cohort with infarct lesion volume by CT at 3 months ( $n=570$ ;  $P=0.05$ ). A fibrinogen-by-treatment interaction was detected at 2 hours ( $P=0.05$ ), with higher fibrinogen levels associated with a higher odds of deterioration by 7 to 10 days in the placebo group (OR=1.77; 95% CI, 0.67 to 4.69 per 100-mg/dL increment) than in the rtPA treatment group (OR=0.74; 95% CI, 0.22 to 2.49 per 100-mg/dL increment). Higher 24-hour levels of fibrinogen were associated with  $\approx 40\%$  increase in odds of death by 90 days (OR=1.42; 95% CI, 1.05 to 1.91 per 100-mg/dL increment;  $n=545$ ; Figures 2 and 3). No other significant associations were detected.

## Discussion

Our findings, based on the NINDS rtPA Stroke Study, demonstrate that hemostatic activation after AIS is associated with poorer clinical outcomes after controlling for potential confounders in patients treated with intravenous rtPA.

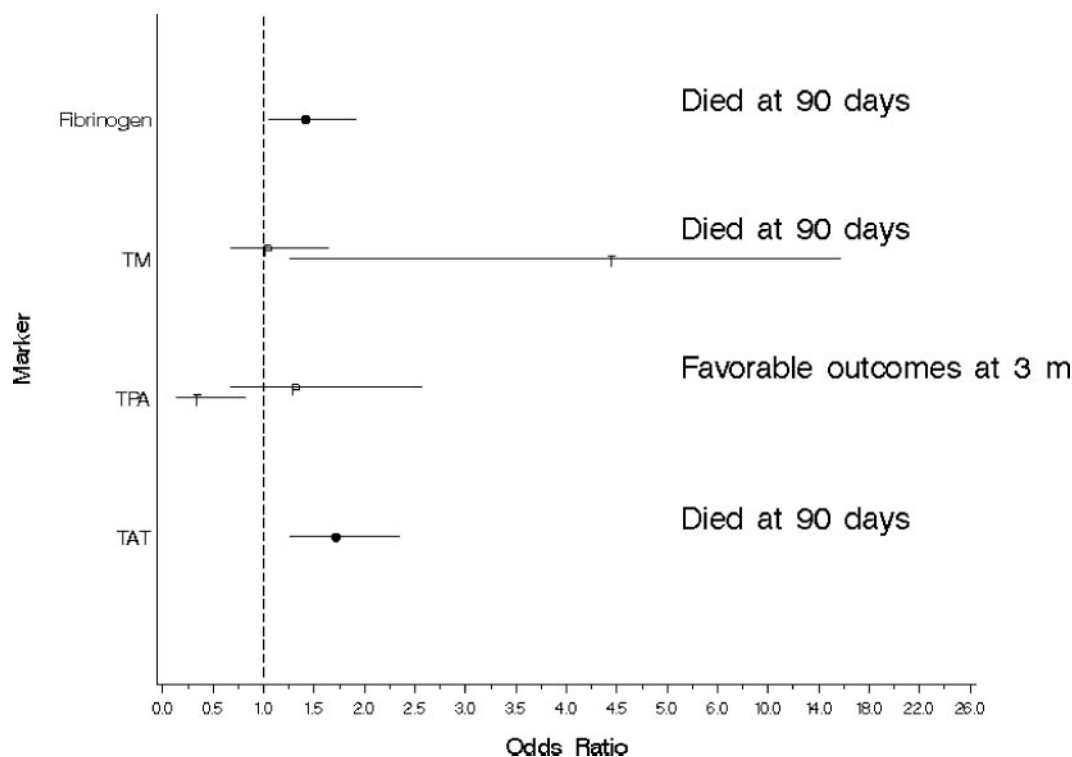
### tPA Antigen

Concentrations of tPA antigen were highest 2 hours after treatment in the rtPA group. This increase likely reflects in part detection of the therapeutically administered exogenous rtPA by the tPA assay, as shown after AMI.<sup>15</sup> Plasma tPA antigen is a grand measure of both inactive and free active tPA, the majority of which is bound to plasminogen activator



**Figure 2.** The associations of 24-hour levels by quartiles of TAT, tPA antigen, thrombomodulin, and fibrinogen with specified outcome measures.  $P$  for the interaction between treatment and tPA antigen on favorable outcomes at 3 months is 0.09 and for the interaction between treatment and thrombomodulin on death at 3 months is 0.035.

inhibitor type 1 (PAI-1), the main physiological inhibitor of tPA, as an inactive circulating complex.<sup>16</sup> Findings of elevated tPA antigen are usually interpreted as reduced net fibrinolytic capacity with relative excess in PAI-1. We did not measure other markers of fibrinolysis, but prior research shows that measurement of PAI activity and antigen levels adds little to the measurement of tPA antigen levels.<sup>16,17</sup> Higher plasma tPA antigen levels were found to predict incident stroke.<sup>17,18</sup> We found an association between high



**Figure 3.** The associations of 24-hour levels of TAT, tPA antigen, thrombomodulin, and fibrinogen with specified outcome measures, adjusted for potential confounders. The ORs correspond to a unit change of the log-transformed marker data for TAT, tPA, and thrombomodulin and 100 mg/dL change for fibrinogen. The lines represent the corresponding ORs and their 95% CIs. Data are presented separately if an interaction of treatment by marker was detected ( $P < 0.10$ ). ■ indicates rtPA and placebo combined; T, rtPA group; and P, placebo group.

