Serum Cellular Fibronectin and Matrix Metalloproteinase-9 as Screening Biomarkers for the Prediction of Parenchymal Hematoma After Thrombolytic Therapy in Acute Ischemic Stroke

A Multicenter Confirmatory Study

Mar Castellanos, MD, PhD; Tomás Sobrino, BSc; Mónica Millán, MD; María García, MD, PhD; Juan Arenillas, MD, PhD; Florentino Nombela, MD, PhD; David Brea, BSc; Natalia Perez de la Ossa, MD; Joaquín Serena, MD, PhD; José Vivancos, MD, PhD; José Castillo, MD, PhD; Antoni Dávalos, MD, PhD

Background and Purpose—Plasma levels of cellular fibronectin (c-Fn) ≥3.6 μg/mL and of matrix metalloproteinase-9 (MMP-9) ≥140 ng/mL have been associated with parenchymal hematoma (PH) after treatment with tissue-type plasminogen activator (t-PA) in patients with acute ischemic stroke. In this prospective study, we sought to validate the predictive capacity of the preestablished cutoff values of these biomarkers for PH in a larger series of patients.

Methods—We studied 134 patients treated with t-PA within 3 hours from symptom onset according to the SITS-MOST criteria (median time to infusion, 152 minutes; median National Institutes of Health Stroke Scale score, 14) in 4 university hospitals. Hemorrhagic transformation was classified according to the European-Australasian Acute Stroke Study II definitions on computed tomography scans performed 24 to 36 hours after treatment. Relevant hemorrhagic transformation was defined as hemorrhagic infarction type 2 or any PH. Serum c-Fn and MMP-9 levels were determined by an ELISA on blood samples obtained before treatment.

Results—Cranial computed tomography showed hemorrhagic transformation in 27 patients (20%), hemorrhagic infarction in 15 (type 2 in 8 patients), and PH in 12 patients (symptomatic in 4). Serum c-Fn and MMP-9 concentrations at baseline were significantly higher in patients with relevant hemorrhagic transformation and PH than in those without (all \( P<0.001 \)). The sensitivity, specificity, and positive and negative predictive values for PH by c-Fn concentrations at baseline were 100%, 60%, 20%, and 100%, respectively, whereas corresponding values were 92%, 74%, 26%, and 99% for MMP-9 levels ≥140 ng/mL. When both biomarkers were at levels above the cutoff points, specificity increased to 87% and the positive predictive value increased to 41%.

Conclusions—This prospective study confirmed the high sensitivity and negative predictive value, with retained good specificity, of c-Fn and MMP-9 for the prediction of PH in patients treated with t-PA. Development of faster analytic methods will prove the applicability of these biomarkers in routine clinical practice.

Key Words: stroke ■ hemorrhage ■ thrombolytic therapy ■ metalloproteinases ■ biomarkers ■ blood-brain barrier

Tissue-type plasminogen activator (t-PA) is an effective treatment for acute ischemic stroke, but the increased associated risk of hemorrhagic transformation (HT) is still of great clinical concern.1-3 Although high blood sugar concentrations, age, and the extent of early infarct signs on cranial computed tomography (CT) or diffusion-weighted magnetic resonance imaging scans have been associated with parenchymal hematoma (PH) and symptomatic HT,4-5 there are no good clinical or neuroimaging predictors of hemorrhagic risk. It is therefore critically important that useful biomarkers linked to the underlying mechanisms of this complication be identified to improve the safety profile and the risk-benefit ratio of t-PA in the treatment of stroke.

