Association of Serial Biochemical Markers With Acute Ischemic Stroke

The National Institute of Neurological Disorders and Stroke Recombinant Tissue Plasminogen Activator Stroke Study

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Background and Purpose—Biochemical markers of acute neuronal injury may aid in the diagnosis and management of acute ischemic stroke. Serum samples from the National Institute for Neurological Disorders and Stroke (NINDS) recombinant tissue plasminogen activator Stroke Study were analyzed for the presence of 4 biochemical markers of neuronal, glial, and endothelial cell injury. These biochemical markers, myelin basic protein (MBP), neuron-specific enolase (NSE), S100, and soluble thrombomodulin, were studied for an association with initial stroke severity, infarct volume, and functional outcome.

Methods—In the original NINDS study, serum samples were drawn from all patients on presentation to the Emergency Department and at 2 and 24 hours after initiation of study therapy. In this analysis, stored serum samples were available for 359 patients; 107 patients had samples for all 3 time points. Serum marker concentrations were measured by ELISA techniques. We examined the relation between serum concentrations of each marker and the degree of baseline neurological deficit, functional outcome, and infarct size on computed tomography at 24 hours and the effect of fibrinolytic therapy.

Results—Higher 24-hour peak concentrations of MBP, NSE, and S100 were associated with higher National Institutes of Health Stroke Scale baseline scores ($r=0.186$, $P<0.0001$; $r=0.117$, $P=0.032$; and $r=0.263$, $P<0.0001$, respectively). Higher peak concentrations of MBP and S100 ($r=0.209$, $P<0.0001$; $r=0.239$, $P<0.0001$) were associated with larger computed tomography lesion volumes. Patients with favorable outcomes had smaller changes in MBP and S100 ($P<0.05$) concentrations in the first 24 hours. Soluble thrombomodulin was not associated with any severity or outcome measure.

Conclusions—This study corroborates previous work demonstrating correlations of MBP, NSE, and S100 with clinical and radiographic features in acute stroke. Despite significantly better outcomes in the tissue plasminogen activator–treated group, we found no difference in the early release of the 4 biomarkers between treatment groups. Further study will define the role of biomarkers in acute stroke management and prognostication. (Stroke. 2006;37:2508-2513.)

Key Words: fibrinolytics $\bullet$ ischemic stroke $\bullet$ myelin basic protein $\bullet$ neuron-specific enolase $\bullet$ S100 $\bullet$ thrombomodulin

The use of serum biochemical markers in diagnosing acute coronary syndromes has dramatically improved patient care. There is growing interest in the possibility of identifying comparable biomarkers of acute cerebral ischemia. This study reports on the utility of 4 markers of cerebrovascular injury in stroke.

The 4 markers used in this study were chosen to represent the various types of cells injured in acute ischemia. Neuron-specific enolase (NSE), a dimeric isoenzyme of the glycolytic enzyme enolase, is localized mainly within neurons and cells of neuroendocrine origin. NSE has been reported to be a useful biochemical marker for neuronal injury from various causes.1–5 Myelin basic protein (MBP) is an abundant myelin membrane proteolipid produced by oligodendroglia cells and may assist in the clinical assessment of multiple sclerosis6–8 and stroke.9,10 S100 is an abundant cytosolic calcium-binding...
protein. The S100β isoform is found primarily in glial and Schwann cells. S100β possesses many of the desirable characteristics of a biochemical marker: low molecular size, high organ specificity, and a high degree of solubility.\textsuperscript{11–15} Thrombomodulin is an endothelial cell membrane–bound glycoprotein that binds thrombin, producing an anticoagulant effect. Serum soluble thrombomodulin (sTM) is a potential marker of endothelial injury. Elevated sTM concentrations have been associated with spontaneous intracerebral hemorrhages in patients receiving oral anticoagulation and have been inversely associated with stroke severity.\textsuperscript{16,17}

We hypothesized that serum concentrations of MBP, NSE, S100β, and sTM obtained from patients in the original National Institute of Neurological Disorders and Stroke (NINDS) recombinant tissue plasminogen activator (rt-PA) Stroke Study\textsuperscript{18} are (1) related to the degree of neurological deficit and infarct size on computed tomography (CT), (2) associated with functional outcome, and (3) associated with the effect of fibrinolytic therapy.

