Novel Diagnostic Test for Acute Stroke

John R. Lynch, MD; Robert Blessing, MSN, ACNP; William D. White, MPH; Hilary P. Grocott, MD, FRCPC; Mark F. Newman, MD; Daniel T. Laskowitz, MD

Background and Purpose—The absence of a widely available and sensitive diagnostic test for acute cerebral ischemia remains a significant limitation in the diagnosis and management of stroke. The objective of this study was to examine the feasibility of developing a diagnostic panel of blood-borne biochemical markers of cerebral ischemia.

Methods—Serial blood samples were obtained from patients (n=65 with suspected ischemic stroke, n=157 control subjects) presenting to an academic medical center emergency department. We analyzed 26 blood-borne markers believed to play a role in the ischemic cascade and created a 3-variable logistic regression model to predict the clinical diagnosis of stroke, defined as persistent neurological symptoms of cerebral ischemia lasting >24 hours.

Results—Of the 26 blood-borne markers analyzed, univariate logistic analysis revealed that 4 were highly correlated with stroke (P<0.001): a marker of glial activation (S100β), 2 markers of inflammation (matrix metalloproteinase-9 and vascular cell adhesion molecule), and 1 marker of thrombosis (von Willebrand factor). When the outcome level was set to a cutoff of P=0.1, our logistic model provided a sensitivity and specificity of 90% for predicting stroke.

Conclusions—A panel of blood-borne biochemical markers may be helpful in identifying patients with acute cerebral ischemia who could benefit from urgent care. Such a test may also be helpful in identifying stroke patients in the prehospital setting so that they could be fast-tracked to an institution equipped to care for patients with acute stroke. (Stroke. 2004;35:57-63.)

Key Words: diagnosis ■ stroke ■ stroke assessment

At present, the absence of a widely available and sensitive diagnostic test for acute cerebral ischemia remains a significant limitation in the diagnosis and management of stroke. In the absence of such a test, the diagnosis of acute ischemic stroke at most hospitals is made solely on clinical grounds after intracranial hemorrhage or mass lesion is ruled out by CT. However, other potential causes for acute focal neurological deficits, including complex migraine, postictal paresis, tumors, demyelinating disease, transient ischemic attack (TIA), or even metabolic disturbances such as hypoglycemia, may be difficult to differentiate from stroke.

At most institutions, CT of the brain is performed as part of the initial evaluation of a patient with suspected stroke. The main advantage of this imaging modality is its widespread availability and sensitivity for hemorrhage. However, it is insensitive to early ischemic changes during acute cerebral ischemia and is usually of little value for establishing the diagnosis of acute stroke. Several technologies based on MRI have shown promise for the early diagnosis of stroke. For example, diffusion-weighted imaging can demonstrate parenchymal changes early in the presentation of stroke. In theory, a full MRI study, including diffusion- and perfusion-weighted imaging, MR angiography, and standard T1- and T2-weighted images, could be performed within 30 minutes.

However, as a practical issue, most hospitals do not have these specialized MRI services available in the acute setting. Thus, without a practical and widely available radiological test, the diagnosis of stroke remains largely a clinical decision. Although several simplified clinical stroke scales have been developed for early evaluation of patients with suspected cerebral ischemia, at present they have limited utility.2,3 Obviously, any clinical neurological screening test will be limited by the training and experience of the examiner. This suggests the need for an adjunctive clinical test that can provide diagnostic information above and beyond screening clinical exams.

Another approach to the diagnosis of acute stroke is the evaluation of blood-borne biochemical markers of tissue injury. This approach is well established in the clinical setting of suspected myocardial ischemia. In acute coronary syndromes, the myocardial isoform of creatinine phosphokinase and troponin play an important role both in treatment decisions and clinical research. Similarly, B-type natriuretic peptide has become a routine part of the assessment of patients with congestive heart failure and dyspnea. In many respects, the ischemic cascade of glial activation and ischemic neuronal injury is far more complex than myocardial ischemia and less amenable to the use of a single biochemical
marker. For example, acute stroke has been associated with serum elevations of numerous inflammatory and anti-inflammatory mediators such as interleukin 6 (IL-6) and matrix metalloproteinase-9 (MMP-9).4–9 markers of impaired hemostasis and thrombosis,10,11 and markers of glial activation such as S100β.12,13 Several of these mediators, including IL-6, are elevated within hours after ischemia and correlate with infarct volume.14,15 However, although highly correlated with cerebral ischemia, such inflammatory markers lack the specificity necessary for a useful diagnostic test. Most clinical studies to date have been limited by low numbers of patients, especially those presenting within the first 6 hours of symptom onset, and by the fact that no individual biochemical marker has been demonstrated to possess the requisite sensitivity and specificity to allow it to function independently as a clinically useful diagnostic marker.