Received January 3, 2007; accepted January 17, 2007.
From the Department of Neurology (M.C., J.S.) and Biostatistics Unit, (M.G.), Hospital Universitari Doctor Josep Trueta, Girona, Spain; the Department of Neurology (T.S., D.B., J.C.), Neurovascular Research Laboratory, Hospital Clínico Universitario, Universidad de Santiago de Compostela, Santiago de Compostela, Spain; the Department of Neurosciences (M.M., J.A., N.P.d.I.O., A.D.), Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Spain; and the Department of Neurology (F.N., J.V.), Hospital Universitario de La Princesa, Madrid, Spain. Correspondence to Dr Antoni Dávalos, Acute Stroke Unit, Department of Neurosciences, Hospital Germans Trias I Pujol, Universitat Autònoma de Barcelona, Ctra de Canyet s/n, 08916 Badalona, Spain. E-mail adavalos.germanstrias@gencat.net
© 2007 American Heart Association, Inc.

Stroke is available at http://www.strokeaha.org

DOI: 10.1161/STROKEAHA.106.481556

1855
The capacity of several biomarkers to predict HT of the ischemic lesion after t-PA administration has been tested. Pretreatment hemostatic markers have failed to predict symptomatic PH in patients receiving t-PA.6 However, an association has been found between secondary bleeding of the cerebral infarct and increased blood levels of biomarkers of extracellular matrix proteolysis and microvascular basal lamina injury, particularly matrix metalloproteinase-9 (MMP-9) and cellular fibronectin (c-Fn).7–10 These findings are in agreement with emerging experimental data that have pointed to the importance of MMP overexpression and blood-brain barrier disruption in the pathogenesis of HT and brain edema development after t-PA reperfusion therapy.11

In a retrospective, hypothesis-generating study, we found that c-Fn plasma levels ≥3.6 μg/mL and MMP-9 values ≥140 ng/mL before t-PA administration in patients with acute ischemic stroke were independently associated with the highest odds of subsequent hemorrhagic infarction type 2 and PH,10 which we shall refer to as “relevant HT.” Furthermore, c-Fn levels achieved high sensitivity and negative predictive value (NPV) for the prediction of PH development, and good specificity was maintained. In this prospective study, we sought to validate the predictive capacity of the preestablished cutoff values of c-Fn and MMP-9 for relevant HT and PH development in a new series of patients.

Patients and Methods

We studied acute ischemic stroke patients consecutively treated with intravenous t-PA within 3 hours of symptom onset in 4 university hospitals. All patients were treated according to the SITS-MOST criteria (available at http://acutestroke.org). For the purpose of this investigation, reasons for exclusion were known infectious, inflammatory, or neoplastic diseases at the time of treatment and nonavailability of blood samples at baseline. Concomitant treatment with NXY-059 versus placebo within the Stroke-Acute Ischemic NXY-059 Treatment I trial was allowed,12 but the present investigation was not a substudy of the Stroke-Acute Ischemic NXY-059 Treatment I. No other drugs under investigation were used. The protocol was approved by the ethics committees of the participating centers, and informed, written consent was obtained from the patients or their relatives.

Patients were continuously monitored in an acute stroke unit. Stroke severity was quantified immediately before t-PA administration and at 24 hours according to the National Institutes of Health Stroke Scale. Early neurologic deterioration was diagnosed when the National Institutes of Health Stroke Scale score worsened by ≥4 points between baseline and 24 hours. CT scans were performed immediately before treatment and at 24 to 36 hours after thrombolytic therapy or on neurologic deterioration. According to the European-Australasian Acute Stroke Study II definitions,3 HT was classified as hemorrhagic infarction type 1 or type 2, and PH, as type 1, type 2, or remote PH. As specified earlier, relevant HT was defined as hemorrhagic infarction type 2 and any type of PH. HT was defined as symptomatic when it was associated with early neurologic deterioration. CT scans were evaluated by investigators who were blinded to the laboratory determinations and clinical outcome.

