Diffusion Tensor MRI Reveals Chronic Alterations in White Matter Despite the Absence of a Visible Ischemic Lesion on Conventional MRI
A Nonhuman Primate Study

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Background and Purpose—The impact of stroke on white matter is poorly described in preclinical investigations mainly based on rodents with a low white (WM)/gray matter ratio. Using diffusion tensor imaging, we evaluated WM alterations and correlated them with sensorimotor deficits after stroke in the marmoset, a nonhuman primate that displays a WM/gray matter ratio close to that of humans.

Methods—Marmosets underwent a transient brain ischemia (3-hour). Eight serial MRI examinations were made during ischemia and up to 45 days after reperfusion. The sensorimotor deficits were evaluated weekly over 45 days. To assess WM alterations, the SD of the angle of the first eigenvector projection was calculated in the cortex and in the internal and external capsules. The fiber-tracking approach was used to measure the number and the length of bundles.

Results—Changes in the apparent diffusion coefficient and the fractional anisotropy values were similar during the temporal evolution of the lesion in the marmoset model of ischemia to that reported in patients with stroke. Despite an absence of visible lesions on T2-MRI and diffusion tensor imaging at the chronic stage, diffusion tensor MRI evidenced alterations in WM by the increase in the standard deviation of the angle of the first eigenvector projection in the cortex, internal and external capsules, and the decrease in the number of bundles of fibers tracked. The disruption of WM was strongly correlated with the chronic sensorimotor deficits.

Conclusions—Despite an absence of a visible ischemic lesion at the chronic stage, diffusion tensor MRI revealed disorganization of WM, which probably underlies the persistence of functional deficits. (Stroke. 2011;42:1412-1419.)

Key Words: diffusion tensor imaging ■ functional recovery ■ nonhuman primate ■ stroke ■ white matter

The impact of stroke-induced brain lesions on functional deficits has been widely addressed in many animal studies. Nonetheless, several authors failed to find any correlation between lesion size and the ultimate functional outcome. This discrepancy could be partly explained by the little attention given to alterations and reorganizations in white matter (WM). Indeed, the most widely used end point is the volumetric measurement of cortical and subcortical infarction without any distinction between WM and gray matter (GM) involvement. This drawback might be attributed to technical difficulties in imaging WM in rodents that display a low WM/GM ratio. However, this issue can be addressed more readily in nonhuman primates. The importance of nonhuman primates in the study of the pathophysiology and the treatment of stroke was highlighted in many recent reviews. Indeed, apart from the similarities that exist between humans and other primates in their cerebrovascular systems, brain metabolism, and a rich behavioral repertoire, nonhuman primates display a higher WM/GM ratio in comparison to rodents. These features allow one to more precisely define the specific alterations in WM and their influence on functional recovery after stroke.

The development of MRI, in particular diffusion tensor imaging (DTI), permits the analysis of the magnitude of the diffusion of water molecules and thus provides quantitative measures of their mobility and their deviation from the isotropic diffusion. The apparent diffusion coefficient (ADC) and diffusion anisotropy indices such as fractional anisotropy (FA) are used to estimate the tissular integrity. Furthermore, DTI can uniquely study the orientation and integrity of WM tracts and is thus an ideal tool to identify WM abnormalities in stroke subjects.

We have taken advantage of the recent development of a focal stroke model in the nonhuman primate and examined...
first, the temporospatial evolution of diffusion abnormalities (ADC and FA) in acute, subacute, and chronic stages of ischemia; and second, the WM alterations at the chronic stage. These parameters were correlated to functional deficits evaluated through the use of a battery of behavioral tests.

Materials and Methods
All procedures were performed according to the European Directive (86/609/EEC) and approved by the Regional Ethics Committee (No. 06-003).

Induction of Cerebral Ischemia
Four male and 4 female marmosets (Callithrix jacchus, 285 to 370 g; 18 to 24 months) bred in the laboratory (Cyceron, Caen, France) were used. The details of the procedure to induce ischemia were described previously. Briefly, in isoflurane-anesthetized marmosets, a nylon thread was inserted into the external carotid artery and gently advanced up to the origin of the middle cerebral artery (MCA). The filament was removed 3 hours later to allow reperfusion.

