Fatty Acid Binding Protein as a Serum Marker for the Early Diagnosis of Stroke

A PILOT STUDY

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No biological marker is currently available for the routine diagnosis of stroke. The aim of this pilot study was to determine whether heart-fatty acid binding protein (H-FABP) could be used as a valid diagnostic biomarker for stroke, as compared with neuron-specific enolase (NSE) and S100B proteins. Using two-dimensional gel electrophoresis separation of cerebrospinal fluid proteins and mass spectrometry techniques, FABP was found elevated in the cerebrospinal fluid of deceased patients, used as a model of massive brain damage. Because H-FABP, a FABP form present in many organs, is also localized in the brain, an enzyme-linked immunosorbant assay was developed to detect H-FABP in stroke versus control plasma samples. However, H-FABP being also a marker of acute myocardial infarction (AMI), troponin-I and creatine kinase-MB levels were assayed at the same time in order to exclude any concomitant heart damage. NSE and S100B levels were assayed simultaneously. These assays were assessed in serial plasma samples from 22 control patients with no AMI or stroke, 20 patients with AMI but no stroke, and 22 patients with an acute stroke but no AMI. Twenty-two out of the 22 control patients and 15 out of the 22 stroke patients were correctly classified, figures much better than those obtained with NSE or S100B, in the same study’s population. H-FABP appears to be a valid serum biomarker for the early diagnosis of stroke. Further studies on large cohorts of patients are warranted. Molecular & Cellular Proteomics 3:66–72, 2004.

Over the last two decades, a number of biological markers (biomarkers) have been studied in the cerebrospinal fluid (CSF) and serum of patients with stroke, including creatine kinase-BB (1), lactate dehydrogenase (2), myelin basic protein (3), S100 protein (4), neuron-specific enolase (NSE) (5), glial fibrillary acidic protein (6), and tau (7). Most of them have proved quite useful indicators of the extent of brain damage and accurate predictors of clinical status and functional outcome. In contrast, the diagnostic value of biomarkers for stroke has been hampered by their late appearance and a delayed peak after the cerebrovascular event, their poor sensitivity and specificity, and the limited understanding of the mechanisms governing the release of these molecules into the CSF and ultimately in the blood. As a result of these limitations, the use of stroke biomarkers is currently limited to research settings, and none has been recommended for routine assessment of stroke (8).

Ideally, a biomarker for the diagnosis, monitoring and prognosis of stroke should include at least the following characteristics: 1) it should be brain specific; 2) because of obvious difficulties to obtain CSF samples in patients with acute stroke, detection in serum is highly desirable; 3) it should appear very early, i.e. hours at the most, after the insult; 4) its peak level, alternatively the area under the curve of sequential concentrations, should reflect the extension of brain damage; 5) it should possibly distinguish between transient and established stroke, between hemorrhage and ischemia and perhaps between the necrotic and penumbra aspect of the brain lesion; finally, 6) it should be indicative of functional outcome. While such an ideal stroke biomarker does not exist yet, we investigate a novel stroke biomarker and compare it with S100 and NSE, the two molecules, which have been most extensively assessed for this purpose.

In this pilot study, we describe how fatty acid binding protein (FABP) has been identified as a novel diagnostic biomarker for stroke using a proteomics-based analysis of CSF from deceased patients as a model of massive brain damage. And we report on results obtained after serum FABP levels have been sequentially determined using an enzyme-linked immunosorbsorbant assay (ELISA) in patients with acute stroke, as compared with S100 and NSE.

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1 The abbreviations used are: CSF, cerebrospinal fluid; AMI, acute myocardial infarction; CK-MB, creatine kinase-MB; FABP, fatty acid binding protein; H-FABP, heart-FABP; NSE, neuron-specific enolase; ELISA, enzyme-linked immunosobrsorbant assay; CT, computed tomography; MRI, magnetic resonance imaging; TIA, transient ischemic attack; 2-DE, two-dimensional gel electrophoresis; DTE, dithioerythritol; IPG, immobilized pH gradient; PBS, phosphate-buffered saline; OD, optical density; ROC, receiver operating characteristic; MS, mass spectrometry; B-FABP, brain-FABP.