**Methods**

**Study Design**

This is a secondary analysis of data and serum samples from the NINDS rt-PA Stroke Study.\textsuperscript{18} The original study was a randomized, double-blind, placebo-controlled study of rt-PA for acute ischemic stroke conducted at 8 clinical centers from January 1991 through October 1994. The study evaluated early changes in neurological deficits within 24 hours from administration of rt-PA and investigated the long-term clinical benefit from receiving rt-PA. All patients were enrolled within 3 hours from symptom onset; 302 were treated within 90 minutes and 322 from 91 to 180 minutes from symptom onset.

**Data and Blood Samples Collected During the Original NINDS rt-PA Stroke Study**

Patient eligibility for the original study was previously published.\textsuperscript{18} Neurological deficits were measured at presentation and at 24 hours and 3 months afterward with the National Institutes of Health Stroke Scale (NIHSS). A favorable 3-month outcome, measured by the modified Rankin Scale (mRS), was defined as a mRS score of 0 to 1. CT scans were obtained at presentation and at 24 hours and 3 months. Baseline scans were reviewed by the coordinating center neuroradiologist for the presence of early ischemic changes.\textsuperscript{19} Repeat scans obtained 24 hours after treatment were reviewed for intracerebral hemorrhage and infarct size.\textsuperscript{20}

All 624 enrolled patients underwent serum sampling at presentation (baseline) and at ~2 and 24 hours after treatment. Venous blood samples were collected in evacuated (Vacutainer) tubes, and the exact date and time of sample collection were noted. Samples were centrifuged as soon as possible after collection, and the serum was frozen at −70°C. All samples were shipped frozen to theHenry Ford Hospital and stored at −70°C. Samples were provided to investigators after NINDS steering committee approval.

**Serum ELISA Testing**

Samples were sent frozen to SYN\textsuperscript{X} Pharma Inc, Mississauga, Ontario, Canada, and analyzed in duplicate with commercially available ELISA test kits to measure serum concentrations of S100β, MBP, NSE, and sTM. The ELISA methodology has been described previously.\textsuperscript{21} Lower limits of detection of the ELISA were 0.02 ng/mL for MBP, 1.0 ng/mL for NSE, 0.01 ng/mL for S100β, and 0.8 ng/mL for sTM. SYN\textsuperscript{X} Pharma personnel were blinded to clinical and CT data.

**Statistical Analyses**

Comparison of baseline covariates between the present study population and the entire NINDS population and the baseline covariates between the patients in each treatment arm in this study were performed with the χ² test, unpaired t test, or Mann-Whitney U test, as appropriate.

Several patients with high serum marker concentrations right-skewed the data; these data were logarithmically transformed after 1 was added to the marker concentrations owing to the presence of data points with a zero (subthreshold) value. Mixed-effects linear models were used to evaluate the change in serum marker concentrations over time. The timing of blood draws at specified intervals was variable; exact times from symptom onset to sample collection were used in the analyses. The mixed-effects linear-model method allows for incorporating the repeated-measures structure of the data and the exact time of sample collection, as well as data from patients without all 3 samples. Various models were constructed to determine the influence of predictor variables on the marker concentrations. Logistic regression was used to evaluate the predictive value of marker concentrations, adjusted for confounding variables.

**Results**

**Patient Population**

Serum samples were available for 359 of the original 624 patients in the study (178 in the treatment arm and 181 in the placebo arm; 208 patients at baseline, 311 at 2 hours, and 177 at 24 hours). Serum samples were available for 1 interval for 128 subjects, 2 intervals for 123 patients, and for all intervals for 108 patients. The ELISA for NSE is sensitive to sample hemolysis, and 91 samples demonstrating gross hemolysis were excluded from NSE analysis.

