Increased Brain Expression of Matrix Metalloproteinase-9 After Ischemic and Hemorrhagic Human Stroke

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Background and Purpose—Abnormal expression of some matrix metalloproteinases (MMP) has shown to play a deleterious role in brain injury in experimental models of cerebral ischemia. We aimed to investigate MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in brain parenchyma in both ischemic and hemorrhagic strokes.

Methods—Postmortem fresh brain tissue from 6 ischemic and 8 hemorrhagic stroke patients was obtained within the first 6 hours after death. Finally, 78 brain tissue samples from different areas (infarct, peri-infarct, perihematoma and contralateral hemisphere) were studied. To quantify gelatinase content we performed gelatin zymograms that were confirmed by Western Blot Analysis, immunohistochemistry to localize MMP source, and in situ zymography to detect gelatinase activity.

Results—Among ischemic cases, gelatin zymography showed increased MMP-9 content in infarct core although peri-infarct tissue presented also higher levels than contralateral hemisphere (P<0.0001 and P=0.042, respectively). Within infarct core, MMP-9 was mainly located around blood vessels, associated to neutrophil infiltration and activated microglial cells. In peri-infarct areas the major source of MMP-9 were microglial cells. Tissue around intracranial hemorrhage also displayed higher MMP-9 levels than contralateral hemisphere (P=0.008) in close relationship with glial cells. MMP-2 was constitutively expressed and remained invariable in different brain areas.

Conclusions—Our results demonstrate in situ higher levels of MMP-9 in human brain tissue after ischemic and hemorrhagic stroke, suggesting a contribution of MMP-9 to ischemic brain injury and perihematoma edema. (Stroke. 2006;37:1399-1406.)

Key Words: MMP-2 • MMP-9 • stroke

Stroke leads within minutes to brain damage and cellular death as a result of an ischemic or hemorrhagic event. Ischemic stroke may also involve a hypoperfused peri-infarct area where cells are still viable although a cascade of apoptotic mechanisms has been activated. Less knowledge exists regarding molecular mechanisms underlying brain hematoma formation and secondary injury after the initial bleeding.

Because most of the stroke research has been conducted on animal models, anatomicopathologic studies evaluating in situ ischemic and hemorrhagic molecular mechanisms in humans are scarce. These types of studies are relevant to show coincident results among species in order to target pathways to improve stroke therapy. This is the case of matrix metalloproteinases (MMP), a family of zinc-dependent proteases involved in the degradation of basal lamina and extracellular matrix components. In fact, important differences have been reported by others regarding MMP-2 and MMP-9 expression related to brain injury between rodents and nonhuman primates.1-3 An abnormal expression of some MMPs, such as MMP-2 (gelatinase A) and MMP-9 (gelatinase B), appears after cerebral ischemia and contributes to infarct extent and blood-brain barrier breakdown.3,6 Animal models of cerebral ischemia have reported that MMPs are produced after ischemia by different cell types, describing a differentially time-dependent expression of these 2 gelatinases.7-9

Regarding humans, investigations with peripheral blood samples have shown high MMP-9 levels in patients with ischemic stroke10 and intracranial hemorrhage.11 Moreover and more importantly, MMP-9 levels are related to poor neurological outcome, suggesting a deleterious role in human brain injury. Only 2 reports have studied MMPs in human brain after stroke12,13 including several-day-old strokes. Those interesting reports showed gelatinases over-expression but did not distinguish among different areas within the infarcted hemisphere. Moreover, MMPs have never been studied in human brain after hemorrhagic stroke.

Received January 27, 2006; final revision received March 21, 2006; accepted March 27, 2006.

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Stroke is available at http://www.strokeaha.org DOI: 10.1161/01.STR.0000223001.06264.af
Therefore, we have studied both MMP-2 and MMP-9 content in brains of human ischemic and hemorrhagic stroke patients by means of gelatin zymography and immunoblotting, and in order to localize gelatinase expression both in situ zymography and immunohistochemistry studies were performed.

