Once diagnosed, the primary therapy for acute ischemic stroke is intravenous tissue plasminogen activator (rt-PA) administration; however, despite better outcome in treated patients, hemorrhagic transformations (HTs) remain all too frequent. In fact, patients who receive rt-PA have a 10-fold increased risk of intracranial bleeding compared with untreated patients. In an attempt to avoid this possible deleterious side effect of rt-PA treatment, it is critical to identify the underlying mechanisms by which this severe complication occurs only in some patients in order to improve the safety profile of thrombolytic agents for stroke management and enable a wider use of this treatment.

Oxidative stress results from an imbalance in redox state in which pro-oxidants overwhelm antioxidant capacity. It is considered to be a potential mechanism implicated in the pathogenesis and disease progression of many unrelated pathological processes, including cardiovascular diseases, diabetes mellitus, and cancer, and may therefore contribute

Fluorescent Molecular Peroxidation Products
A Prognostic Biomarker of Early Neurologic Deterioration After Thrombolysis

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Background and Purpose—Fluorescent molecular peroxidation products (FMPPs) are considered potential markers of molecular oxidative damage and may provoke increased permeability and disruption of the blood–brain barrier. This study aimed to determine the value of FMPPs as a biomarker to predict neurological worsening related to early hemorrhagic transformation.

Methods—Baseline FMPP levels were measured in 186 consecutive acute ischemic stroke patients before tissue plasminogen activator treatment was administered. A serial FMPP profile (baseline before tissue plasminogen activator treatment, and 1, 2, 12, and 24 hours from treatment) was determined in a subset of 100 patients. Computed tomographic scans were performed at admission and repeated at 24 to 48 hours or after neurological worsening occurred. Symptomatic intracranial hemorrhage was defined as blood at any site in the brain associated with neurological deterioration.

Results—Patients who worsened had higher median FMPP levels compared with those who did not (59.68 [48.63–85.73] versus 44.87 [36.37–58.90] Uf/mL; P=0.035) at baseline. After logistic regression multivariate analysis, FMPP >48.2 Uf/mL together with age, hypertension, and systolic blood pressure remained baseline predictors of worsening at 48 hours. Moreover, baseline FMPP determination helped to distinguish between patients who worsened and those who did not (Integrated Discrimination Improvement index, 5.7%; P=0.0004). Finally, within patients who had worsened at 48 hours, those with symptomatic intracranial hemorrhage had higher FMPP levels (P=0.038).

Conclusions—FMPPs might be a valuable biomarker of poor early neurologic outcome and be related to the appearance of symptomatic intracranial hemorrhage in tissue plasminogen activator–treated patients, one of the most feared neurological complications after thrombolytic treatment of acute ischemic stroke. (Stroke. 2014;45:432-437.)

Key Words: biomarkers ▪ stroke
significantly to disease mechanisms. Reactive oxygen species (ROS) are highly reactive to any or all of the following molecular targets: lipids, proteins, nucleic acids, or carbohydrates, thereby modifying their chemical structure and generating oxidation-derived products. These could be markers of molecular oxidative damage and, as a consequence, contribute to disease development.8–10

Increased ROS production has been found in ischemic stroke, during both ischemia and reperfusion. However, despite being the main clinical aim of stroke treatment, reperfusion might trigger adverse effects because oxidative stress can rapidly take place on reoxygenation.6,7 The production of ROS during brain ischemia is followed by vasodilatation, eradication of endothelium-dependent responses, and endothelial cell dysfunction, which promote an increase of permeability and disruption of the blood–brain barrier.6–10

ROS-mediated lipid peroxidation produces lipid hydroperoxides, which further decompose into several lipid peroxidation products such as 2-propanal, isoprostanes, hydroxyl-2-nonenal, and aldehydes such as malondialdehyde.11 Oxidative stress also promotes the formation of age pigments by the polymerization of lipids and proteins.12 All these species are considered fluorescent molecular peroxidation products (FMPPs) and have been found to be increased in certain diseases, such as diabetes mellitus13 and multiple sclerosis.14 However, to our knowledge, they have never been tested in the acute phase of stroke.

We hypothesized that FMPPs could promote blood–brain barrier degradation, contributing to rt-PA–related HT and, therefore, being involved in early neurological deterioration after ischemic stroke and thrombotic treatment. Therefore, our main aim was to ascertain the usefulness of plasma FMPP levels, determined before thrombolysis, as a biomarker to predict early neurological deterioration related to HT.

