Oxidative Stress and Matrix Metalloproteinase-9 in Acute Ischemic Stroke

The Biomarker Evaluation for Antioxidant Therapies in Stroke (BEAT-Stroke) Study

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Background and Purpose—Experimental stroke studies indicate that oxidative stress is a major contributing factor to ischemic cerebral injury. Oxidative stress is also implicated in activation of matrix metalloproteinases (MMPs) and blood-brain barrier injury after ischemia-reperfusion. Plasma biomarkers of oxidative stress may have utility as early indicators of efficacy in Phase 2 trials of antioxidant therapies in human stroke. To date, a valid biomarker has been unavailable. We measured F2-isoprostanes (F2IPs), free-radical induced products of neuronal arachadonic acid peroxidation, in acute ischemic stroke. We aimed to determine the change in plasma F2IP levels over time and relationship with plasma MMP-9 in tPA-treated and tPA-untreated stroke patients.

Methods—We performed a case–control study of consecutive ischemic stroke patients (25 tPA-treated and 27 tPA-untreated) presenting within 8 hours of stroke onset. Controls were individuals without prior stroke from a primary care clinic network serving the source population from which cases were derived. Infarct volume was determined on acute diffusion-weighted MRI (DWI) performed within 48 hours using a semi-automated computerized segmentation algorithm. Phlebotomy was performed at <8 hours, 24 hours, 2 to 5 days, and 4 to 6 weeks. F2IPs were measured by gas chromatography/mass spectrometry and MMP-9 by ELISA. Prestroke antioxidant dietary intake was measured by the 24-hour recall method.

Results—In 52 cases and 27 controls, early (median 6 hours postonset) F2IPs were elevated in stroke cases compared with controls (medians 0.041 versus 0.0295 pg/mL, P = 0.012). No difference in F2IPs was present at later time points. Early plasma F2IPs correlated with MMP-9 in all patients (P = 0.01) and the tPA-treated subgroup (P = 0.02). No correlation was found with NIHSS, DWI infarct volume, 90-day Rankin score, or C-reactive protein (P > 0.05 for all).

Conclusions—In early human stroke we found evidence of increased oxidative stress and a relationship with MMP-9 expression, supporting findings from experimental studies. (Stroke. 2008;39:100-104.)

Key Words: cerebrovascular disorders • metalloproteinase • oxidative stress

Data from cell culture studies and animal stroke models indicate that free-radical induced oxidative stress is of key importance in the pathogenesis of ischemic brain injury.1,2 Despite these data, the study of oxidative stress in human stroke has been limited by the unavailability of a valid biomarker which may be detected in plasma or urine.3 The Stroke Therapy Academic Industry Roundtable (STAIR) collaborators have emphasized the potential importance of biomarkers which may act as early indicators of therapeutic efficacy in Phase 2 studies.4 Although Phase 3 clinical trials are inconclusive, antioxidant compounds such as NXY-059 have shown promise as neuroprotective agents in animal models and clinical studies.5 A valid biomarker of oxidant stress in acute stroke may have potential to improve selection of patients most likely to benefit from acute antioxidant therapies in Phase 3 trials.

Plasma levels of malondialdehydes, thiobarbituric acid reactive (TBAR) substances, and lipid hydroperoxides have been used as markers of oxidant stress–induced cerebral lipid peroxidation after ischemic brain injury.6–9 However these...
markers lack specificity, precision, and reproducibility for detection of oxidative stress in vivo. In contrast, F2-isoprostanes (F2IPs) are prostaglandin-like products of non-cyclooxygenase free radical–induced peroxidation of arachidonic acid.\textsuperscript{10} Detected in plasma and urine, they are stable, sensitive, and specific markers of oxidative stress–induced lipid peroxidation when directly compared with other markers in rat models of oxidative injury.\textsuperscript{11} Despite increasing recognition as the best available index of oxidant stress in vivo,\textsuperscript{10,11} few data exist on F2IP metabolism in human stroke.