**Materials and Methods**

**Brain Tissue Samples**

Several samples from 14 patients who had a stroke within the previous 5 days were included in the study: 8 of them were hemorrhagic and 6 ischemic (see Table). On autopsy and during macroscopic examination, morphological features and last available neuroimages were used to guide brain tissue sampling from ischemic ipsilateral hemisphere (“infarcts”, “peri-infarcts”) or from contralateral hemisphere (“contralaterals”). Infarcted area was delimited by an experienced neuropathologist (mainly through the consistence and color of the parenchyma) and 1 cm³ of the contiguous tissue was obtained as peri-infarct. Afterward, histopathological microscopic examination confirmed that we were not in front of an infarcted-necrotic area. Hence, peri-infarct tissue was defined as noninfarcted tissue situated in the infarct boundary and suspected to experience metabolic changes, although we cannot ensure whether it corresponds to ischemic penumbra. One single sample from an hemorrhagic transformation was collected in case 1. In hemorrhagic strokes blood mass was removed, and 1 cm³ of adjacent parenchyma was obtained as “perihematomas” together with contralateral tissue. Sampling was done within the first 6 hours after death to avoid tissue degradation. Finally, 32 different samples were obtained from hemorrhagic cases (16 perihematomas and 16 contralaterals) and 46 from ischemic cases (18 infarcts, 13 peri-infarcts and 15 contralaterals). Brain tissue samples from 2 patients, age-matched with stroke patients, that died because of a noninflammatory pathology (nontumoral, noninfectious and nonautoimmune) were also used as “controls.”

All brain parenchyma was snap-frozen in liquid nitrogen and stored at −80°C. This study was approved by the Ethics Committee of the hospital, and informed consent was acquired from relatives before the autopsy. Homogenates were prepared for gelatin zymography and Western Blot Analysis with lysis buffer containing protease inhibitors as previously described and protein content of supernatants determined by bicinchoninic acid (BCA) assay. Standard Hematoxylin-Eosin staining was used to identify morphological brain damage.

**Gelatin Zymography**

Substrate-specific zymography for determination of gelatinolytic activity of MMP-2 and MMP-9 was performed as others in SDS-PAGE gels (10%) containing 0.1% gelatin. Prestained molecular weight standard (Bio-Rad) and 2 µL of human gelatinase control (Chemicon) were also run with samples (20 µg). Enzymatic bands were visualized after staining for 1 hour with Amido Black 0.1%, distained and enzymatic activities ensured by incubating identical zymograms with the addition of 20 mmol/L EDTA. To measure gelatinase activities, gels were read using Gel Logic 440 Imaging System (Kodak) and the intensity of the bands (arbitrary units) was normalized to human gelatinase control (pro-MMP-9 band) to allow comparisons between gels (1D Image Analysis Software; Kodak).

**Immunoblotting**

To verify zymograms findings, MMP-9 expression was detected by Western Blot Analysis. Briefly, 20 µg of total protein were loaded in SDS-PAGE (10%) and transferred to a polyvinylidene difluoride (PVDF) membrane. Nonspecific bindings were blocked, and membranes were incubated for 2 hours with mouse antihuman MMP-9

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**Studied Patients and Case Material**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age / Sex</th>
<th>Time (hours) stroke-exitus</th>
<th>Time (hours) exitus-autopsy</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>60 / male</td>
<td>108</td>
<td>4</td>
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<tr>
<td>2</td>
<td>80 / female</td>
<td>88</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>78 / female</td>
<td>77</td>
<td>4</td>
</tr>
<tr>
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<td>59 / male</td>
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<tr>
<td>5</td>
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</tr>
<tr>
<td>6</td>
<td>72 / male</td>
<td>37</td>
<td>3</td>
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<td>5</td>
</tr>
<tr>
<td>8</td>
<td>68 / male</td>
<td>18</td>
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<td>9</td>
<td>88 / male</td>
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<td>10</td>
<td>77 / male</td>
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<td>11</td>
<td>92 / male</td>
<td>12</td>
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<td>12</td>
<td>84 / male</td>
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<tr>
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<td>64/female</td>
<td>-</td>
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</tr>
<tr>
<td>C2</td>
<td>82/male</td>
<td>-</td>
<td>6</td>
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</table>