Thus, the absence of a rapid, objective, clinically accurate, available diagnostic tool remains a major obstacle to optimal care of stroke patients. At many community hospitals, a formal stroke team or vascular neurologist may not be available, and the lack of a confirmatory diagnostic test may contribute to a physician’s reluctance to initiate thrombolytic therapy. In addition, a rapid diagnostic tool would also be valuable in clinical trials evaluating novel therapeutic interventions to improve functional outcome after stroke. Given the absence of a single peripheral diagnostic marker of stroke, an alternative approach is the creation of a panel of serum biomarkers.16,17 In this study, we examine the feasibility of developing a novel panel of biochemical markers, taking into account the complexity of the ischemic cascade to diagnose acute stroke.

Patients and Methods

After approval from the Duke University Medical Center Institutional Review Board was received, written, informed consent was obtained from study participants or their legal designates. The primary end point in this study was the presence of clinical stroke defined by focal neurological signs or symptoms thought to be of vascular origin that persisted for >24 hours. Ischemic strokes were confirmed by CT and/or MRI before discharge. Blood samples from 65 patients admitted to the emergency department between November 21, 1999, and February 26, 2001, with suspected stroke were stratified into 2 categories based on the latency from symptom onset to blood draw: <6 hours (n=24) and 6 to 24 hours (n=41). Of the 65 patients initially suspected of having a stroke, 44 had persistent residual neurological deficit at 24 hours and met the clinical criteria for stroke; the remaining 21 patients served as the initial control group, which included patients with TIA (n=15), syncpe (n=1), and other conditions (n=7). This initial control group was later enriched with blood samples taken from age- and sex-matched patients without vascular disease (n=157).

Blood drawn from each patient was centrifuged (10 000g), and the resulting supernatant was immediately frozen at −70°C until analysis was completed as described previously.18,19 Measurements of 26 biochemical markers involved in the ischemic cascade were performed by Biosite Inc. All assays were performed in a 10-μL reaction volume in 384-well microplates, with the amount of bound antigen detected by means of alkaline phosphatase–conjugated secondary antibodies and AttoPhos substrate (JBL Scientific).

Statistical Analysis

Descriptive statistics—including frequencies and percentages for categorical data, as well as mean and SD, median, first and third quartiles, and minimum and maximum values for continuous variables—were calculated for all demographic and sample assay data. Demographic variables were compared by the Wilcoxon test (age) or χ2 test for categorical variables. Two data sets were created, representing serum collected from patients whose blood was drawn within 6 hours (the acute group) and those whose blood was drawn 6 to 24 hours (the subacute group) after admission to the emergency department. Markers of glial activation and inflammation, as well as markers of acute cerebral ischemia, including apoptosis, myelin breakdown and peroxidation, thrombosis, and cellular injury, were assayed in the blood of patients presenting with suspected cerebral ischemia.

Univariate logistic regression was initially performed for each marker. The association of stroke by marker levels at a given sample period was tested in stages to minimize overtesting in this exploratory study. A logistic regression model was then created from the data set for each of the 2 groups of patients. To preserve independence in each analysis, multiple samples from the same patient were not used in the same analysis. When multiple samples were available from the same patient within the same time period, only the sample closest to the start of the period was used.

To create this logistic model, we first analyzed the predictive discrimination of each individual variable in the univariate data set. This allowed us to eliminate most variables that had a poor correlation with our end point. On the basis of the collinearity and known biological effects of these markers, we created a 6-variable logistic regression model and performed a stepwise, backward-elimination model to arrive at a 3-variable model with the highest predictive power. To confirm these 3 variables, we repeated the process using a forward stepwise approach in which variables were added sequentially, starting with the variables that were most predictive in the univariate analysis. Correlations among the included markers were checked to avoid collinearity, and influence statistics (change in χ2) were examined to guard against an undue influence of any single observation. In this manner, separate models were developed for 2 periods of marker sampling during which sufficient numbers of stroke samples were available: <6 hours and 6 to 24 hours.

The full study population was used in the model development process, and the predictive performance of the model was internally validated through bootstrap analysis. Fifty test data sets of the same size as the analysis data set were generated by random selection with replacement from the analysis data set. The model was then fit on each bootstrapped data set, with the results inspected for consistency. The measure of predictive discrimination used to characterize model performance in both our original sample and the validation bootstrapped samples was the concordance index. In our particular case in which there is a binary end point of stroke/no stroke, the concordance index is equivalent to the area under the receiver-operating characteristics (ROC) curve in which sensitivity is plotted as a function of (1−specificity).