Comparisons of baseline covariates between the treatment arms (placebo versus t-PA) in the 359 patients studied showed that the 2 groups were similar, with the exception of platelets/mm\(^3\), weight, baseline fibrinogen concentration, and aspirin use before randomization (data not shown). The differences in weight and aspirin use were also observed in the total cohort.\textsuperscript{22}

**Serum Marker Concentrations Over Time**

Serum marker concentrations at baseline, 2 hours, and 24 hours after treatment by treatment arm are shown in Table 1. Concentrations of MBP and S100β increased with time (P=0.008), whereas NSE and sTM remained largely unchanged. Marker concentrations at baseline tended to be higher in the placebo group than in the treatment group. There were no significant differences in marker concentrations between the treatment and placebo groups at 2 and 24 hours after treatment was initiated. At baseline, S100β, NSE, and MBP were positively correlated (ρS100β-NSE=0.242; ρS100β-MBP=0.255; ρNSE-MBP=0.401). sTM was not correlated with other markers. At 2 hours, similar results were observed (ρS100β-NSE=0.388; ρS100β-MBP=0.232; ρNSE-MBP=0.292). At 24 hours, sTM was also correlated with NSE (ρS100β-NSE=0.377; ρS100β-MBP=0.616; ρNSE-MBP=0.276; ρNSE-sTM=0.168).

**Relation Between Serum Marker Concentration and Degree of Neurological Deficit, Presence of Early Ischemic Changes on Baseline CT, and Infarct Size**

Baseline serum marker concentrations were not associated with baseline NIHSS, early ischemic changes on baseline CT images,
or lesion volumes at 24 hours or 3 months. Based on Spearman’s rank correlation coefficients, baseline NIHSS was positively associated with 24-hour CT lesion volume ($r_s = 0.527$, $P<0.0001$). In these patients, the assumption of a linear relation was not violated, and the correlation is similar to that previously reported for the entire cohort.23 Higher peak marker concentrations were statistically associated with greater baseline NIHSS for MBP ($r_s = 0.187$, $P<0.0001$), NSE ($r_s = 0.117$, $P=0.032$), and S100β ($r_s = 0.263$, $P<0.0001$) and with larger lesion volumes on 24-hour CT scan for MBP ($r_s = 0.212$, $P<0.0001$) and S100β ($r_s = 0.238$, $P<0.0001$).

**Relation Between Marker Concentrations and 3-Month Functional Outcome**

A history of hypertension, stroke subtype (cardioembolic or large-vessel occlusive), presence of early ischemic changes on baseline CT, higher baseline NIHSS, and higher admission systolic blood pressure were individually associated with outcomes in univariable logistic-regression models. Baseline marker concentrations were not predictive of outcome. Despite this lack of association, marker concentrations were forced into the multivariable models. After adjusting for treatment arm, history of hypertension, NIHSS at baseline, early ischemic changes on initial CT, and admission systolic blood pressure, none of the baseline marker concentrations were associated with outcome ($P>0.247$). The association between marker concentrations at 2 and 24 hours and outcomes was also tested. There was a trend for a greater association as time increased from onset to sample draw; lower 24-hour MBP concentrations were marginally associated with more favorable outcomes (odds ratio, 0.991; 95% confidence interval, 0.983, 1.00; $P=0.051$). In addition, we considered logistic-regression models that included all 4 markers as predictors of favorable outcomes. At no time point were biomarkers in a combined model significant predictors of outcome.

Changes in marker concentrations over time are shown in the Figure. Various models were computed to determine the factors influencing the change in marker concentrations over time. Baseline NIHSS, presumed stroke subtype determined at baseline, and treatment arm were considered (Table 2). S100β and MBP concentrations were positively associated with time, NSE was negatively associated with time, and sTM did not vary significantly with time. Additionally, S100β concentrations were positively associated with a higher baseline NIHSS.

The same mixed models were expanded to include outcomes with an mRS of 0 or 1 at 3 months as a favorable outcome. Patients with a favorable outcome had smaller changes in S100β and MBP concentrations over time (Table 3). Changes in NSE and sTM concentrations over time were not associated with outcome.