Magnetic Resonance Imaging
Each marmoset underwent 8 MRI examinations (7 T; Pharmascan, Bruker, France) as shown in Figure 1. The parameters of the sequences were: DTI MRI: number of shots = 2, 30 diffusion directions, $b = 1000 \text{s/mm}^2$ (number of experiment = 1), $b = 0 \text{s/mm}^2$ (number of experiments = 5); echo time/repetition time = 41.1/5000 ms, matrix = 128×128, field of view = 50×50 mm, 21 contiguous slices (1.5 mm); fast T2-weighted imaging (RARE factor 8); number of experiments = 4; echo time/
repetition time=60/5000 ms; matrix=256×192, field of view=50×50 mm, 21 contiguous slices (1.5 mm), T2*-weighted imaging:
echo time/repetition time=11/400 ms, matrix=256×256, field of view=50×50 mm, 21 contiguous slices (1.5 mm).

Behavioral Assessment
The following behavioral tests were performed as previously described8: neurological score, tactile stimulation, hill and valley staircases, and adhesive removal. The animals were trained on behavioral tasks for the week preceding MCA occlusion, then evaluated on all tasks daily for 1 week, and thereafter at 5-week intervals.

Histology
At Day 45, animals were deeply anesthetized with isoflurane (5%) and transcardially perfused with a heparinized solution of saline followed by a solution of 4% paraformaldehyde in phosphate buffer. The brains were removed and cut in the coronal plane at 50-μm thickness. One section in every 5 was mounted for Luxol fast blue staining.

Data Analyses
ADC and FA maps were calculated from the DTI MRI (Paravison software). To identify the areas with a decreased ADC, ADC maps were thresholded at the mean minus twice the SD of the contralateral values (in-house macros on ImageJ). In each lesion so identified, the mean absolute ADC and FA values were quantified. At Day 45, because there was no apparent diffusion abnormality, we analyzed FA and WM alterations in restricted regions of interest. Circular regions of interest (0.6 mm² on 2 consecutive slices) were positioned on the WM bundles of internal and external capsules, which are included in the lesion in acute and subacute stages of ischemia. Additionally, we have included a region of interest in the superior temporal cortex (supplemental methods). Three analyses were performed in these regions of interest to obtain 3 quantifiable indices: measurement of mean FA, measurement of the intervoxel homogeneity of the preferential direction of diffusion (assessed by calculating the SD of the angle projected in the slice plane of the first eigenvector [AFE] and visualized on maps of the local homogeneity of the AFE) and fiber tracking (MedINRIA software; supplemental methods and Supplemental Figure II).

Statistical Analyses
The MRI data are presented as mean±SEM. The behavioral data are presented as percentage of deficit compared with the reference values (defined at Day-1). Wilcoxon test was used in most instances. Spearman test was used to correlate the chronic alterations of WM
with the persistent functional deficits (ie, at 45 days; supplemental methods). Values of $P<0.05$ were considered significant.

**Results**

At Day 0, arterial pressure, heart rate, blood gases, and temperature remained stable within physiological limits. In addition, we observed no gender effect on the lesion volume or behavioral performances (data not shown).

**MRI Data**

MR angiography confirmed MCA occlusion and reperfusion. T2* MRI failed to reveal any cerebral hemorrhage at any time point analyzed (data not shown).

**Evolution of the ADC-Defined Lesion**

At 30 minutes after MCA occlusion, a decrease in ADC was observed in the MCA territory (Figure 1A). At this time, the tissue volume with reduced ADC was $458.2 \pm 97.8$ mm$^3$ and increased over 2 hours to $823.4 \pm 146.1$ mm$^3$ and stabilized at $708.4 \pm 166.7$ mm$^3$ at 180 minutes of MCA occlusion. Immediately after reperfusion, this volume significantly decreased ($P<0.05$ versus volume measured during MCA occlusion; Figure 1A–B). At Day 8, the volume of ADC-identified lesion was $409.6 \pm 111.7$ mm$^3$. At Day 45, no abnormal signal was observed on ADC maps (Figure 1A).