Results

There was no significant difference in age between patients with and without clinical stroke in either data set. For both the acute and the subacute groups, a greater proportion of patients with than without stroke were female. A greater proportion of stroke patients in both data sets were black and had had a prior incidence of myocardial infarction (Table 1).

Of the 26 biochemical markers involved in pathogenesis of stroke and neuronal injury, univariate logistic analysis demonstrated that 4 markers were highly correlated with stroke (P<0.001) at both time periods (Table 2). These included 1 marker of glial activation (S100β), 2 markers of inflammation (matrix metalloproteinase-9 [MMP-9] and vascular cell adhesion molecule [VCAM]), and 1 marker of thrombosis (von Willebrand factor [vWF]). In addition, several markers were differentially upregulated as a function of time. For
The overall model likelihood ratio was 95.1 (P<0.0001); goodness of fit was confirmed at P=0.2134 (Hosmer-Lemeshow test); and the concordance index was 95% (0.953). With the outcome level set to a cutoff of P=0.1, this model provided a sensitivity and specificity of 90% for discriminating stroke (Figure 4). The bootstrapping validation showed all 50 trials with model P<0.0001, and 49 of the concordance indexes were greater than 91%. S100β was significant (P<0.05) in 47 of 50 samples; VCAM, in 45 of 50; and vWF, in 49 in 50.

### Discussion

The search for a rapid serum diagnostic test of ischemic brain injury remains critically important in the management of acute stroke. In fact, several point-of-care platforms are currently available for the diagnosis of myocardial ischemia. For example, the availability of immediate biomarker information is routine in the management of chest pain. An objective serum marker is now commonly used for diagnosis of heart failure by measuring B-type natriuretic peptide and provides diagnostic information at the point of care within ~15 minutes. The immediate availability of a diagnostic biomarker panel for patients with stroke symptoms would be enormously valuable for increasing clinical confidence and diagnostic accuracy in the early time window for therapeutic decision making. Unlike the current research tools incorporating MR-based technology, a major advantage to such an approach would be its potential cost-effectiveness and widespread availability in settings such as doctors’ offices, long-term care nursing facilities, or ambulances, thus facilitating rapid patient transport to a hospital with an integrated stroke team. A reliable biochemical marker of cerebral ischemia could also potentially provide additional information for primary care providers who are considering the administration of thrombolytic therapy and could facilitate the design of neuroprotective trials with a short therapeutic window. Such a diagnostic test might also be helpful in evaluating tissue at risk and guiding the management of patients with clinical deterioration after acute cerebral ischemia.

In this study, we demonstrate the feasibility of creating a diagnostic test for acute stroke based on a panel of biochemical markers, including vWF, S100β, VCAM, and MMP-9. These markers reflect the role of astrocyte activation (S100β), inflammation and upregulation of adhesion molecules (MMP9 and VCAM) as early events after cerebral ischemia. Although several of these markers have previously been

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**TABLE 1. Patient Demographics for the Data Set in Which Blood Was Collected Acutely (Within 6 Hours of Symptom Onset) and Subacutely (Between 6 and 24 Hours After Symptom Onset)**

<table>
<thead>
<tr>
<th></th>
<th>Acute Stroke (n=16)</th>
<th>Acute No Stroke (n=165)</th>
<th>P</th>
<th>Subacute Stroke (n=38)</th>
<th>Subacute No Stroke (n=176)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD)</td>
<td>62 ± 15</td>
<td>63.3 ± 8</td>
<td>NS</td>
<td>63 ± 5</td>
<td>62.9</td>
<td>NS</td>
</tr>
<tr>
<td>Female sex, %</td>
<td>62.5</td>
<td>32.3</td>
<td>0.026</td>
<td>57.9</td>
<td>32.0</td>
<td>0.005</td>
</tr>
<tr>
<td>History of MI, %</td>
<td>30.8</td>
<td>1.2</td>
<td>&lt;0.001</td>
<td>37.1</td>
<td>2.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Race, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>37.5</td>
<td>91.9</td>
<td></td>
<td>44.7</td>
<td>89.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Black</td>
<td>62.5</td>
<td>3.8</td>
<td></td>
<td>52.6</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>4.4</td>
<td></td>
<td>2.6</td>
<td>4.1</td>
<td></td>
</tr>
</tbody>
</table>

Age is expressed as mean ± SD. For the categorical characteristics, percents are given as proportion of patients with or without stroke who had the characteristics.