Material and Methods

Study Population

Our target group consisted of patients who had had an acute ischemic stroke and were admitted within the first 3 hours after symptoms onset. One hundred eighty-six consecutive patients with a nonlacunar stroke involving the middle cerebral artery or the basilar artery territories were evaluated. All patients underwent urgent carotid ultrasound and transcranial Doppler examinations. All patients received rt-PA (alteplase; Actilyse; Boehringer, Germany) in a standard 0.9 mg/kg dose. Patients with a known inflammatory or malignant disease were not included.

Clinical Protocol

A detailed history of vascular risk factors was obtained from each patient. To identify potential mechanism of cerebral infarction, a set of diagnostic tests was performed. With this information, and the neuroimaging data, previously defined etiologic subgroups were determined as described previously.15 Clinical examination was performed on admission and at 12, 24, and 48 hours from symptom onset. Stroke severity was assessed by using the National Institutes of Health Stroke Scale (NIHSS) score. Neurological improvement was defined as a decrease in NIHSS score by ≥4 points and neurological deterioration as either death or an increase in score by ≥4 points at 48 hours.16 This study was approved by the ethics committee of the hospital, and all patients or relatives gave informed consent. Clinical data were collected blinded to biomarker results.

Computed Tomography

All patients underwent a baseline computed tomography within the first 3 hours of stroke onset, before any treatment was started, which was repeated after 24 to 48 hours (or earlier when rapid neurological deterioration occurred) to evaluate the presence of HT.

Computed tomography scans were reviewed by a neuroradiologist with extensive experience in acute stroke who was blinded to FMPP results. Presence and type of HT were defined according to the European Cooperative Acute Stroke Study (ECASS) criteria.17 Symptomatic intracranial hemorrhage (SICH) was defined as blood at any site in the brain on the computed tomography scan and documentation of neurological deterioration.

No patient with an initial hypodensity involving >33% of the middle cerebral artery territory received rt-PA in this study.

Assay of FMPPs

Peripheral blood samples were drawn from all patients (n=186) at study entry (before rt-PA administration). In a subset of patients (n=100), serial samples were obtained to study FMPP temporal profile at baseline (before rt-PA administration), at 1, 2, 12 hours post-rt-PA, and at 24 hours after symptom onset.

Ethylendiaminetetraacetic acid (EDTA) tubes were used for blood collection and posterior FMPP measurements, and plasma was immediately separated by centrifugation at 1500g for 15 minutes and stored at −80ºC. Determination of plasma FMPP was based on spectrofluorometric detection according to Shimasaki.18 FMPP levels were measured in duplicate and coefficient of variation was <5% in all samples. Quinine sulfate in diluted 0.1 N H2SO4 was used for standard solutions and to calculate the relative fluorescence intensities of samples (given as Uf/mL). The mean interassay coefficients of variation were <10%. Determination of FMPP was performed by personnel blinded to clinical and neuroimaging data.

Primary and Secondary End Points

Univariate and multivariate analysis, as well as Net Reclassification Improvement (NRI) and Integrated Discrimination Improvement (IDI) indexes, was performed with worsening at 48 hours as primary end point. The appearance of SICH was considered a secondary end point.

Statistical Analyses

SPSS statistical package 15.0 was used, unless contrary is stated.

Normality was assessed by Kolmogorov–Smirnov test. Normally distributed variables (P>0.05) were analyzed by Student t test or ANOVA and mean and SD values are given, whereas for non-normally distributed variables, Mann–Whitney U or Kruskal–Wallis test were used and median and interquartile range (IQR) are reported.

Temporal profile modifications were assessed by Friedman test. Wilcoxon analysis was performed among baseline, 1, 2, 12, and 24 hours. After paired comparison, P values were adjusted by Bonferroni correction. In the univariate analysis, intergroup differences were assessed by Pearson χ2 test for categorical variables. FMPP cut-off points with the optimal accuracy (both sensitivity and specificity) to predict end point were obtained from receiver-operator characteristic curves. To build predictive models, all clinical variables that were associated with outcome at P<0.05 in the univariate analysis were included in a forward stepwise multivariate logistic regression analysis. For those independent variables, adjusted odds ratio (ORadj) and 95% confidence interval (CI) were adjusted by NIHSS score at admission and age. Afterward, FMPP was added by Enter method to the clinical models. The predictive models were checked for possible interactions between FMPPs and modifying factors such as age, hypertension, and systolic blood pressure (SBP).