24-hour levels of tPA antigen with lower odds of favorable outcome at 3 months, specifically after rtPA therapy, but this association was relatively weak. Further studies are required to examine the correlates of increased tPA antigen and to test whether the observed association might reflect the need to maintain recanalization.

### Thrombin Generation

Failure to achieve reperfusion and artery reocclusion hamper the efficacy of thrombolysis in AIS.<sup>2,3</sup> In patients with AMI, markers of elevated thrombin generation predict poorer clinical outcomes, failure to reperfuse, and increased hemorrhagic events.<sup>7,8,10</sup> We found a surge of thrombin generation after rtPA therapy for AIS. High thrombin generation at 24 hours is associated with a higher overall odds of mortality. These findings, consistent with observations in AMI, suggest that increased thrombin generation may reflect more complicated and thrombogenic atherosclerotic lesions or a sustained hypercoagulable state that threatens the integrity of cerebral reperfusion. Thrombin may also act as proinflammatory mediator and may promote neuronal apoptosis.<sup>19</sup>

Fibrinogen is involved in primary hemostasis, platelet aggregation, and leukocyte-endothelial cell interactions and is a key determinant of blood viscosity and an acute-phase protein. Fibrinogen levels predict stroke risk, and elevated levels after AIS may be associated with a worse outcome.<sup>20,21</sup> In the present study, higher 24-hour levels of fibrinogen were independently associated with higher odds of mortality irrespective of rtPA therapy. Thrombomodulin is an integral

vascular endothelial cell membrane glycoprotein that has a major role in the regulation of intravascular coagulation. Thrombomodulin binds thrombin, changes thrombin conformation, and allows thrombin to activate protein C and thrombin-activating fibrinolysis inhibitor. The physiological significance of soluble thrombomodulin is controversial, but it is used as a surrogate marker of vascular endothelial injury.<sup>22</sup> Increased levels of soluble thrombomodulin may be associated with a reduced risk of brain infarction in subjects with no previous vascular disease, but after stroke it was shown to predict higher mortality.<sup>23</sup> We have found that higher soluble thrombomodulin levels at 24 hours were associated with a 4.5-fold increased odds of death at 3 months, particularly after rtPA therapy. The underlying pathophysiological mechanisms underlying this observation are not clear.

### Strengths and Limitations

Our study has several strengths. First, it is the first investigation of hemostatic markers in a randomized trial of a thrombolytic agent in AIS. It is unique in that it is based on the rigorous methodology and outcome assessment of the NINDS rtPA Stroke Trial. Second, we measured hemostatic markers at 3 specified time points and thus could evaluate changes in the levels of these markers over time. Not all patients were included in this analysis, but the characteristics of those included were overall representative of the entire NINDS rtPA Stroke Trial patients. Third, we adjusted for imbalances in important variables that are known to be associated with stroke outcome. Because no anticoagulant or



antiplatelets treatments were allowed within the first 24 hours after rtPA or placebo, our results are not affected by such medications. A limitation of our study is that it relied on measurement of hemostatic markers from plasma samples that were kept frozen at  $-70^{\circ}\text{C}$  for 6 to 9 years. Longitudinal stability in the biochemical properties of frozen plasma for coagulation, fibrinolysis, and inflammation factors, however, has been shown.<sup>24</sup> Indeed, the elevation of TAT and lower fibrinogen concentrations at 2 hours were as expected biologically, indicating that the tests were reliable in detecting true biological effects. Second, direct data on recanalization and reocclusion, important for elucidating the underlying pathophysiological processes, were not available in this trial. Finally, this was an exploratory, post hoc analysis. We assessed for multiple associations, and many of them were negative. Given the observed variances and the available sample size, we had reasonable power to detect differences on most outcome measures. However, because we tested for multiple associations and did not adjust our significance level for multiple testing, some of the associations may have been identified by chance alone and require further confirmatory studies. Specifically, our findings need to be confirmed prospectively in a separate group of patients treated with rtPA.

Hemostatic markers provide important insight about the pathophysiological mechanisms underlying thrombolytic therapy for AIS and may be useful in refining future protocols aiming at more effective and safe treatment. Our findings illustrate the limitations of current thrombolytic treatment paradigms, which affect stroke within a narrow time window, while thrombin generation, inflammation, and impaired endogenous fibrinolysis may persist to worsen outcomes. Early and effective reperfusion may not only limit the infarct size but also attenuate the hemostatic response, limiting vascular injury and inflammation, leading to a reduced release of these biomarkers. Alternatively, these findings may suggest that patients may have a higher propensity for reocclusion after recanalization or reperfusion injury. Preliminary studies suggest that antithrombotics after rtPA therapy may be safe and efficacious in AIS.<sup>25</sup> Activated protein C has anticoagulant and profibrinolytic as well as anti-inflammatory properties and may add to the effectiveness of rtPA for AIS.<sup>26</sup> On the basis of these findings, there is a rationale to investigate the safety and efficacy of protocols in which rtPA is complemented by agents that are antithrombotic and enhance fibrinolysis.

## Appendix

### Confounders Considered in the Analysis

Age (years)  
 NIH Stroke Scale at baseline  
 Age $\times$ NIH Stroke Scale at baseline  
 Admission mean blood pressure  
 Blood glucose level at baseline  
 History of diabetes  
 Early CT findings  
 Early CT findings (without thrombus)  
 Smoking in year before this stroke  
 Prior aspirin use  
 FDP at baseline  
 Center

Old lesion volume at baseline  
 Presumptive diagnosis at time of treatment

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### Disclosures

None.

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