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For example, caspase 3 values were 10.9 ± 3.9 ng/mL when measured >3 hours from symptom onset, 18.2 ± 9.8 ng/mL when measured 3 to 6 hours from symptom onset, 39.6 ± 18.6 ng/mL at 6 to 12 hours, and 88.9 ± 33.6 at 12 to 24 hours from symptom onset.

To maximize the sensitivity of a diagnostic test utilizing these markers, we created a 3-variable panel of stroke biomarkers using multivariable logistic regression as described above. For patients presenting within 6 hours of symptom onset, sensitivity and specificity were optimized with the variables MMP-9, vWF, and VCAM (Figure 1). Each of these variables contributed to the model significantly and independently (Table 3). The overall model likelihood ratio χ² for this logistic model was 71.4 (P<0.0001); goodness of fit was confirmed at P=0.9317 (Hosmer-Lemeshow test); and the concordance index was almost 98% (0.979). When the outcome level was set to a cutoff of P=0.1, this model provided a sensitivity and specificity of 90% for predicting stroke (Figure 2). Stroke was defined clinically by the presence of focal neurological symptoms resulting from cerebral ischemia lasting >24 hours. Strokes were confirmed by CT and/or MRI before discharge. Three of the 13 patients without residual deficit who were classified as having TIA had MRI findings suggestive of subcortical stroke. The bootstrapping validation showed all 50 trials with model P<0.0001 and all 50 concordance indexes >94%. MMP-9 was significant (P<0.05) in 43 of 50 samples; VCAM, in 43 of 50; and vWF, in 35 of 50.

In similar fashion, we next developed a logistic regression model for patients with subacute symptoms (6 to 24 hours from symptom onset to blood draw). For this group, sensitivity and specificity were optimized using the variables S100β, VCAM, and vWF (Figure 3), each of which contributed to the model significantly and independently (Table 3). The overall model likelihood ratio χ² for this logistic model was 95.1 (P<0.0001); goodness of fit was confirmed at P=0.2134 (Hosmer-Lemeshow test); and the concordance index was 95% (0.953). With the outcome level set to a cutoff of P=0.1, this model provided a sensitivity and specificity of 90% for discriminating stroke (Figure 4). The bootstrapping validation showed all 50 trials with model P<0.0001, and 49 of the concordance indexes were >91%. S100β was significant (P<0.05) in 47 of 50 samples; VCAM, in 45 of 50; and vWF, in 49 in 50.
Markers of thrombosis

- vWF, ng/mL
  - 0–6 h: <0.001
  - 6–24 h: <0.001

Inflammatory mediators

- MMP-9, ng/mL
  - 0–6 h: <0.001
  - 6–24 h: <0.001
- VCAM, μg/mL
  - 0–6 h: <0.001
  - 6–24 h: <0.001
- IL-6, pg/mL
  - 0.039
  - 0.008
- Intracellular adhesion molecule, ICAM, ng/mL
  - NS
  - 0.019
- Tumor necrosis factor, pg/mL
  - 0.016
  - 0.039
- Neuronal cell adhesion molecule, ng/mL
  - NS
  - NS
- IL-1 receptor antagonist, pg/mL
  - NS
  - NS
- Neuronal cell adhesion molecule, ng/mL
  - NS
  - NS
- Tumor necrosis factor, pg/mL
  - 0.016
  - 0.039
- Intracellular adhesion molecule, ICAM, ng/mL
  - NS
  - 0.019
- Vascular cell adhesion molecule, VCAM
  - 0.660
  - 2.423
  - 1.417
  - 4.380
  - 0.0020

Markers of cellular injury and myelin breakdown

- Creatinine phosphokinase, brain band, ng/mL
  - 0.03
  - 0.04
- Tissue factor, pg/mL
  - NS
  - 0.013
- Myelin basic protein, ng/mL
  - NS
  - NS
- Proteolipid protein, RU
  - NS
  - NS
- Malondialdehyde, μg/mL
  - 0.02
  - 0.02

Markers of apoptosis, growth factors, miscellaneous

- Brain natriuretic peptide, pg/mL
  - 0.019
  - <0.001
- Caspase 3, ng/mL
  - NS
  - 0.002
- Calbindin-D, pg/mL
  - NS
  - NS
- Heat shock protein 60, ng/mL
  - NS
  - NS
- Cytochrome C, ng/mL
  - NS
  - NS

The odds ratio for each of the covariates is presented per unit of 1 SD.