**Evolution of Absolute ADC Values**

The ADC values in the contralateral hemisphere remained stable over 45 days ($827.1 \pm 15.5$ $\mu$m$^2$/s). In the ischemic hemisphere, ADC values dramatically decreased ($502.1 \pm 8.3$ $\mu$m$^2$/s at 30 minutes) in the ADC-defined lesion and remained at this level throughout the MCA occlusion period (Figure 1C). As early as 5 minutes after the reperfusion, the ADC partially recovered but remained lower in comparison to contralateral values ($P<0.05$ versus 30 minutes of occlusion). At Day 8, the ADC values decreased and were comparable to the ADC measured during the MCA occlusion. At Day 45, the ADC fully normalized in the ipsilateral hemisphere ($807.3 \pm 14.4$ $\mu$m$^2$/s; Figure 1C).

**Evolution of FA**

FA increased during MCA occlusion in the ADC-defined lesion but was not normalized by reperfusion ($P<0.05$ versus contralateral FA; Figure 1D). However, at Day 8, we observed a decrease in FA values compared with those during MCA occlusion and in the contralateral hemisphere ($P<0.05$). At Day 45, the FA values measured in restricted regions of interest in WM and GM were not different from those in the contralateral side (Figure 2A).

**Volume of the T2-Defined Lesion**

At 120 minutes of occlusion, no abnormal signal was observed on T2 MRI (Figure 1A). However, at Day 8, we observed a hyperintensity affecting both cortical and subcortical regions ($361.6 \pm 130.8$ mm$^3$; Figure 1A). This volume was correlated to that measured on ADC maps ($R^2=0.88$, $P=0.019$). At Day 45, no abnormal signal was observed (Figure 1A). However, an atrophy of the ipsilateral hemisphere was found ($-3.89% \pm 1.47%$ versus contralateral hemisphere, $P=0.03$).

**Variability of the AFE**

An alteration of the homogeneity of the preferential direction of the diffusion was visible on the local homogeneity of AFE maps (Figure 2B) and was demonstrated by the calculation of the standard deviation of the AFE (Figure 2B). Indeed, there was an increase in the SD of AFE in the ipsilateral cortex and internal and external capsules when compared with the contralateral side ($P<0.05$).

**Fiber Tracking**

The analyses of the length of bundles of fibers showed a decrease in the number of small bundles tracked in the...
internal and external capsules ($P<0.05$ versus the contralateral side; Figure 2C–D).

**Behavioral Data**
Before MCA occlusion, all animals showed normal behavior with the tasks used.

**Neurological Score**
After MCA occlusion, all animals exhibited a long-lasting contralateral neurological deficit (Figure 3A).

**Tactile Stimulation**
A long-lasting decrease in tactile perception in the contralateral side was observed with a partial recovery after Day 8 ($P=0.026$ compared with Day 1; Figure 3B).

**Hill and Valley Staircase**
The hill staircase test revealed a long-lasting contralateral hemiparesis (Figure 4A) with some partial recovery. Similarly, the valley staircase revealed a contralateral deficit (Figure 4C). The valley staircase test evidenced a contralateral perception deficit because a decrease in the ipsilateral score (ie, contralateral space) was observed at Week 1 and Week 2 (Figure 4D).

**Adhesive Removal**
After MCA occlusion, a contralateral tactile deficit was observed (Week 1: deficit $=7500\% \pm 152\%, \ P=0.02$) with a partial recovery from Week 4 (deficit $=872\% \pm 319\%, \ P=0.01$) compared with Week 1.

A bilateral motor coordination deficit was observed. Time to remove adhesive labels was increased compared with preoperative measurements (Week 1: time $=1628\% \pm 675\%$ for the contralateral side; and deficit $=4615\% \pm 680\%$ for the ipsilateral side, $P<0.05$ compared with Day-1). On the ipsilateral and contralateral sides, we observed a partial recovery from Week 4 (ipsilateral deficit $=1015\% \pm 491\%$, contralateral deficit $=1325\% \pm 717\%, \ P<0.05$ compared with Week 1).

**Histology Data**
At Day 45, Luxol fast blue staining revealed an alteration of fine networks of fibers as shown in the posterior part of the external capsule (Figure 5A). The large network of fibers seems unaltered. The microscopic analyses showed a changed orientation of the bundles of myelin in the internal and external capsules (Figure 5B).

**Correlations Between Behavioral Deficits and WM Alterations**
The significance of the crosscorrelations between behavioral deficits and WM abnormalities are summarized in the Table. The strongest correlation was found between alterations in the internal capsule (SD of AFE and the significant decreased
number of tracked fibers in the ipsilateral hemisphere) and the chronic motor and sensory deficits.