Experimental data indicate that oxidative stress may be an early trigger of matrix metalloproteinase (MMP) upregulation after cerebral ischemia-reperfusion.\textsuperscript{12–14} MMPs, particularly MMP-9, are important mediators of microvascular blood-brain barrier injury and hemorrhagic transformation (HT) after ischemic stroke.\textsuperscript{15,16} We investigated plasma F2IP metabolism in acute ischemic stroke. We aimed to establish the temporal change in plasma concentrations, relationship with key clinical parameters, and relationship with plasma MMP-9 in tPA-treated and tPA-untreated patients.

### Methods

#### Subjects
Cases were consecutive patients admitted to a single hospital with neuroimaging-proven acute ischemic stroke within 8 hours of symptom onset. Exclusion criteria were: (1) Primary intracerebral hemorrhage, post-seizure neurological deficit, brain abscess, or tumor; (2) clinical or laboratory findings consistent with acute infection (pneumonia, urinary tract, other site); (3) myocardial infarction, surgery, trauma in previous 30 days; (4) absent neuroimaging; (5) specified comorbidities (end-stage renal/hepatic disease, metastatic malignancy). Controls were age- and sex-comparable subjects, included if they attended a large primary care network serving the population from which cases were derived. Control exclusion criteria were identical to those for cases, but potential controls were also excluded if there was clinical, ultrasound, or radiographic evidence of previous stroke, transient ischemic attack, or carotid stenosis.

National Institutes of Health Stroke Scale (NIHSS) was measured in cases within 8 hours of onset. 90-day outcome was measured by telephone follow-up with patients or family members using the modified Rankin Score. The study was approved by our Institutional Review Board, and all subjects (or approved proxy) signed an informed consent form.

#### Neuroimaging
Stroke protocol MRI (including diffusion weighted imaging [DWI]) was performed on a 1.5T scanner within 48 hours. DWI infarct volume was calculated using a semi-automated segmentation algorithm (ALICE, Hayden Software) with manual editing to conform to anatomic boundaries. Infarct volume was calculated as the sum of (area of axial DWI hyperintensity×slice thickness).

#### Laboratory Methods
Venous blood was collected in EDTA tubes using a butterfly to minimize shear stress at <8 hours, 24 hours, 2 to 5 days, 4 to 8 weeks (convalescent phase). Tubes were gently inverted twice to ensure mixing, placed on ice, and centrifuged within 60 minutes. Plasma for F2IP quantification was immediately frozen at −80°C for later measurement by gas chromatography/mass spectrometry. Total plasma MMP-9 (active pluszymogen) was measured by ELISA (R&D Systems). As a marker of acute inflammatory response, the initial white blood cell count (within 8 hours) was recorded, and plasma CRP was quantified by immunonephelometry.

### Table 1. Clinical Characteristics of Subjects

<table>
<thead>
<tr>
<th></th>
<th>Cases With Stroke (n=52)</th>
<th>Controls (n=27)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD)</td>
<td>70.1 (14.6)</td>
<td>68.2 (9.9)</td>
<td>ns</td>
</tr>
<tr>
<td>Gender, % female</td>
<td>53.4</td>
<td>40.7</td>
<td>ns</td>
</tr>
<tr>
<td>Current Smoking, % (n)</td>
<td>9.6 (5)</td>
<td>0 (0)</td>
<td>NA</td>
</tr>
<tr>
<td>Hypertension, % (n)</td>
<td>57.6 (30)</td>
<td>40.7 (11)</td>
<td>ns</td>
</tr>
<tr>
<td>Diabetes mellitus, % (n)</td>
<td>23 (12)</td>
<td>3.7 (1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Atrial Fibrillation, % (n)</td>
<td>36.5 (19)</td>
<td>25.9 (7)</td>
<td>ns</td>
</tr>
<tr>
<td>Hyperlipidemia, % (n)</td>
<td>36.5 (19)</td>
<td>40.7 (11)</td>
<td>ns</td>
</tr>
<tr>
<td>Previous Stroke, % (n)</td>
<td>11.5 (6)</td>
<td>0 (0)</td>
<td>NA</td>
</tr>
<tr>
<td>Acute (&lt;48 hours) DWI</td>
<td>84%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Stroke subtype (TOAST), % (n)</td>
<td>11.5 (6)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Large artery</td>
<td>1.9 (1)</td>
<td></td>
<td></td>
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<tr>
<td>Small artery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardioembolism</td>
<td>59.6 (31)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other determined cause</td>
<td>7.7 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undetermined cause</td>
<td>19.2 (10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interval between stroke onset and first phlebotomy (hours), mean (SD)</td>
<td>6.2 (2.85)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>MRI infarct volume (cc), mean (SD)</td>
<td>38.84 (64.4)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>NIHSS mean (range, SD)</td>
<td>13 (2–29, 6.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 month Rankin, mean (SD)</td>
<td>2.9 (1.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tPA treated, % (n)</td>
<td>49 (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin C intake (median, 25–75% IQR), mg/d*</td>
<td>89.4 (24.6–172)</td>
<td>84.7 (52.3–161.3)</td>
<td>ns</td>
</tr>
<tr>
<td>Vitamin E intake (median, 25–75% IQR), mg/d*</td>
<td>6.59 (2.0–31.6)</td>
<td>7.85 (5.83–22.3)</td>
<td></td>
</tr>
</tbody>
</table>