**TABLE 3. CI for Odds Ratios in Units of 1 SD of Predictor**

<table>
<thead>
<tr>
<th>Time of Blood Draw, h</th>
<th>Unit (1 SD)</th>
<th>Odds Ratio</th>
<th>CI Lower Limit</th>
<th>CI Upper Limit</th>
<th>P</th>
</tr>
</thead>
</table>
| 0–6                  | MMP-9       | 137.0      | 13.202         | 3.085         | 98.035 | 0.0026
|                      | VCAM        | 0.5900     | 4.104          | 1.793         | 12.721 | 0.0045
|                      | vWF         | 1462.0     | 3.581          | 1.590         | 9.450  | 0.0036
| 6–24                 | S100β       | 65.0       | 6.371          | 2.225         | 26.246 | 0.0024
|                      | VCAM        | 0.660      | 2.423          | 1.417         | 4.380  | 0.0020
|                      | vWF         | 1621.0     | 3.180          | 1.934         | 5.67   | <0.0001

VCAM indicates vascular cell adhesion molecule.

The odds ratio for each of the covariates is presented per unit of 1 SD.
bral perfusion and minimizing secondary injury (ie, relaxing blood pressure parameters and minimizing febrile and hyperglycemic episodes). Finally, a peripheral marker of cerebral injury might play an adjunctive role in determining neurological prognosis and making end-of-life decisions after global cerebral ischemia.13

Another critically important piece of information that would help guide the management of patients with acute and subacute cerebral ischemia is the volume of ischemically injured but potentially viable (penumbral) tissue. Although time from symptom onset is a reasonable surrogate for salvageable brain tissue, there is most likely a subgroup of patients who would still respond favorably to delayed reperfusion.21 However, a sensitive and widely available diagnostic test to identify patient subgroups that might benefit from delayed intervention is still lacking. At present, the most promising tool that we have to identify volume of tissue at risk is diffusion/perfusion mismatch as demonstrated by MRI techniques.22 Although a promising research tool, this MR-based technology is unlikely to become widely available for most patients presenting to nonacademic facilities with acute stroke syndromes. Thus, an additional benefit of obtaining a panel of markers might be a biochemical fingerprint of

Figure 1. Unadjusted (univariate) relations between each continuous variable used in logistic regression model for patients whose blood was drawn within 6 hours of symptom onset. Each panel depicts probability of stroke as function of vWF (A), VCAM (B), and MMP-9 (C), with 95% CIs (dotted curves) as function of baseline variable.

Figure 2. A, Observed rates of stroke vs predicted rate of stroke for patients in whom blood was drawn within 6 hours of symptom onset on the basis of logistic regression model using the variables vWF, VCAM, and MMP-9. Patients were divided into groups of 10 according to probability of stroke as defined by this model. Actual stroke rate for each group is plotted vs average model prediction. Dashed diagonal line represents perfect calibration of the model predictions. B, ROC curve demonstrating sensitivity as a function of 1—specificity. This logistic model has a sensitivity and specificity of 90%.
irreversibly injured tissue versus at-risk but still salvageable penumbral tissue. We observed that caspase 3, a marker of apoptosis, increased as a function of time from symptom onset and possibly represents volume of irreversible tissue injury. Although these results are preliminary, this approach may ultimately enable the clinician to identify the subset of patients who may benefit from reperfusion therapy delayed >3 hours from symptom onset. One obvious limitation of trying to establish a biochemical fingerprint of unsalvageable tissue in the present study is the absence of a gold standard. In the present study, we used time from symptom onset as a surrogate for tissue at risk. However, a follow-up study validating these markers with more sophisticated approaches such as perfusion diffusion-mismatch may be a viable approach in the future.

One limitation of this pilot study is the relatively small number of initial control subjects, ie, patients who initially presented with suspected stroke but lacked stroke as the ultimate diagnosis. For this reason, and before any analysis, we enriched the control group with age-matched normal
control subjects. Ultimately, the sensitivity and specificity of this diagnostic test should be validated exclusively on patients with suspected stroke. Because the number of true stroke cases was too small in this exploratory study to support a rigorous predictive multivariable model adjusting for covariables such as age or race, we limited the number of predictors in the multivariable tests and obtained strong, consistent, and apparently stable evidence of the marker relationships, which was well corroborated by the bootstrap validation analysis. Nevertheless, to externally validate the use of this model as a diagnostic tool, we are now in the process of collecting additional samples from multiple sites.

Conclusions
In summary, our data support the use of a panel of blood-borne biochemical markers to aid in the diagnosis of acute cerebral ischemia. Such a test would have potential utility in enabling physicians to fast-track patients with cerebral ischemia to an institution with a stroke team and may ultimately play a role in guiding patient management decisions and designing future clinical research trials testing new therapeutic interventions.

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References
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