Using R software (Hmisc and PredictABEL packages), NRI and IDI indexes were calculated to assess the added value of FMPPs to the clinical predictive model.19,20 In NRI test, prespecified clinically relevant thresholds of predicted risk (<10% and >90%) were used to calculate reclassification of patients into risk outcome groups.

In all cases, a P value <0.05 was considered statistically significant.
Results

The mean age of the patients included was 71.52±11.9 years, and 46.2% were women. All demographic factors are indicated in Table 1. A positive correlation was identified between baseline levels of FMPP and age, SBP, and diastolic blood pressure (Table 1). No difference was found on FMPPs regarding statins or antiinflammatory treatment (ie, aspirin) before admission (data not shown).

The temporal profile of FMPPs was studied in a subset of patients after stroke (Figure 1). Plasma FMPPs decreased from baseline to 24 hours. Significant differences were found among different time points (P<0.001) and also when different times were compared by paired analysis (P<0.001).

Concerning neurological status, clinical assessment revealed that 16 (8.6%) patients worsened, 104 (55.9%) improved, and 58 (31.2%) remained stable during the first 48 hours after admission. Higher baseline levels of FMPPs were observed in patients who had neurological worsening at 48 hours (59.68 [48.63–85.73] versus 44.87 [36.37–58.90] Uf/mL; P=0.035; Figure 2). We identified a cut-off point for FMPPs at 48.2 Uf/mL for worsening at 48 hours with a sensitivity and specificity of 80% and 57.9%, respectively. Main baseline characteristics of patients who worsened at 48 hours are shown in Table 2. Hypertension and SBP >180 mmHg were related to worsening at 48 hours (P=0.045 and 0.02, respectively). After this univariate analysis, a binary logistic regression was performed adding hypertension, SBP >180 mmHg, and FMPP >48.2 Uf/mL into the regression and adjusting by age and NIHSS score at admission. Age, SBP, hypertension, and

Table 1. Plasma FMPP Levels and Demographic Factors

<table>
<thead>
<tr>
<th>Qualitative data</th>
<th>Frequency, N (%)</th>
<th>Baseline FMPP Levels by Presence of the Indicated Factor</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Female sex</strong></td>
<td>86 (46.2)</td>
<td>50.0 (36.2–74.7) vs 44.1 (37.0–55.0)</td>
<td>0.102</td>
</tr>
<tr>
<td>Hypertension</td>
<td>101 (55.2)</td>
<td>48.5 (36.9–66.5) vs 44.1 (36.3–55.6)</td>
<td>0.129</td>
</tr>
<tr>
<td>Dyslipemia</td>
<td>60 (32.8)</td>
<td>45.8 (37.3–60.0) vs 47.3 (36.3–63.0)</td>
<td>0.899</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>35 (19)</td>
<td>49.7 (39.8–70.0) vs 44.8 (36.2–58.6)</td>
<td>0.137</td>
</tr>
<tr>
<td>Previous stroke</td>
<td>26 (14.2)</td>
<td>50.6 (39.1–86.4) vs 45.0 (36.2–85.9)</td>
<td>0.150</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>73 (39.9)</td>
<td>47.7 (37.3–67.4) vs 44.7 (34.7–58.6)</td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>35 (20)</td>
<td>49.6 (42.0–58.5) vs 44.9 (36.1–63.0)</td>
<td></td>
</tr>
<tr>
<td>Proximal occlusion</td>
<td>131 (72)</td>
<td>45.0 (36.5–59.9) vs 46.7 (37.1–61.0)</td>
<td></td>
</tr>
<tr>
<td><strong>TOAST</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardioembolic</td>
<td>90 (49.2)</td>
<td>46.7 (36.1–65.4) vs ...</td>
<td>0.111</td>
</tr>
<tr>
<td>Aterothrombotic</td>
<td>47 (25.7)</td>
<td>43.2 (34.7–51.6) vs ...</td>
<td>0.319</td>
</tr>
<tr>
<td>Undetermined</td>
<td>43 (23.5)</td>
<td>44.4 (34.8–65.1) vs ...</td>
<td>0.856</td>
</tr>
<tr>
<td>Other</td>
<td>3 (1.6)</td>
<td>60.7 (59.8–62.8) vs ...</td>
<td>0.134</td>
</tr>
</tbody>
</table>

Quantitative data were compared by the Mann–Whitney U test. Quantitative data were compared with the Pearson test. DBP indicates diastolic blood pressure; FMPP, fluorescent molecular peroxidation product; NIHSS, National Institutes of Health Stroke Scale; SBP, systolic blood pressure; and TOAST, Trial of Org 10172 in Acute Stroke Treatment.