IC indicates infarcted core; PI, peri-infarct; PH, perihematoma; C, contralateral.
Immunohistochemistry
To localize MMP-9, tissues were embedded in optimal cutting temperature (OCT) compound and 12 µm sections placed on precoated poly-lysine slides. Sections were fixed in cold acetone and rehydrated with Tris Buffered Saline (TBS) for 5 minutes before normal goat serum applied 1:10 for 30 minutes. Incubation with primary antibody, mouse antihuman MMP-9 1:50 (Chemicon, Temecula, Calif), was carried out for 2 hours. Secondary antibody (goat antimouse biotin conjugate, 1:500; Calbiochem; San Diego, Calif) was applied during 1 hour. Afterward, endogenous peroxidase activity was blocked with 3% H2O2 for 30 minutes and subsequent incubation with streptavidin-HRP (1:1000) performed for 1 hour. Immunoreactive sites were developed with diaminobenzidine (DAB) solution and sections counterstained with Mayer’s Hematoxylin, dehydrated and mounted in DPX. As negative controls, primary antibody omission was performed in some criosections.

In Situ Zymography
In situ zymography was carried out on criosections as previously described7 including some sections incubated with a metalloproteinase inhibitor (10 nM/ml of 1,10-phenanthroline) as controls. Reaction products were examined with fluorescence microscope (Zeiss).

Statistical Analyses
Statistical analyses were performed by use of the SPSS 12.0 package. Mean values and standard error of mean (SEM) were calculated for all studied areas, and statistical significance for intergroup was assessed by Student’s t test or ANOVA followed by Bonferroni tests for intergroup comparisons. The Spearman coefficient was used to study correlations between continuous variables. P<0.05 was considered statistically significant.

Results
Clinical Features and Histopathological Findings
Our study group presented typical characteristics of stroke patients: 10 men (71%), mean age 76.5, 3 patients had hypertension (85%), 1 diabetes (7.1%), 1 dyslipidemia (7.1%), 3 atrial fibrillation (21.4%) and 3 patients had a previous stroke (21.4%). Stroke severity was assessed using the National Institutes of Health Stroke Scale (NIHSS) and baseline median value was 21.12–23 Noninfectious, inflammatory or malignant diseases were known. All patients received standard treatment except case 3 that was treated with tissue plasminogen activator (t-PA). Cause of death of all patients was brain herniation that was related to hemorrhagic transformation in cases 1 and 3.

Microscopic appearance of the ischemic infarcts showed clear evidence of acute ischemic damage. In gray matter shrunken cell bodies, neuronal eosinophilia, pyknosis and nucleioli were lost, and these findings were associated with widespread vacuolation of the adjacent neuropil. In some areas cellularity was sparse, necrotic neurons appeared as eosinophilic ghost cells and neutrophilic infiltration began specifically in those infarcts with an antemortem interval of 2 to 5 days together with early microglial activation. In subcortical white matter, degenerating oligodendrocytes appearance was the most important finding. Peri-infarct zones and brain parenchyma around hematomas did not show any significant morphological change.

Gelatinase Expression
Zymograms showed a great variability on MMP-9 band intensities (both pro- and cleaved forms), whereas no alteration on MMP-2 (both pro- and cleaved forms) was detected and a third gelatinase band around 145 kDa appeared strongly related with pro-MMP-9 variations (at 95 kDa) in all samples (Figure 1A and 1B). Among samples from ischemic strokes, pro-MMP-9 was found highly expressed in infarcted tissue as compared with the peri-infarct (P=0.009) and contralateral hemisphere (P<0.0001). Moreover, peri-infarct samples also showed higher pro-MMP-9 levels than contralateral hemisphere (P=0.042; Figure 1C). MMP-9 intensity was maximal in the hemorrhagic transformation sample as Figure 2 shows. A band corresponding to a cleaved MMP-9 form appeared in all infarcted samples. The mean intensity value obtained in the infarcted core of the t-PA–treated patient was 1.1, whereas mean value of all studied samples was 1.4. Regarding hemorrhagic strokes, MMP-9 increased levels in perihematoma tissue were detected as compared with the contralateral hemisphere (P=0.002) as shown in Figure 1D. No differences were found between pro-MMP-2 levels neither in ischemic nor in hemorrhagic strokes (Figure 1C and 1D).