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Eight CSF samples were obtained at autopsy from deceased patients with no pathology of the central nervous system. Four others were collected by lumbar puncture from living patients who had a neurological work-up for benign conditions unrelated to stroke (ataxic headache and idiopathic peripheral facial nerve palsy). CSF samples were centrifuged immediately after collecting, aliquoted, frozen at -80 °C, and stored until analysis.

The population used for the assessment in serum of the various markers detailed below included a total of 64 prospectively studied patients (Table I) equally distributed into three groups: 1) a control group including 14 men and 8 women aged 65 years (ranges: 34–86 years) with no known peripheral or central nervous system condition; 2) a group of patients with acute myocardial infarction (AMI group) including 14 men and 6 women aged 65 years (ranges: 29–90 years); the diagnosis of AMI was established in all cases by typical electrocardiography modifications and elevated levels of creatine kinase-MB (CK-MB) (above a cut-off value of 2 ng/ml); 3) a group of patients with acute stroke (stroke group) including 14 men and 8 women aged 65 years (ranges: 30–87 years); the diagnosis of stroke was established by a trained neurologist and was based on the sudden appearance of a focal neurological deficit and the subsequent delineation of a lesion consistent with the symptoms on brain computed tomography (CT) or magnetic resonance imaging (MRI) images, with the exception of transient ischemic attacks (TIAs), where a visible lesion was not required for the diagnosis. The stroke group was separated according to the type of stroke (ischemia (16 patients) or hemorrhage (6 patients)), the location of the lesion (brainstem (4 patients) or hemisphere (18 patients)), and the clinical evolution over time (TIA when complete recovery occurred within 24 h (5 patients) or established stroke when the neurological deficit was still present after 24 h (11 patients)). The diagnostic of TIA was further supported by the absence of any recent lesion on the CT or MRI scans in all five TIA patients, whereas all 11 patients with established ischemic stroke had a definite and recent lesion on neuroimaging exams. The size of the lesion varied from lacune to large infarct.

For each patient of the three groups, a blood samples was collected at the time of admission in dry heparin-containing tubes. After centrifugation at 1500 × g for 15 min at 4 °C, plasma samples were aliquoted and stored at -20 °C until analysis. For the stroke group, three additional blood samples were collected after the neurological event: <24 h; <48 h; and >48 h. In this group, the time interval between the neurological event and the first blood draw was 185 min (ranging from 40 min to 3 days). This parameter was taken into account in the data analysis. Each patient or patient’s relatives gave informed consent prior to enrolment.

CSF Two-dimensional Gel Electrophoresis (2-DE)—All reagents and apparatus used have been described in detail elsewhere (9). Two hundred fifty microleters of CSF was mixed with 500 μl of ice-cold acetone (−20 °C) and centrifuged at 10,000 × g at 4 °C for 10 min. The pellet was mixed with 10 μl of a solution containing 10% SDS (w/v) and 2.3% dithioerythritol (DTE) (w/v). The sample was heated to 95 °C for 5 min and then diluted to 60 μl with a solution containing 8 M urea, 4% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid (CHAPS) (w/v), 40 mM Tris, 85 mM DTE, and a trace of bromphenol blue. The whole final diluted CSF sample corresponding to 45 μg was loaded in a cup at the cathodic end of the immobilized pH gradient (IPG) strips, 2-DE was performed as described previously (10). In brief, the first dimensional protein separation was performed using a commercial 18-cm nonlinear IPG going from pH 3.5 to 10 from Amersham Biosciences (Uppsala, Sweden). The second dimensional separation was performed onto in-house manufactured vertical gradient slab gels (9–16% T, 2.6% C). Analytical gels were then stained with ammoniacal silver staining (11). Gels were scanned using a laser densitometer (Amersham Biosciences). 2-DE computer image analysis was carried out with the MELANIE 3 software package (12). Spots were detected and quantified automatically. The optical density (OD), the area, and the volume were computed and directly related to protein concentration. The relative OD and relative volume were also calculated in order to correct for differences in gel staining. Differential analysis (~200% and Student t test p < 0.05) using the relative volume of each spot (~0.05%) allowed the detection of significantly overexpressed polypeptides with a minimum ratio of 2.