*P<0.05.

Figure 1. Temporal profile of fluorescent molecular peroxidation products (FMPPs) at baseline, 1, 2, 12, and 24 hours (P<0.001; Friedman test). After paired analysis, significant differences were found between plasma levels of FMPPs at different times (n=100). rt-PA indicates tissue plasminogen activator. **P<0.001.
FMPP >48.2 Uf/mL remained as the main baseline predictors of worsening after 48 hours from rt-PA treatment and confirmed our predictive model (Table 3). There was no interaction between FMPPs and any of these clinical variables (data not shown).

By determining plasma FMPP concentrations, we were able to increase the discrimination significantly between subjects who worsened at 48 hours and those who did not (IDI index, 5.7%; \( P=0.0004 \)). However, FMPPs did not reclassify patients into higher-risk categories (NRI index, 7.4%; \( P=0.0004 \)). Although IDI was not shown.

Among different HT subtypes, parenchymal hematoma type 2 and remote parenchymal hematoma had the highest FMPP levels, although not significantly (Figure 3A). From those patients who worsened at 48 hours, 31.25% of patients had SICH and showed statistically significant higher levels of FMPPs (98.6 [81.9–110.2] versus 51.43 [44–61.7] Uf/mL; \( P=0.038 \); Figure 3B).

**Discussion**

The present study shows, for the first time to our knowledge, that plasma FMPP levels, measured in acute stroke patients before thrombolytic therapy, predict early neurological deterioration at 48 hours.

The temporal profile of FMPPs is similar to the one previously described for malondialdehyde, advanced oxidation protein products, and myeloperoxidase by Domínguez et al.\(^5\) with a peak at baseline followed by a clear decrease along the first 24 hours. Measurement of FMPPs encompasses the determination of all these species, thereby increasing sensitivity compared with when measured alone. In fact, FMPP has been considered a nonspecific measurement of molecular oxidation because it reflects a mixture of oxidation products from lipids, proteins, carbohydrates, and DNA instead of a specific oxidation product.\(^2^1\)

In our study, we found a positive correlation between FMPPs and blood pressure parameters (SBP and diastolic blood pressure) and a trend with hypertension. These results follow the evidence of the previously published relation between oxidative stress and hypertension.\(^2^2\)

In our analysis, we have applied statistical tests IDI and NRI. IDI reflects the increase in the discrimination of true-positive and true-negative cases when a marker is added to the predictive model, whereas NRI shows the capability of a marker added to the clinical model to reclassify patients among different risk categories compared with the clinical model alone. Plasma FMPP determination was able to increase the discrimination ability of the clinical model, identifying the events (ie, worsening) better than reducing the false-positive rates in a mild, though significant, way. These might aid in the detection of patients more prone to early neurological deterioration at 48 hours and their maintenance in stroke units, where they might be better managed.

Other biomarkers such as matrix metalloproteinase 9 (MMP-9),\(^2^3,2^4\) vascular adhesion protein 1 and its semicarbazide-sensitive amino oxidase activity,\(^2^5\) and activated protein C\(^2^6\) have been found to be related to poor outcome associated with the appearance of HT.

Whether combining the information from all those markers with FMPPs might improve predictive value, as well as discriminative and reclassification capacities, is an interesting working hypothesis that needs to be addressed in future studies with larger cohorts containing a greater number of HT cases and especially more SICH, the most clinically relevant hemorrhagic complication.

We identified SICH as the cause of neurological deterioration of one third of patients who worsened at 48 hours. Results from previous in vitro and in vivo studies suggest a clear relation between ROS production and endothelial cell dysfunction and increase in blood–brain barrier permeability.\(^8,1^0\) These results might explain the relation of raised FMPPs at baseline in patients with SICH, suggesting an underlying impairment
in the redox balance leading to the endothelial function boosting this type of bleeding after rt-PA treatment. Despite several reported biomarkers associated with poor outcome in ischemic stroke,\textsuperscript{27} it is important to notice that the main cause of neurological deterioration is not always identified. To be useful, a biomarker that predicts poor outcome after stroke should be related to a specific cause of neurological worsening, allowing physicians to prescribe the most suitable treatment for each type of complication. Because HT is 1 of the main causes of poor outcome in some rt-PA–treated patients and a major concern for physicians at the time of prescribing this therapy, a rapid test that could identify patients with a subsequent risk of HT is of interest. Proper identification of these patients might aid in the implementation of new patient management approaches such as blood pressure lowering.