A correlation study between pro-MMP-9 and the upper band (at 145 kDa) demonstrated a strong relation between these 2 gelatinase forms (r=0.912; P<0.0001; Figure 3A). Western Blot Analysis confirmed MMP-9 findings identified in zymograms. Moreover, the upper band was positive for MMP-9 (Figure 3B).

MMP-9 Tissue Localization
Immunohistochemistry showed different expression patterns depending on the studied region. In samples from infarcted cores, MMP-9 was mainly located around blood vessels (endothelial cell/periendothelial layer) together with the presence of perivascular immunoreactive neutrophils (Figure 4A through 4C). A reduced cellular localization was found within the necrotic parenchyma probably attributable to the presence of disintegrated cell bodies and sparse cellularity. In that area, major MMP-9 positive cells were activated microglia (Figure 4D). On peri-infarct samples, MMP-9 immunoreactivity was predominantly located in several parenchymal cell types such as activated microglial cells and neutrophils, whereas the abundance and intensity of positive vascular structures decreased in comparison to the infarcted tissue. In all ischemic samples, isolated neuronal MMP-9 reactivity was detected together with some immunoreactive macrophages (Figure 4E and 4F). On hemorrhagic stroke sections, perihematoma tissue presented intense MMP-9 immunoreactivity from glial cells, and no positive vessels were detected both in contralateral and control samples. Moreover, a diminished cellular immunoreactivity appeared in those locations (Figure 4G through 4I).

In Situ Gelatinase Activity
In situ zymography showed abundant gelatinase activity (green fluorescence) around blood vessels and at cellular level
Figure 1. A and B, Zymograms corresponding to 3 representative ischemic and 1 hemorrhagic patients. Note that infract areas (and HT one) presented the MMP-9 cleaved form. C and D, Mean values and standard error of mean (SEM) of pro-MMP-9 (in black) and pro-MMP-2 (in gray) of each studied area in all ischemic and all hemorrhagic strokes are represented in bar graphs. Normalized band intensity was calculated as a ratio to human gelatinase control (pro-MMP-9 band). *P<0.05. PH indicates perihematoma; C, contralateral; IC, infarcted core; PI, peri-infarct; HT, Hemorrhagic Transformation; C1/C2, control cases.
in infarcted and peri-infarcted tissue, whereas a weak signal was detected in contralateral samples (Figure 5A through 5C). In hemorrhagic strokes perihematoma sections presented again increased fluorescence as compared with contralateral sections or control samples (Figure 5D through 5F). Samples counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue signal) revealed the presence of DNA of cell bodies, and gelatinase activity from cells (Figure 5G) could be identified where a merge signal appeared (not shown). Incubation with an MMP inhibitor 10 mmol/L of 1,10-phenanthroline largely abolished fluorescence signal, confirming that most gelatinase activity was attributable to MMPs (Figure 5H and 5I).

Discussion

This study demonstrates an in situ brain expression and activity of MMP-9 after human stroke. This over-expression is differentially induced in a cell- and region-dependent manner and in close relation to brain injury. However, MMP-2 remained invariable in our study time period.

Animal models of cerebral ischemia have previously demonstrated that MMP-9 is early upregulated in injured tissue suggesting a detrimental role and its involvement in neuronal death and brain damage. Moreover, MMP-9 inhibition through knockout models or drug treatments reduce infarct volume. A second wave of expression of MMP-2 has been reported from the fourth day after ischemia by infiltrating inflammatory cells such as microglia and macrophages. Similarly, immediate increases of MMP-9 followed by MMP-2 upregulation in postmortem human brain tissue have been reported from 2 days to several years after stroke.