Mass Spectrometry Identification—Differentially expressed spots were found through the comparison of analytical gels of deceased versus healthy CSF (n = 4). Spots of interest were analyzed by peptide mass fingerprinting using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Voyager STR MALDI-TOF-MS; PerSeptive Biosystems, Framingham, MA) (10) and identified through database using the PeptIdent tool (www.expasy.ch/sprot/peptident.html).

FABP Measurement—Heart-FABP (H-FABP) levels were measured in plasma by a sandwich ELISA. Specificity of anti H-FABP antibodies was tested by Western 2-DE blots. They specifically detected the H-FABP spot and not the other forms (data not shown). A 96-well polystyrene microplate (Nunc, Polylabo, Switzerland) was coated with 100 μl/well polyclonal goat anti-human muscle FABP (Spectral Diagnostics HC, Ontario, Canada), 20.4 μg/ml in carbonate buffer 0.1 M, pH 9.6, overnight at 4 °C. The plate was automatically washed with phosphate-buffered saline (PBS; 15 mM Na2PO4-120 mM NaCl-2.7 mM KCl, pH 7.4; Sigma, St. Louis, MO) on a BioRad Novaphath™ washer (Hercules, CA). Every washing step was performed with fresh PBS. Nonspecific binding sites were blocked with 200 μl/well 2%
Casein (w/v) in carbonate buffer for 2 h at 37 °C. After the washing step, the crude samples were pipetted in duplicate at 100 μl/well. The plate was incubated 2 h at 37 °C. After the washing step, 100 μl/well of mouse anti-human H-FABP (clone 66E2; HyCult Biotechnology b.v., Uden, Netherlands), 0.3 μg/ml in PBS-1% BSA (w/v), were incubated for 1 h at room temperature with shaking. After the washing step, 100 μl/well of phosphatase-labeled anti-mouse immunoglobulins (Dako, Glostrub, Denmark), 15 μg/ml in PBS, were incubated 1.5 h at room temperature with shaking. After the washing step, 50 μl/well of phosphatase substrate, 1.5 mg/ml paranitrophenylphosphate in diethanolamine, were incubated 30 min. Reaction was stopped with 100 μl/well NaOH 1 M. Color development was assessed with a microplate reader, Milenia™ kinetic analyzer (Diagnostic Products Corporation, Los Angeles, CA), at a wavelength of 405 nm.

CK-MB and Troponin-I Measurement—Plasma samples were centrifuged at 1500 × g for 15 min, and aliquots were stored at −20 °C. Serum CK-MB and troponin-I levels were determined using a fluorescent microparticle enzyme immunoassay with an automated chemical analyser AxSYM™ system (Abbott Laboratories, Abbott Park, IL). The formation rate of fluorescent products was directly proportional to the amount of troponin-I in the sample. The detection limit for troponin-I was 0.3 μg/liter. CK-MB measurement is proportional to the amount of fluorescent probes, and the detection limit was 0.7 μg/liter.

NSE and S100 Measurement—Similar to H-FABP measurements, NSE and S100B were assayed in the four serial plasma samples of the stroke group. The SMART S100 and SMART-NSE ELISA kits were used and commercialized both by Skye PharmaTech Inc. (Ontario, Canada). The detection limits for NSE and S100B were 1 μg/liter and 0.01 μg/liter, respectively.

Statistical Analysis—H-FABP levels were expressed in OD values as mean ± SD. Because recombinant H-FABP was not available, external calibration and limit of sensitivity could not be performed to express results as concentration units (ng/ml). Troponin-I and CK-MB levels were expressed in ng/ml. Because plasma H-FABP, troponin-I, and CK-MB concentrations did not fulfill the criteria for a Gaussian distribution in neither of the normal, stroke, and AMI populations according to the Kolmogorov-Smirnov test, comparisons between the three groups was carried out using the nonparametric Kruskall-Wallis test with the post hoc Dunn’s procedure. Comparisons between the stroke subgroups defined above were made by means of the Mann-Whitney U test, and longitudinal assessment of H-FABP concentrations over time were analyzed using the repeated measures analysis of variance. Reference limits for H-FABP aiming at distinguishing stroke versus normal patients were delineated using receiver operating characteristic (ROC) curves (Analyze-It™ software for Microsoft Excel™; Microsoft, Redmond, WA) (13). Statistical significance was set at p < 0.05.