Laboratory Determinations

Serum samples were taken immediately after admission (within 3 hours of stroke onset and before t-PA treatment) and stored at −80°C. c-Fn and MMP-9 levels were determined with commercially available quantitative sandwich ELISA kits obtained from Biosite Pte Finland and Biotrack Amersham Pharmacia, UK, respectively. The interassay variability was 5.5% for c-Fn and 5.1% for MMP-9.

| TABLE 1. Baseline Vascular Risk Factors, Clinical Characteristics, Stroke Subtype, Biochemical Parameters, and Neuroimaging Findings in Patients Included in the Study |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Age (y)                                         | Male (n (%))    | Time from stroke onset to treatment (min) | Clinical characteristics | Stroke subtype (n (%)) | Cardioembolic | Large-artery atherosclerosis | Small-vessel disease | Underdetermined etiology | Other (n (%)) | Prior antiplatelet therapy (n (%)) | Cotreatment with NXY-059/placebo (n (%)) | History of vascular risk factors (n (%)) | Hypertension | Diabetes mellitus | Current smoking habit | Dyslipidemia | Prior stroke (n (%)) | Biochemistry and vital signs at admission | Systolic blood pressure (mm Hg) | Diastolic blood pressure (mm Hg) | Axillary temperature (°C) | Plasma glucose (mg/dL) | Platelet count (10⁵/mm³) | Leukocyte count (10⁹/mm³) | Activated partial thromboplastin time (s) | Neuroimaging findings | Early signs of infarction (n (%)) | Hypodensity > 1/3 of middle cerebral artery territory (n (%)) | Volume of hypodensity at 24–36 hours (cm³) |
| 67 (12)                                         | 88 (65.7)       | 152 [125, 170] | 14 [9, 19] | 58 (43.3) | 26 (19.4) | 9 (6.7) | 38 (20.6) | 3 (2.2) | 38 (28.4) | 23 (17.2) | 66 (49.2) | 25 (18.7) | 20 (14.9) | 43 (32.1) | 11 (8.2) | 152 (23) | 82 (13) | 36.2 (0.5) | 137 (52) | 241 (92) | 8.8 (2.4) | 28 (10) | 45 (33.6) | 6 (4.5) | 19 [3, 80] |

Continuous variables are expressed as mean (SD) or median (quartiles). Clinical investigators were unaware of the laboratory results until the end of the study, once the database had been closed.

Statistical Analyses

Continuous variables are expressed as mean±SD or median [quartiles] and were compared with Student’s t test, and the Mann–Whitney U test or Kruskal–Wallis test, as appropriate. Categorical variables are presented as percentages and were compared with the χ² test. To confirm our hypothesis, the sensitivity, specificity, NPV, and positive predictive value (PPV) of the preestablished cutoff values of the biomarkers were calculated for the prediction of relevant HT and PH. The importance of the biomarkers’ preestablished cutoff values in the development of PH after the administration of t-PA was also determined by logistic-regression analysis after adjusting for those variables related to PH in the univariate analysis. A sensitivity analysis was performed in patients not randomized in the Stroke-Acute Ischemic NXY-059 Treatment I trial. In a post hoc explanatory analysis, receiver

Downloaded from http://stroke.ahajournals.org/ by guest on July 13, 2017
operating characteristic curves were constructed to determine the best cutoff value of the biomarkers in this new series of patients for the prediction of PH after t-PA administration.

**Results**

A total of 151 patients consecutively treated with intravenous t-PA during the study period (which varied between 5 and 26 months, depending on the center) were evaluated. Eleven patients were excluded from the analysis owing to nonavailability of blood samples at baseline, and 6 patients were excluded because of cancer, current infection, or inflammatory disease, so 134 patients were finally included in the study. Table 1 shows the baseline characteristics of the patients included in the study. At 24 hours, 8 patients (6%) showed neurologic deterioration, whereas 58 patients (43%) had neurologic improvement. CT at 24 to 36 hours showed no signs of infarction in 14 (10%) patients. The median hypodensity volume was 19 [3, 80] cm³. In 108 patients (80%), the infarction was located in the middle cerebral artery territory. HT was observed in 27 (20%) patients (hemorrhagic infarction type 1 in 7 and type 2 in 8; PH type 1 in 7, type 2 in 4, and remote in 1), and it was symptomatic in 4, all of whom had PH.