\*n=24. NA indicates not applicable; NS, not significant (P≥0.05).
Comparisons of case and control F2IPs (corrected significance where a Bonferroni correction was applied to correct for multiple analyses) were performed on SAS software. A probability value of 0.05 (2-tailed) was taken to indicate statistical significance, except for the correlation coefficient, which was calculated using Pearson's test as appropriate. The linear association between continuous variables was determined by Pearson correlation coefficient, following log-transformation of variables which were not normally distributed. Differences in F2IPs over time were compared by repeated measures analysis of variance. Receiver operating characteristic (ROC) analysis was performed to determine the ability of different F2IP thresholds to discriminate between case and control groups. All analyses were performed on SAS software. A probability value of <0.05 (2-tailed) was taken to indicate statistical significance, except where a Bonferroni correction was applied to correct for multiple comparisons of case and control F2IPs (corrected significance threshold <0.0125).

Dietary Intake
Dietary intake of antioxidants and other nutrients in the 24 hours before each blood sample was determined by the validated 24-hour recall method administered by a trained nutritionist.17

Statistical Analysis
Categorical variables were compared using the $\chi^2$ test and continuous variables using the t test or Wilcoxon rank-sum (Mann–Whitney) test as appropriate. The linear association between continuous variables was determined by Pearson correlation coefficient, following log-transformation of variables which were not normally distributed. Differences in F2IPs over time were compared by repeated measures analysis of variance. Receiver operating characteristic (ROC) analysis was performed to determine the ability of different F2IP thresholds to discriminate between case and control groups. All analyses were performed on SAS software. A probability value of <0.05 (2-tailed) was taken to indicate statistical significance, except where a Bonferroni correction was applied to correct for multiple comparisons of case and control F2IPs (corrected significance threshold <0.0125).

Results
Clinical Characteristics
52 ischemic stroke cases and 27 controls were included (Table 1). No difference in age or gender was present between groups. Five (9.6%) patients with stroke were current smokers, compared with no current smokers among controls. All cases had brain CT within 8 hours of symptom onset, with stroke protocol MRI (including DWI) performed on 84% within 48 hours. 25 cases (48%) were treated with thrombolytic therapy, all of whom had initial phlebotomy of 6.2 hours. Plasma F2IPs in cases were elevated in the acute (median 6 hours) phase compared with controls ($P=0.012$); Figure 1, Table 2). No case–control difference was found at other acute time points (24 and 72 hours) or in the convalescent phase (median 36 days). No difference in plasma F2IPs was found in the acute phase compared with later time-points by repeated measures ANOVA (Figure 1).

On receiver-operating characteristic (ROC) analysis of acute plasma F2IPs, the area under the ROC curve (AUC) was 0.68 (SE 0.062, 95% CI 0.55 to 0.8; Figure 2). A threshold of at least 0.34pg/mL was associated with the best combination of sensitivity (66%) and specificity (69.2%) to discriminate cases from controls, with 67% of acute stroke cases correctly classified. A threshold of 0.43pg/mL was highly specific (88.5%) with fair sensitivity (46%) to discriminate between stroke and control groups.