Our results are in accordance with these findings because gelatin zymograms show high MMP-9 levels in infarcted and in perihematoma areas, and MMP-2 levels remained invariable both in ischemic and hemorrhagic strokes <5 days old.

Regarding different brain areas, we found MMP-9 mainly located around perivascular tissue as previously described. Specifically, we have identified both MMP-9 positive endothelial cells and peripheral neutrophils crossing the vessel wall. Because disruption of the basal lamina has been postulated to be the primary cause of microvascular hemorrhage after an ischemic event, a very close relationship between blood-brain barrier leakage and MMP-9 expression has been reported in animal models of cerebral ischemia. As

Figure 2. Gelatinase expression after hemorrhagic transformation: computed tomography image, fresh brain tissue sample displaying HT and corresponding in situ zymography (magnification 100x) and gelatin zymography showing greater MMP-9 signal in HT than in infarct and peri-infarct.

Figure 3. A, A strong correlation was found ($r=0.912, P<0.0001$) between pro-MMP-9 and 145 kDa bands. B, Immunoblotting for MMP-9 confirming gelatin zymography findings.
we know, ongoing extravasation of plasmatic constituents to brain tissue (as MMP-2 and MMP-9) could contribute to MMP-9 increase in brain parenchyma. But, if a plasmatic protein contribution occurs in the studied samples, and mainly in a hemorrhagic transformation one, it is minimal because MMP-2 remains constitutively expressed without changes, as gelatin zymography demonstrates.

New MRI techniques, such as diffusion-weighted imaging, lead us to a topographic assessment of tissue injury by which others have demonstrated that exists a different pattern of molecular mechanisms in infarcted core than in peri-infarct tissue. Moreover, we have recently reported that baseline plasma MMP-9 level is strongly correlated to diffusion-weighted imaging measured lesion growth in t-PA–treated patients, suggesting its ultra-early role as a predictor of brain damage. Although in our study peri-infarct areas could not be definitively associated with ischemic penumbra because neuroimages were obtained several hours before patient’s death, we have identified distinctive biochemical signatures of those areas. Taking together both histopathological findings and MMP-9 expression assessed by gelatin zymography and Western Blot Analysis, we were able to describe areas surrounding infarcted tissue where necrosis has not started but MMP-9 is over-expressed both in cells and to a lesser extent around capillary blood vessels. These findings show an MMP-9 expression previous to a possible cell death within ischemic parenchyma, supporting its participation on ongoing brain injury. This fact reinforces the possible therapeutic role of inhibiting this protease as an attempt to protect surrounding salvageable ischemic tissue. However, other ipsilateral areas far from the infarct should be studied in the future to elucidate whether this MMP-9 expression exists in all the

Figure 4. MMP-9 immunohistochemistry: brown immunoreactivity in infarcted tissue (A and B) surrounding the endothelial/periendothelial layer associated to neutrophils infiltration (arrowheads in B and C). Detail of a capilar vessel (C) displaying endothelial immunoreactivity (thin arrow) and neutrophil infiltration from peripheral blood containing MMP-9. MMP-9 citoplasmatic stains (arrows) in neurons (D), activated microglial cells (E), and reactive macrophages (F). Sparse cellular immunoreactivity was detected in contralateral samples (G) whereas blood vessels (v) remained unreactive for MMP-9 both in contralateral hemisphere (H) and control brain (I).
ischemic hemisphere, is limited to the infarct boundary and closely related to infarct extent, or is attributable to a local inflammatory process even under nonischemic conditions.