The severity of neurologic deficit at admission was significantly higher (median National Institutes of Health Stroke Scale score = 18 [13, 20] versus 14 [8, 19]; \( P=0.050 \)) and

**Serum biomarker concentrations by HT subtype.** Relevant HT group includes patients with PH. Boxplots show median values (horizontal line inside the box), quartiles (box boundaries), and the largest and smallest observed values (lines drawn from the end of the box) of c-Fn (A) and MMP-9 (B) levels before t-PA treatment. The dotted line represents prespecified cutoff points of c-Fn and MMP-9.
early CT signs of infarction were significantly more frequent (55% versus 30%; \( P = 0.039 \)) in patients with relevant HT compared with those without. No other baseline clinical, radiologic, or biochemical (glucose level, leukocyte and platelet counts, and coagulation tests) characteristics were found to be significantly different between patients with and without relevant HT. When comparisons were made between patients with and without PH, baseline temperature (36.0[0.5] versus 35.0[0.6]; \( P = 0.044 \)) and the volume of hypodensity at 24 to 36 hours of evolution (127[137] versus 52[87]; \( P = 0.011 \)) were significantly higher in patients with PH. At baseline, serum c-Fn and MMP-9 concentrations were significantly higher in patients with relevant HT and PH than in those without (the Figure). Median c-Fn concentrations were 3.2 [1.9, 4.2] \( \mu \)g/mL in patients with no or nonrelevant HT and 6.5 [5.3, 8.2] \( \mu \)g/mL in those with relevant HT (\( P < 0.001 \)), whereas median MMP-9 values were 93.7 [63.0, 140.3] and 194 [138.3, 216.1] \( \mu \)g/mL, respectively (\( P < 0.001 \)). In patients with PH, median c-Fn and MMP-9 levels were 7.9 [6.6, 8.6] and 204.7 [161.6, 235.3] \( \mu \)g/mL, respectively (\( P < 0.001 \) for both comparisons with the non-PH group). The sensitivity, specificity, NPV, PPV, and global accuracy of c-Fn levels \( \geq 3.6 \) \( \mu \)g/mL and MMP-9 levels \( \geq 140 \) \( \mu \)g/mL predicted the development of PH after t-PA administration with a sensitivity of 100% (70% to 99%), a specificity of 88% (80% to 93%), a PPV of 45% (26% to 64%), an NPV of 100% (96% to 100%), and an accuracy of 89% (82% to 93%).

### Discussion

This multicenter, prospective study replicates the high sensitivity and NPV of predefined levels of c-Fn and MMP-9 for the prediction of relevant HT and particularly for PH.\(^9\) Hence, this study confirms the validity of these compounds as screening biomarkers for the prediction of secondary cerebral bleeding in patients with acute stroke treated with t-PA.

Whereas hemorrhagic infarction has no impact on clinical outcome and is probably not associated with the t-PA effect,\(^13,14\) PH is often clinically relevant, causes a mild to significant space-occupying effect, and is probably not associated with the t-PA effect.\(^13,14\)

### Table 3. Adjusted Odds Ratios of PH

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.94 (0.88–1.00)</td>
<td>0.080</td>
</tr>
<tr>
<td>National Institutes of Health Stroke Scale score at admission</td>
<td>1.12 (0.94–1.32)</td>
<td>0.210</td>
</tr>
<tr>
<td>Temperature at admission (°C)</td>
<td>0.44 (0.11–1.74)</td>
<td>0.244</td>
</tr>
<tr>
<td>Volume of hypodensity at 24–36 hours</td>
<td>1.00 (0.99–1.00)</td>
<td>0.816</td>
</tr>
<tr>
<td>c-Fn ( \geq 3.6 ) ( \mu )g/mL and MMP-9 ( \geq 140 ) ( \mu )g/mL</td>
<td>67 (6.9–649)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
ated with neurologic worsening and poor functional outcome, and is strongly related to t-PA administration. The present study confirms that c-Fn $\geq 3.6 \mu g/mL$ is an accurate biomarker of severe HT, because all cases with this level were found to develop PH. In combination with MMP-9 $\geq 140 ng/mL$, c-Fn levels achieved good specificity and were associated with a 67-fold increased risk of PH (Table 3). Interestingly, no other independent clinical predictors of PH development were identified in the logistic-regression analysis. However, this study also showed that the best cutoff point of c-Fn levels for the prediction of severe bleeding has yet to be defined, because receiver operating characteristic curves yielded a better cutoff value than the previously reported one. The use of a different ELISA kit might at least in part explain the variability in the c-Fn cutoff point.