**Relation Between Serum Marker Concentration and Administration of rt-PA**

There were no differences between the rt-PA and placebo groups in 2- or 24-hour marker concentration, peak marker concentration, or change in marker concentration. The placebo group tended to have higher baseline marker concentra-
Peripheral markers of central nervous system injury may assist in the diagnosis and management of acute ischemic stroke. We investigated the association of these markers with initial stroke severity, functional outcome, and treatment effect. In contrast to other studies of these markers, we used samples collected very early in the course of the acute stroke; baseline blood draw occurred within 3 hours from symptom onset, half within 90 minutes from onset. Furthermore, this study had a placebo arm, allowing us to investigate the impact of treatment and reperfusion on marker release. Few studies have been able to incorporate these features into their analyses.

S100β concentrations have been reported to peak 2 to 3 days after onset in nonfibrinolytic-treated stroke patients.

<table>
<thead>
<tr>
<th>TABLE 2. Models Showing the Change in Serum Marker Concentrations Over Time</th>
<th>95% CI</th>
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| Dependent Variable | Independent Variable | β | Lower | Upper | P  
| Ln(S100β*1000 + 1) | β₀ | 2.2681 | 1.9868 | 2.5495 | <0.0001  
| Time, h | 0.0228 | 0.0138 | 0.0317 | <0.0001  
| NIHSS | 0.0284 | 0.0119 | 0.0449 | 0.0008  
| Ln(NSE+1) | β₀ | 2.8076 | 2.6588 | 2.9563 | <0.0001  
| Time, h | -0.0040 | -0.0087 | 0.0006 | 0.0908  
| NIHSS | 0.0106 | 0.0017 | 0.0194 | 0.0195  
| Ln(MBP*100 + 1) | β₀ | 0.8888 | 0.5955 | 1.1822 | <0.0001  
| Time, h | 0.0600 | 0.0495 | 0.0704 | <0.0001  
| NIHSS | 0.0236 | 0.0065 | 0.0407 | 0.0069  
| Ln(sTM) | β₀ | 3.9383 | 3.8882 | 3.9885 | <0.0001  
| Time, h | 0.0004 | -0.0014 | 0.0021 | 0.6700  

**Discussion**

Peripheral markers of central nervous system injury may assist in the diagnosis and management of acute ischemic stroke. We investigated the association of these markers with initial stroke severity, functional outcome, and treatment effect. In contrast to other studies of these markers, we used samples collected very early in the course of the acute stroke; baseline blood draw occurred within 3 hours from symptom onset, half within 90 minutes from onset. Furthermore, this study had a placebo arm, allowing us to investigate the impact of treatment and reperfusion on marker release. Few studies have been able to incorporate these features into their analyses.

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<table>
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<th>TABLE 3. Models Showing the Change in Serum Marker Concentrations Over Time, Dependent on the Occurrence of Favorable Outcome</th>
<th>95% CI</th>
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</table>
| Dependent Variable | Independent Variable | β | Lower | Upper | P  
| Ln(S100β*1000 + 1) | β₀ | 2.4203 | 2.1190 | 2.7216 | <0.0001  
| Time*poor outcome | 0.0364 | 0.0247 | 0.0480 | <0.0001  
| Time*favorable outcome | 0.0060 | -0.0082 | 0.0203 | 0.4072  
| Poor vs favorable outcome | -0.0888 | -0.4145 | 0.2369 | 0.5922  
| NIHSS | 0.0214 | 0.0023 | 0.0406 | 0.0281  
| Ln(NSE+1) | β₀ | 2.8228 | 2.6630 | 2.9825 | <0.0001  
| Time*poor outcome | -0.0036 | -0.0096 | 0.0023 | 0.2324  
| Time*favorable outcome | -0.0058 | -0.0130 | 0.0014 | 0.1159  
| Poor vs favorable outcome | -0.1002 | -0.2766 | 0.0762 | 0.2645  
| NIHSS | 0.0136 | 0.0032 | 0.0241 | 0.0106  
| Ln(MBP*100 + 1) | β₀ | 1.1881 | 0.8839 | 1.4923 | <0.0001  
| Time*Poor outcome | 0.0764 | 0.0628 | 0.0901 | <0.0001  
| Time*Favorable outcome | 0.0299 | 0.0132 | 0.0466 | 0.0005  
| Poor vs favorable outcome | -0.0941 | -0.4231 | 0.2349 | 0.5742  
| NIHSS | 0.0071 | -0.0126 | 0.0268 | 0.4799  
| Ln(sTM) | β₀ | 3.9630 | 3.8837 | 4.0432 | <0.0001  
| Time*poor outcome | 0.0004 | -0.0017 | 0.0025 | 0.6926  
| Time*favorable outcome | 0.0000 | -0.0025 | 0.0026 | 0.9713  
| Poor vs favorable outcome | -0.0417 | -0.1444 | 0.0611 | 0.4259  