RESULTS

In order to delineate proteins differentially expressed in the CSF of deceased (n = 4) versus healthy (n = 4) subjects, CSF samples were separated by 2-DE and the expression (relative volume) of all detectable protein spots (1682 spots) was compared between both groups. Eighteen spots were found elevated (p < 0.05) in the CSF map of deceased patients as compared with healthy subjects. Two protein spots (Fig. 1) were identified as epidermal- and adipocyte-FABPs by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MS) and tandem MS sequencing (data not shown), thus establishing FABP as a potential marker of brain necrosis to be further assessed for the diagnosis of stroke.

Individual results of the H-FABP assay in the three populations, expressed in OD units, are graphically shown in Fig. 2. Mean plasma H-FABP concentration was 0.221 ± 0.134 OD in the control group, 1.079 ± 0.838 OD in the stroke group, and 2.340 ± 0.763 OD in the AMI group. The intra-assay coefficient of variation found for this ELISA was 5.8% ± 3.8. Using the Kruskall-Wallis test, all three concentrations were found significantly different (p < 0.001) from each other. The best cut-off value to discriminate between the control and the stroke groups was set at OD > 0.531 as determined by the ROC curves for H-FABP level (data not shown). Using this cut-off value, validity measures of H-FABP for the...
TABLE II

Values of serum markers for stroke diagnosis in the control, AMI, and stroke group

H-FABP was expressed in OD value measurement at 405 nm. Troponin-I and CK-MB were expressed in international units (ng/ml). The significance level refers to the Mann-Whitney U test, with respect to control group (SD, standard deviation; NS, nonsignificant).

| Group     | Control | AMI | Stroke |
|-----------|---------|-----|--------|
| H-FABP    | Mean    | 0.221 | 2.434 | 1.079 |
|           | SD      | 0.134 | 0.638 | 0.838 |
|           | Significance | <0.001 | <0.001 |        |
| Troponin-I| Mean    | 0.0   | 164.6 | 0.5   |
|           | SD      | 0.1   | 205.6 | 1.3   |
|           | Significance | <0.001 | NS     |        |
| CK-MB     | Mean    | 1.3   | 63.8  | 7.9   |
|           | SD      | 0.9   | 51.5  | 21.3  |
|           | Significance | <0.001 | NS     |        |

diagnosis of stroke were as follows: sensitivity was 68.2% with 15 out of 22 stroke patients above the cut-off, specificity was 100% with all of the 22 control subjects below the cut-off.

To discriminate, at the biological level, between patients from the AMI and the stroke groups, troponin-I and CK-MB were further assayed in each group with cut-off values set at 2 ng/ml for the AxSYM troponin-I assay and 3.8 ng/ml for the AxSYM CK-MB assay (Table II). As expected, the concentrations of these AMI markers were significantly higher ($p < 0.01$) in the AMI group as compared with both the control and the stroke groups. No difference was found between the last two groups, thus confirming that troponin-I and CK-MB do not increase as a result of a brain insult and that stroke patients did not sustain a concomitant AMI at the time of their stroke.

Taken together, H-FABP, troponin-I, and CK-MB concentrations allowed a correct discrimination between AMI (increase of all three markers) and stroke (increase of H-FABP with normal troponin-I and CK-MB) in all the 20 AMI patients and in 15 stroke patients, with the exception of one stroke patient showing, along with increased H-FABP levels, moderately elevated levels of troponin-I and CK-MB in the absence of electrocardiogram modifications, all of which being consistent with a concomitant non-AMI heart damage.

In the stroke group, seven false negative results were found with H-FABP levels below the cut-off value of OD 0.531 at any time point following the neurological event. Of these seven patients, two had a negative MRI and a rapid and complete recovery of their neurological deficits within 24 h consistent with a TIA, and two have had a lacunar stroke on MRI images, one located in the brainstem. While TIA and lacunar stroke may explain false negative results in a majority of patients, no explanation was consistently found for the three remaining stroke patients with low H-FABP levels.