As previously described, the levels of c-Fn had a greater predictive value for relevant HT than did MMP-9 levels. This may be due to the fact that we determined total MMP-9 levels, which included not only activated MMP-9 levels but also pro-MMP-9 released as a result of ischemia. However, because c-Fn is almost exclusively located at the endothelium, c-Fn levels probably more accurately reflect the acute microvessel ischemic injury secondary to the activated MMP-9 and inflammatory molecule release. Although MMP-9 concentrations may be higher in serum than in plasma samples, given that platelets and leukocytes contain MMP-9, we found median serum values similar to those obtained in plasma samples in our previous study.

The limited sample size of the present study and consequently the low number of PHs weaken the strength with which we may draw our conclusions. The low PPVs of the biomarkers are also a limitation of the present study, because they do not currently allow patients to be selected for denial of thrombolytic treatment in this way. However, because knowledge that a patient has no risk of severe HT is a relevant clinical datum, the high specificity and NPV are still very significant. Finally, ELISA kits can only be either accepted or are valid as screening analytic tests, because these technique are slow and expensive and are therefore not applicable in daily clinical practice. However, the robustness of the results gives support to the need to develop a faster analytic method for determining the best cutoff points and for measuring serum c-Fn and MMP-9 at emergency departments and to test their accuracy in a large multicenter trial.

Sources of Funding
This project was partially supported by a grant from the Spanish Ministry of Health (Instituto de Salud Carlos III) and by the RETICS-RD06/0026.

Disclosures
Dr Joaquín Serena, Dr Antoni Dávalos, Dr Mar Castellanos, and Dr José Castillo are joint patent holders of US Patent No. 2007005261 with the title “Cellular fibronectin as a diagnostic marker in stroke and methods of use thereof.”

References


Serum Cellular Fibronectin and Matrix Metalloproteinase-9 as Screening Biomarkers for the Prediction of Parenchymal Hematoma After Thrombolytic Therapy in Acute Ischemic Stroke: A Multicenter Confirmatory Study

Mar Castellanos, Tomás Sobrino, Mónica Millán, María García, Juan Arenillas, Florentino Nombela, David Brea, Natalia Perez de la Ossa, Joaquín Serena, José Vivancos, José Castillo and Antoni Dávalos

Stroke. 2007;38:1855-1859; originally published online May 3, 2007;
doi: 10.1161/STROKEAHA.106.481556

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/38/6/1855

An erratum has been published regarding this article. Please see the attached page for:
/content/38/8/e76.full.pdf
In the article entitled “Serum Cellular Fibronectin and Matrix Metalloproteinase-9 as Screening Biomarkers for the Prediction of Parenchymal Hematoma After Thrombolytic Therapy in Acute Ischemic Stroke: A Multicenter Confirmatory Study” by Castellanos et al,1 the authors inadvertently omitted a conflicts-of-interest disclosure statement. The authors regret this error. The Disclosures section of the article should state the following:

“Dr Joaquín Serena, Dr Antoni Dávalos, Dr Mar Castellanos, and Dr José Castillo are joint patent holders of US Patent No. 20070005261 with the title ‘Cellular fibronectin as a diagnostic marker in stroke and methods of use thereof.’ The other authors have nothing to disclose.”

The corrected version of this article can now be viewed online at http://stroke.ahajournals.org.