**Discussion**

Through the use of DTI MRI in a pertinent and robust model of stroke in the nonhuman primate, the present study documented the evolution of ischemia-induced brain damage not only in GM, but also WM. The major finding is that pathological changes in WM were detectable even in the absence of a visible lesion on conventional MRI parameters (ie, T2-weighted imaging and diffusion-weighted imaging). These structural changes were correlated with functional deficits and could explain, in part, the persistence of stroke-induced functional deficits. Because the imaging paradigms used in our study can be readily applied to clinical situations, our data argue for the importance of the analyses of WM in patients with stroke. Moreover, the data also plead for the systematic analysis of WM integrity after therapeutic interventions in both animal models and patients.

The use of DTI MRI allows the analysis of the evolution of ADC and FA in acute, subacute, and chronic stages of ischemia. These parameters, which reflect the integrity of brain tissue, were reported in several animal models and in human pathology.9,10 However, their temporospatial evolution is not fully characterized. In our studies, the absolute ADC values are comparable to those reported in rodents, nonhuman primates, and humans, both in healthy and injured tissues. The volume of ADC-defined lesion progressively increased during the first 2 hours of occlusion with a slight reduction at 180 minutes, which may be attributed to the activation of the collateral cortical circulation. The absolute ADC values in the lesion remained stable over MCA occlusion, indicating that the severity of the cytotoxic edema did not evolve during the occlusion. However, immediately after reperfusion, the ADC values recovered partially but still remained below the normal values in the contralateral hemisphere. At Day 8, the presence of reduced ADC in the ipsilateral hemisphere suggests that the evolution of the lesion is slower in the marmoset in comparison to that reported in rodents. Indeed, the acute reduction of ADC values for rodents normalizes, in general, 4 days after the occlusion.11 The ADC evolution in the marmoset was closer to that in humans and other nonhuman primates, in which the ADC was described to normalize 10 days after ischemia.10,12 At the chronic stage of ischemia (ie, Day 45) despite a slight hypointensity possibly consecutive to astroglisis, ADC val-
subjects of change in earlier but not in chronic phases of ischemia. To ensure analytic accuracy, we quantified the SD of AFE in areas of the temporal cortex as well as the internal and external capsules, all of which initially displayed a homogenous preferential direction of the diffusion in the healthy hemisphere. This approach made apparent a dramatic increase in the SD of AFE in all these regions, suggesting a disorganization of the brain tissue.

To further analyze the alterations in WM, we used the fiber tracking approach using MedINRIA software. This approach, which is increasingly used in the examination of WM in the brain and spinal cord, revealed a marked disorganization of the fibers in the internal and the external capsules and was clearly visible on tractographs and on histological sections. This profile of changes is in concordance with that reported in primates, including humans. Interestingly, when bundles of fibers were analyzed as a function of their length, short fibers were preferentially altered. The significance of this alteration is not known but it may underlie the persistent sensorimotor deficits described in the present and previous studies.

This experimental approach is characterized by a long-lasting contralateral hemiparesis, hemianesthesia, and a transient hemineglect. The persistence of functional deficits is a multifactorial process including the neuronal loss and astrogliosis, as suggested by the correlation between the cerebral atrophy and the chronic deficits in the neurological score ($P=0.02$, $R^2=0.87$). Despite a limited area of investigation, the WM alterations may be more closely involved in the chronic deficits, in particular alterations in the internal capsule, as suggested by the multiple correlations with the chronic functional deficits. Overall, the data show first that DTI MRI reveals alterations in the corticospinal tract despite pseudonormalization of conventional MRI-derived parame-

### Table. Crosscorrelations Between Functional Deficits and Alterations in White Matter at the Chronic Stage of Ischemia (ie, Day 42 or Week 7)