Regarding hemorrhagic strokes, this is the first report describing perihematoma gelatinase brain content. We found that in this injured area MMP-9 was over-expressed and may contribute to both hematoma extent and brain edema after the initial hemorrhagic event. This deleterious effect of MMP-9 would take place in the secondary injury after initial bleeding and would be integrated in a complex cascade involving inflammatory processes. In fact, a collagenase-induced intracranial hemorrhage model has demonstrated upregulation and close implication of other MMPs such as MMP-12 in inflammatory mechanisms surrounding parenchyma hematomas.16

Interestingly, a strong correlation was found between MMP-9 and a 145 kDa band identified by immunoblotting suggesting that other MMP-9 forms or complexes appear within the neuroimmunflammatory response. These complexes have been identified as participating in enzyme activity in tumor extracts mainly related to their endogen tissue inhibitors (TIMPs),2 and TIMPs are known to be present in the nervous system.22 Although the nature of these complexes in the ischemic brain is still unknown, these preliminary results suggest that MMP-9/TIMP interactions need further investigation to better understand the homeostasis of this MMP after stroke.

Complementary to gelatin zymograms and immunohistochemistry detection, in situ zymography reveals a source of gelatinase activity on brain cells and blood vessels together with an extracellular activity both in infarcted and peri-infarcted samples. Immunohistochemistry failed to detect the same amount of cellular MMP-9 in parenchymal cells such as neurons maybe attributable to different sensitivities of in situ

Figure 5. In situ zymographies: A through C are representative zymograms from an ischemic stroke (A: infarct; B: peri-infarct; C: ischemic contralateral); D and E, representative zymograms from a hemorrhagic stroke (D: perihematoma, and E: hemorrhagic contralateral); F, control. Magnifications 100x. G through I, Higher magnifications (400x) from a representative ischemic case showing gelatinase activity sourcing from cell bodies (G) and a detail from a blood vessel (H) and inhibitors of 10 mmol/L-phenanthroline effect in the same vessel (I).
zymography versus immunohistochemistry. Similar differences between these 2 techniques have been shown previously for hippocampal remodeling after kainate lesions. On gelatin zymograms a MMP-9 cleaved band was observed only in infarced samples whereas in situ zymography showed activity in other areas. This discrepancy previously reported by other authors in animal models could be explained by other nonproteolytic cleavage mechanisms of pro-MMP activation. Among those, MMP-activation mechanisms related to oxidative stress such as peroxinitrite-dependent pro-MMP activation have to be considered in future research. Another fact that could explain this issue is that other metalloproteinases such as MMP-1, MMP-3, MMP-7 or MMP-13 could also digest gelatin substrates contributing to gelatinase activity detected in our samples. In fact, the MMP inhibitor of 1,10-phenanthroline could not completely abolish in situ activity.

A low expression of both gelatinases was found in contralateral samples and in control tissue. We think that this finding could be in part attributable to the effect of global cerebral ischemia that may occur within the first hours after death until the tissue is removed and stored, leading to a minimal MMP-9 expression as others have described that could affect in the same manner both hemispheres.

Although the small number of patients of our study could be a limitation, we want to emphasize the very short time permitted in the study to obtain brain parenchyma (within the first 6 hours after death) and on the short time passed from stroke to death offering powerful information in a time-period that has never been studied before in some cases. Better definition of MMP-9 immunopositive cells identified in brain samples remains to be elucidated in a future study. It is very likely that double immunostaining of MMP-9 and cell markers would be useful to clarify this issue. A final limitation is the lack of information about the arterial permeability at death moment. This fact might clarify this issue. A final limitation is the lack of information about the arterial permeability at death moment. This fact might clarify this issue.

In conclusion, our investigation reveals a real in situ MMP-9 over-expression in infarcted parenchyma mainly located around blood vessels and for the first time around the injured area, both in ischemic and hemorrhagic strokes suggesting its relation to brain cell death and ischemic brain injury. Future studies should be addressed to inhibit this MMP in an attempt to protect surrounding tissue damage and to improve neurological outcome after stroke.

Acknowledgments

A.R. is the recipient of a grant from the Institu de Recerca, Hospital Vall d’Hebron. We are grateful to Manolo Quintana for statistical advice and to all neurologists and residents from the Neurovascular Unit and technical staff from the Neuropathology Unit who helped in performing this study. This study was supported in part by the Spanish neurological diseases network (RED CIEN) and by FIS 05/322.

References


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Stroke. 2006;37:1399-1406; originally published online May 11, 2006;
doi: 10.1161/01.STR.0000223001.06264.af

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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