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Using ultraearly blood sampling times, we demonstrated increasing MBP and S100B concentrations within the first 24 hours from stroke onset. This supports the temporal concentration changes for the first days after stroke observed by others, including Lynch and Laskowitz,25 who demonstrated significantly increased levels within 6 hours from stroke onset compared with controls. We also noted an early peak in NSE concentration, as was described by Anand et al,26 with 24-hour concentrations declining from previously described peaks.8,24 Overall, sTM concentrations as measured by this assay did not change over the first 24 hours.

The relation of peak MBP, NSE, and S100B marker concentrations and the change in these marker concentrations over 24 hours to baseline NIHSS and CT volumes at 24 hours supports similar findings for NSE and S100B concentrations at 48 to 96 hours from stroke onset.3,8,27 Similarly, Herrmann et al27 found that the greatest correlation between marker concentration and outcome was noted in samples drawn several days from onset. Generally, studies using samples drawn at later time points demonstrated stronger associations between concentrations and clinical and radiographic stroke measures.

Relatively little is known about the kinetics of soluble sTM in the early stages of stroke. We did not demonstrate a significant change in concentration during the first 24 hours or an association with infarct volume, baseline neurological deficits, or early ischemic changes. Similarly, Kozuka et al22 did not demonstrate an early increase in sTM concentrations, although sTM may be increased in the subacute phase of stroke, 1 month from onset. Our data suggest limited usefulness for sTM as a marker for predicting outcome in acute ischemic stroke.

Worse outcomes were noted in patients with faster rates of change of marker concentrations. It might be inferred from this that a faster increase predicts poorer outcomes. This would suggest early changes in marker concentrations may provide an earlier predictor of outcome than measuring marker concentrations several days after the onset of stroke.

We did not demonstrate differences in marker concentrations, change in concentrations, or peak concentrations in patients treated with rt-PA compared with placebo, even though the treatment group had better outcomes. Using individual samples drawn at least 48 hours from stroke onset, Foaerch et al28 recently described the utility of the peak S100B concentration and the total S100B released as defined by the area under the kinetic curve as surrogate markers for early recanalization in middle cerebral artery occlusions. We did not find a similar association, likely attributable to the ultraearly time window of our samples and insufficient samples to calculate total marker release.

This secondary analysis has several limitations. Although it is unknown whether protein degradation occurred in our samples, they were stored frozen at −70°C for 6 to 9 years before biomarker measurement, and similar sample storage has been shown to adequately preserve similar biomarkers for later assay.23,29 Although we performed a preliminary analysis to investigate the effect of treatment on marker concentrations, true “washout” or reperfusion effects manifested by marker concentration change requires multiple, frequent samples to better calculate the total amount released.28,30 We had only 3 time points available for analysis, all within the first day from symptom onset. Several studies have shown peak serum concentrations of these markers occur well beyond 24 hours; thus, additional sampling both within and beyond 24 hours may be better suited for providing prognosis and identifying treatment effect.

Conclusions

This study corroborates previous work demonstrating correlations of MBP, NSE, and S100B with clinical and radiographic features in acute stroke. S100B and MBP become elevated within the first 24 hours after stroke, although they do not peak until some days after stroke. Serum biomarkers show promise for their role in acute stroke management and prognostication.

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Disclosures

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