Plasma F2IPs and Dietary Antioxidant Intake
Stroke cases and controls were similar for intake of ascorbic acid and $\alpha$-tocopherol in the 24 hours before initial F2IP measurement (Table 1). Regular vitamin C supplement use was more common among cases than controls (36% versus 15%, $P=0.05$), but Vitamin E supplement use was similar between groups (Table 1). No correlation was present between acute plasma F2IPs and dietary ascorbic acid ($r=0.23$, $P=0.05$), but Vitamin E supplement use was similar between groups (Table 1). No correlation was present between acute plasma F2IPs and dietary ascorbic acid ($r=0.24$, $P=0.34$) or $\alpha$-tocopherol ($r=0.04$, $P=0.8$) intake in the 24 hours before sampling, indicating that early elevation of plasma F2IPs was not attributable to lower intake of dietary antioxidants among stroke patients.

Plasma F2IPs, Inflammation, and MMP-9
Acute F2IPs correlated with plasma MMP-9 in all stroke patients ($r=0.41$, $P=0.01$), and the tPA-treated subgroup ($r=0.5$, $P=0.02$) but not in those who were tPA-untreated ($r=0.23$, $P=0.4$; Figure 3). In contrast, no relationship was found between plasma F2IPs and the acute poststroke inflammatory response, measured by CRP ($r=0.11$, $P=0.5$) and initial WBC ($r=0.2$, $P=0.22$).

Plasma F2IPs, Stroke Severity, Risk Factors, and Outcome
No relationship was found between acute plasma F2IPs and current smoking ($P=0.4$), other vascular risk factors ($P>0.2$ for all), stroke severity ($r=0.1$, $P=0.6$), MRI infarct volume ($r=0.1$, $P=0.6$), or 3-month Rankin score ($r=0.3$, $P=0.1$).

Table 2. Plasma F2IPs and MMP-9 in Subjects Across Time Points, According to tPA Status

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Non-tPA T1 (Median 6 Hours)</th>
<th>tPA T1 (Median 6 Hours)</th>
<th>Non-tPA T2 (Median 25 Hours)</th>
<th>tPA T2 (Median 25 Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2IPs, pg/mL</td>
<td>0.03 (0.023–0.037)</td>
<td>0.042 (0.026–0.072)</td>
<td>0.04 (0.029–0.049)</td>
<td>0.031 (0.025–0.056)</td>
<td>0.03 (0.023–0.053)</td>
</tr>
<tr>
<td>MMP-9, ng/mL</td>
<td>27 (25–30)</td>
<td>29 (16.4–36.3)</td>
<td>46 (29–86)</td>
<td>26 (18–35)</td>
<td>32 (21–38.5)</td>
</tr>
</tbody>
</table>

(Continued)
dietary antioxidant intake was usually not measured, it has limitations of these markers for in vivo measurement of oxidative stress. Some clinical studies have found reduced circulating antioxidant markers after stroke but, because no correlation was found with measures of these potential confounders. Further, the similar dietary antioxidant intake in the 24 hours before initial sampling in cases and controls suggests that it is unlikely that higher early F2IPs reflects diminished antioxidant capacity and greater prestroke oxidative stress among cases.

Animal stroke studies of antioxidant agents and transgenic mice under- and overexpressing the protective antioxidant enzyme superoxide dismutase (SOD) indicate that increased generation of reactive oxygen and nitrogen molecules has an important role in the pathogenesis of ischemic brain injury. A few studies of human stroke have found elevation of plasma indices of lipid peroxidation such as malondialdehydes, TBAR substances, and lipid hydroperoxides, but the interpretation of these findings has been unclear because of limitations of these markers for in vivo measurement of oxidative stress. Some clinical studies have found reduced circulating antioxidant markers after stroke but, because dietary antioxidant intake was usually not measured, it has been unclear whether these findings reflect poorer antioxidant intake in stroke patients, depletion of antioxidant capacity because of oxidative stress, or both.