<table>
<thead>
<tr>
<th>Behavioral Test</th>
<th>Methods of White Matter Analysis</th>
<th>Internal Capsule</th>
<th>External Capsule</th>
<th>Temporal Superior Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurological score</td>
<td>SD of the AFE</td>
<td>0.94*</td>
<td>0.77</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>FB</td>
<td>0.75</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>Tactile stimulation</td>
<td>SD of the AFE</td>
<td>0.89*</td>
<td>0.46</td>
<td>0.88*</td>
</tr>
<tr>
<td></td>
<td>FB</td>
<td>0.82*</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>Hill staircase</td>
<td>SD of the AFE</td>
<td>0.95*</td>
<td>0.47</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>FB</td>
<td>0.99*</td>
<td>0.85*</td>
<td></td>
</tr>
<tr>
<td>Valley staircase</td>
<td>SD of the AFE</td>
<td>0.90*</td>
<td>0.46</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>FB</td>
<td>0.99*</td>
<td>0.92*</td>
<td></td>
</tr>
<tr>
<td>Adhesive removal</td>
<td>Contralateral contact</td>
<td>0.73</td>
<td>0.72</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>SD of the AFE</td>
<td>0.57</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FB</td>
<td>0.84*</td>
<td>0.40</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Contralateral removal</td>
<td>0.90*</td>
<td>0.91*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD of the AFE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FB</td>
<td></td>
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</tr>
</tbody>
</table>

AFE indicates angle of the first eigenvector; FB, fiber tracking.

* $P<0.05$, Spearman correlation.
ters such as T2, ADC, and FA; second, these alterations could be related to the sensorimotor deficits. Consequently, our results argue for the importance to include multiparameter WM analyses as an ancillary investigation not only in patients with stroke, but also in animal models, in which therapeutic strategies expected to improve functional recovery are tested.

**Sources of Funding**

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**Disclosures**

None.

**References**

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SUPPLEMENTAL MATERIAL

Supplemental methods

Methods to define the location of regions of interest (ROI)
To place the ROI on the contralateral hemisphere, we used maps in which the preferential direction of diffusion was color-coded (green=anterior-posterior, blue=dorso-ventral and red=latero-median orientation). These maps allowed us to define three ROIs in the contralateral hemisphere in which the preferential direction of diffusion was homogeneous. These ROIs were then mirror-copied on the ipsilateral hemisphere based only on structural landmarks visible on b=0 images, so as to avoid any influence of the homogeneity of the preferential direction of the diffusion, which might bias the placement of the ROI on the ipsilateral side (see supplemental figure S2 http://stroke.ahajournals.org).

Analysis of the homogeneity of the local preferential direction of diffusion
For the analysis of the preferential direction of diffusion, we analyzed the DTI-MR images using an approach similar to that developed by Wu and colleagues. To obtain a quantifiable index of the preferential direction of diffusion, we generated maps of the Angle of projection of the First Eigenvector (AFE) in the coronal plane (see supplemental figure S2 http://stroke.ahajournals.org). The ipsi- and contralateral ROIs, previously defined on the color maps, were transposed on these AFE maps and the SD of these angles within the ROIs were calculated.

Maps of the local homogeneity of the AFE
The SD of the AFE in the neighborhood of each pixel was calculated (where the local area includes the pixel itself and its 8 neighborhood). The color scale of the resultant maps is inverted so that its intensity matches that of FA maps (the clearest values represent a lower SD values in contrast with the darker pixels which present a higher SD).

Fiber tracking parameters
To determine a quantifiable index of the impact of localized alterations in the WM on the fibers network, we performed a fiber-tracking analysis with MedINRIA® software. We have fixed the FA threshold to 0.2, the minimal length of bundles to 10mm, and the smoothness at 20. The number of tracked bundles so defined was classified according to their length.

Correlations
For the correlation study between white matter (WM) alterations and behavioral deficits, we have used first the significant persistent deficits determined by the behavioral tests in the chronic stage (D41 or W7, in % of deficits). These data were correlated to significant WM disturbances at the chronic stage (the SD of AFE in the ipsilateral ROIs and the significant numbers of bundles lost compared to the contralateral one).
Supplemental figure 1: Experimental design of MRI protocol. MCAO= middle cerebral artery occlusion
Supplemental figure 2: A colors maps: indicated the preferential direction of diffusion (green=anterior-posterior, blue=dorso-ventral and red=latero-median orientation). B: representation of the projection of the first eigenvector $\varepsilon_1$ on the coronal plan which defines the angle $\theta$. C: $\theta$ values maps on which the standard deviation (SD) of $\theta$ was calculated.
Supplemental references