Our study has some limitations. The determination of FMPP plasma levels is not a specific assay to measure oxidative damage of a particular molecule, but a global marker of molecular damage. In addition, it has to be taken into account that FMPPs may not only reflect oxidation because there are other non–oxidation-related products that could also generate fluorescence. Apart from hypertension, interindividual

<table>
<thead>
<tr>
<th>Logistic regression</th>
<th>Clinical Model, OR\textsubscript{adj} (95% CI)</th>
<th>Clinical Model Plus FMPPs, OR\textsubscript{adj} (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIHSS at admission</td>
<td>1.038 (0.924–1.166); \textit{P}=0.532</td>
<td>1.020 (0.908–1.146); \textit{P}=0.74</td>
</tr>
<tr>
<td>Age\textsuperscript{*}</td>
<td>0.944 (0.897–0.994); \textit{P}=0.028</td>
<td>0.943 (0.895–0.993); \textit{P}=0.027</td>
</tr>
<tr>
<td>SBP\textsuperscript{*}</td>
<td>4.029 (1.082–14.997); \textit{P}=0.038</td>
<td>3.408 (0.861–13.429); \textit{P}=0.08</td>
</tr>
<tr>
<td>Hypertension\textsuperscript{*}</td>
<td>4.632 (1.029–20.846); \textit{P}=0.046</td>
<td>4.860 (1.010–23.375); \textit{P}=0.049</td>
</tr>
<tr>
<td>FMPPs\textsuperscript{*}</td>
<td>...</td>
<td>5.203 (1.338–20.240); \textit{P}=0.017</td>
</tr>
</tbody>
</table>

Categorical NRI

- NRI events...
- NRI nonevents...
- NRI...

\textit{P} value

- Ref

IDI statistics

- IDI events...
- IDI nonevents...
- IDI...

\textit{P} value\textsuperscript{*}

- Ref

*\textit{P}<0.05.

Table 3. Comparison Between Predictive Models for Worsening at 48 Hours

| Logistic regression models were adjusted by age and NIHSS at admission. SBP was added to both models with a cut-off point of 180 mm Hg, and FMPPs with a cut-off of 48.2 Uf/mL. NRI comprised ≤10%, 10% to 90%, and >90% as risk categories. The percentage of reclassification for events, nonevents, and the sum are indicated with 95% CI. IDI is indicated for events, nonevents, and the sum of both with 95% CI. CI indicates confidence interval; FMPPs, fluorescent molecular peroxidation products; IDI, Integrated Discrimination Improvement; NIHSS, National Institutes of Health Stroke Scale; NRI, Net Reclassification Improvement; OR\textsubscript{adj}, adjusted odds ratio; and SBP, systolic blood pressure.

*\textit{P}<0.05.

Figure 3. FMPP levels in plasma among main subtypes of HT patterns. In our cohort, HT was present in 56 (30.4%) patients (19 [10.3%] HI-1, 13 [7.1%] HI-2, 14 [7.6%] PH-1, 8 [4.3%] PH-2, 2 [1.1%] PH-R; \textit{P}=0.046). A. Plasma levels of FMPPs in patients who worsened at 48 h and had a SICH. SICH was identified in 6 cases, 5 of which worsened at 48 h (B). FMPPs indicates fluorescent molecular peroxidation products; HI-1, hemorrhagic infarct type 1; HI-2, hemorrhagic infarcts type 2; HT, hemorrhagic transformation; PH-1, parenchymal hematoma type 1; PH-2, parenchymal hematoma type 2; PH-R, remote parenchymal hematoma; and SICH, symptomatic intracerebral hemorrhage. *\textit{P}<0.05.
differences in FMPP plasma levels might be also influenced by smoke habits and diabetes mellitus, which have been previously related to oxidative stress increase,28 although we did not find these associations in our cohort. Finally, comparing with other published reports, there were a small number of patients with early neurological deterioration in our study,29,30 as well as patients with SICH after thrombolysis31; therefore, the results of this study should be interpreted with caution.

In conclusion, we report FMPPs as a valuable biomarker of poor early neurological outcome related to the appearance of SICH, one of the main causes of neurological impairment after thrombolysis. The measurement of this easy-to-assay biomarker in acute stroke settings might improve the safety profile of thrombolytic agents for stroke treatment and widen the use of this treatment in those patients at lower risk.

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Disclosures
None.

References
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