Sequential determinations of H-FABP level after stroke showed that 10 out of 15 (67%) H-FABP-positive stroke patients had a very early increase of H-FABP levels (<12 h). Moreover, as shown in Fig. 3, when all stroke patients were considered, the mean H-FABP concentrations decreased steadily after the insult, the highest value being found before 12 h. The differences between the initial measurement and the <48 h and afterward measurements were significant (analysis of variance, $p < 0.05$). When H-FABP levels were compared between the different subgroups of the stroke group, no statistically significant differences were found. H-FABP levels were similar for ischemia (0.955 ± 0.668, n = 15) versus hemorrhage (1.346 ± 1.139, n = 7) strokes, and for hemispheric (0.987 ± 0.783, n = 18) versus brainstem (1.493 ± 1.080) strokes, but the statistical power of the analyses was limited by the small size of the samples to be compared. However, when comparing established strokes versus TIA, the former (1.200 ± 0.892) showed nearly twice as high H-FABP levels as the latter (0.652 ± 0.499), although this difference failed to reach significance (Mann-Whitney U test, $p = 0.24$). Interestingly, H-FABP levels were found moderately increased in three of the five TIA patients, despite the absence of any detectable lesion on the CT or MRI scans, suggesting that the marker may be sensitive to extremely short periods of ischemia.

Finally, NSE and S100B were assayed in the control and the stroke groups, and results were compared with the H-FABP assay. The cut-off values using the SMART-NSE and SMART S100B protein ELISA tests for the diagnosis of stroke were 10 ng/ml for NSE and 0.02 ng/ml for S100B. NSE and S100B levels were slightly increased in the stroke groups (14.12 ng/ml and 0.010 ng/ml, respectively) as compared with the control group (15.88 ng/ml and 0.004 ng/ml, respectively). As shown in Table III, specificity and sensitivity for the diagnosis...
of stroke were found much lower for NSE and S100B than for H-FABP. These differences are relevant because the three markers have been tested in the same samples at the same time points.

**DISCUSSION**

In this pilot study including a small but prospective sample of patients, we identified for the first time and established H-FABP as an early and potentially robust biomarker for the diagnosis of stroke.

In a first step, identification of H-FABP was carried out using an original approach based on proteomics analysis of CSF samples obtained at autopsy from deceased patients, as a model of massive brain damage, which were compared with CSF from healthy living subjects. Comparisons of 2-DE maps showed two distinct spots highly expressed in the CSF of deceased patients, both of which being definitely identified by various MS techniques as two forms of FABP. The FABP family encompasses at least eight different proteins (14) functionally involved in intracellular transport systems, oxidation of fatty acids, and membrane lipids trafficking (15, 16). Brain-FABP (B-FABP) is localized exclusively in neuronal and glial cells (17), whereas H-FABP has a more widespread distribution including, in addition to brain, myocardial muscle, endothelial cells, lung, and kidney (18). B-FABP and H-FABP have a 69% sequence homology. In fact, several forms of FABP with variable degree of sequence homology are present in both the brain and the heart, but they seem to have distinct distribution and different functions.

The second step of the study was to develop and to apply a quantitative FABP detection method in the serum for the diagnosis of stroke. The study was designed to discriminate stroke patients not only from normal controls but also from patients with AMI, because H-FABP has been recently demonstrated to increase in this condition. A sandwich ELISA was therefore developed using a human anti-H-FABP (clone 66E2) (19) monoclonal antibody in combination with a polyclonal anti-human FABP antibody. In another study, the H-FABP assay has been shown to allow adequate detection of FABP in the serum after heart injury (20). Ideally, anti-B-FABP antibodies would have been preferred, but those antibodies are currently not commercially available. When applied to two groups of clinically well-characterized patients, including patients with stroke and subjects for whom a blood draw was performed for unrelated conditions, the H-FABP demonstrated a specificity of 22 patients out of 22 (cut-off at OD 0.531), a sensitivity for the diagnosis of stroke of 15 patients out of 22, when controls were taken as the comparative variable. H-FABP was compared with the two other well-established markers for stroke in the same samples of patients, NSE and S100B proteins.