In contrast, F2IPs are produced by free radical peroxidation of arachidonic acid which is abundant in cerebral tissue. Plasma F2IPs provide a specific, precise, and highly-reproducible index of in vivo oxidative stress when measured by mass spectrometry. One prior study found elevated plasma F2IPs at 2 to 5 days after stroke, but interpretation of these findings is unclear. It is possible that the differences relate to differences in timing of sampling, differences in pre- or poststroke antioxidant intake, or inherent variability related to small sample size (52 in our study, 15 in the earlier study). Strengths of our study are that sampling was performed in the early hours after cerebral ischemia when oxidative injury is likely to be at its peak, serial measures were performed to examine poststroke oxidative stress across acute and convalescent phases, and prestroke dietary antioxidant intake was measured to control for its potential confounding effect. Mass spectrometry, the most accurate available method for F2IPs quantification, was used which avoided problems with specificity and precision associated with newer immune-based assays.

We found that acute plasma F2IPs were correlated with plasma MMP-9 in tPA-treated ischemic stroke patients. To our knowledge, this is the first reported association of oxidative stress and MMP metabolism in human stroke. This finding supports experimental stroke studies indicating that oxidative stress may be an early stimulus for MMP activation, blood-brain barrier injury, and HT. Data from clinical trials are conflicting, however. Although the SAINT-1 trial found lower rates of HT in patients treated with combined tPA and NXY-059, early reports indicate no difference in symptomatic intracerebral hemorrhage among subjects receiving combination therapy in SAINT-2. Recent studies indicate that protease activity of exogenous tPA may directly promote MMP activation via proteolytic cleavage of the inactive proform. Although we did not directly measure reperfusion, one interpretation of the F2IP/MMP-9 association in the tPA-treated group is that it may have comprised a higher proportion of patients with reperfusion, thus indirectly augmenting MMP-9 activation via a second mechanism mediated by oxidative stress. A third possibility also exists, that both MMP-9 and oxidative stress were increased inde-

**Table 2. Continued**

<table>
<thead>
<tr>
<th>T3 (Median 73 Hours)</th>
<th>T4 (Median 36 Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-tPA</strong></td>
<td><strong>tPA</strong></td>
</tr>
<tr>
<td>0.036 (0.016–0.063)</td>
<td>0.027 (0.021–0.045)</td>
</tr>
<tr>
<td>25 (19.5–33)</td>
<td>20 (17–28)</td>
</tr>
</tbody>
</table>

**Figure 2.** Receiver-operating characteristic curve for acute (T1) F2IPs in cases and controls. Area under curve = 0.68 (SE 0.062, 95% CI 0.55 to 0.8). Plasma F2IP level of 0.34pg/mL (upper right arrow) provided the best combination of sensitivity (66%) and specificity (69.2%) to discriminate cases (67% of cases correctly classified). Plasma F2IP level of 0.43pg/mL (lower left arrow) provided high specificity (88.5%) and fair sensitivity (46%) to discriminate cases.

**Figure 3.** Correlation between acute F2IPs and acute MMP-9 in tPA-treated stroke. All points refer to initial samples (T1, median 6 hours). (Pearson r = 0.5, P = 0.02, F2IP units pg/mL, MMP-9 units ng/mL).
pendently of each other in tPA-treated patients, resulting in the observed association. To confirm their validity and specificity for stroke, we caution that our findings require verification in further studies, including other control groups with other acute conditions. Other limitations include the small size of the tPA-treated subgroup and insufficient statistical power to examine the relationship between F2IPs and HT.

The STAIR Collaborators have emphasized the need for development of valid surrogate end points for Phase IIb studies of acute stroke therapies. They have specified that such surrogates should be easily-measured, sensitive to change, reproducible, and resistant to observer bias. Ideally, they should also reflect the biological activity of the drug under study, thus providing “proof of concept” data in humans, refining optimal dose selection, and improving identification of the target stroke population likely to benefit most from the drug.

When judged against these STAIR criteria, plasma F2IPs are a promising potential surrogate end point in Phase IIb efficacy studies of antioxidant stroke treatments. To be validated, they must first be rigorously evaluated in patients similar to those in whom antioxidant neuroprotective agents are intended for use. Such validity studies should determine their relationship to clinical outcome and stroke severity in tPA-treated and untreated patients, and should include measures in the first 6 hours, the likely window of opportunity for neuroprotective therapeutic benefit. Our study provides an initial detailed evaluation of these parameters in human stroke. Further studies are required to verify our findings, to examine the change in F2IPs caused by experimental antioxidant agents, and to determine whether a reduction in plasma levels in response to antioxidant therapy is predictive of improved clinical outcome.

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