S100 is an acidic, calcium-binding protein present in the brain, striated muscle, heart, and kidney. The isoform S100B is a major cytosolic constituent of glial and Schwann cells. S100 protein has been reported as a useful marker for stroke, head injury, brain metastases, and central nervous system complications of cardiac surgery (21–23). NSE is a dimeric glycolytic enzyme that catalyzes the interconversion of phosphoglycerate and phosphoenolpyruvate. It is found primarily in the cytoplasm of neurons, as γ-NSE, and neuroendocrine cells (24). NSE has been reported as a useful marker not only for stroke and head injury but also for the prognosis of neuroblastoma (25), neuroendocrine tumors (26), and lung carcinoma (27). Several studies have shown that S100 and NSE are elevated not only in the CSF but also in the blood of stroke patients. Moreover, in a recent comparative study (28), Fassbender and colleagues have demonstrated that S100 and NSE significantly increased in the serum of stroke patients as early as 8 h following the cerebrovascular event, though only in a small fraction of cases. Unlike NSE, S100 levels correlated with the size of the infarct, as estimated by CT-scan-based volumetry, and with clinical outcome. In contrast, an increased level of S100 within 24 h was found only in 14 out of 22 patients yielding a sensitivity of 64% at day 1 post-stroke. Overall, in another study (29), sensitivity and specificity for stroke have been found to range from 44 to 67% for S100.

In comparison to NSE and S100, all validity measures turned out to be much higher for H-FABP with the exception of the specificity of S100B, which was found almost as high as that of H-FABP. The kinetics of FABP release in the blood, as determined by serial blood sampling after stroke, showed that FABP increases very early after the cerebrovascular event, even within a few hours in some patients. This early rise seems consistent because elevated levels of H-FABP were detected within 24 h in 80% of the H-FABP-positive stroke patients and a delayed H-FABP peak after 24 h was observed in only a few cases. Although this aspect was not fully explored in the present study, NSE and S100B having been measured only at admission, it appears that the very early, that is within 12 h, elevation of H-FABP has been found by others in only a minority of patients with S100B and in none with NSE (28, 30). In that respect, H-FABP has clearly the potential of being not only a valid but also a very early marker of stroke.

Unsurprisingly, the H-FABP levels did not correlate with either the type of stroke (hemorrhage or ischemia) or its location (hemisphere or brainstem), but it is noteworthy that the number of patients was very small in some categories. In contrast, although the size of the lesion was not quantitatively assessed, we found that more than half of the H-FABP falsely negative stroke patients have suffered from either TIAs or

| Table III |
|----------------|
| Statistical values of sensitivity and specificity in percentage units for H-FABP, NSE, and S100B tests found on the tested population |
| H-FABP | NSE | S100B |
|---|---|---|
| Sensitivity | 68.2 | 55 | 15 |
| Specificity | 100 | 36.4 | 95.5 |
small lacunar lesions. There was a trend for TIAs (n = 5) to have lower H-FABP levels than established strokes (n = 11). Taken together, these preliminary results prompt further studies looking at potential correlation between H-FABP levels and lesion size to be undertaken.

Because AMI patients have also elevated levels of H-FABP (20), the assessment of stroke patients must exclude a concomitant acute heart condition, which may contribute to the net value of H-FABP and yield erroneous clinical conclusions. Thus, the H-FABP assay has to be performed along with a dosage of troponin-I (31) or CK-MB. Because the latter is not heart-specific and may increase in response to any muscle damage (32), we propose to combine the H-FABP assay with the troponin-I assay. In this study, we demonstrated that this combination could clearly separate the AMI from the stroke population with almost no overlap, except in rare instances where the two conditions may develop simultaneously.

We believe that the H-FABP assay in its present form still needs further refinements before a more widespread use for the diagnosis of stroke. First, the ELISA was performed in 8 h, which is slower than the S100B assay (29). The use of a rapid microparticle-enhanced turbidimetric immunoenasasy, already developed for the assessment of H-FABP levels in AMI (33), should drastically shorten the availability of the results at bedside. The full automation in a widely used clinical chemistry analyzer like the COBAS MIRA Plus system from Roche (Rotkreuz, Switzerland) or the AxSYM system from Abbott Laboratories should be possible and applied for routine testing. Second, the additional need of the troponin-I assay might become unnecessary provided an ELISA using antibodies directed toward the B-FABP form be manufactured in the future. Finally, further clinical studies are still warranted to definitely confirm and validate the preliminary data presented here. These studies will have to include a large and well-categorized cohort of patients, to establish the sensitivity of the test using MRI-based volumetry of stroke lesion and perhaps to approach the early prognosis of any vascular brain insult.

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