Early Predictive Biomarkers for Lesion After Transient Cerebral Ischemia

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Background and Purpose—Despite the improving imaging techniques, it remains challenging to predict the outcome early after transient cerebral ischemia. The aim of this study was thus to identify early metabolic biomarkers for outcome prediction.

Methods—We modeled transient ischemic attacks and strokes in mice. Using high-field MR spectroscopy, we correlated early changes in the neurochemical profile of the ischemic striatum with histopathologic alterations at a later time point.

Results—A significant increase in glutamine was measured between 3 hours and 8 hours after all ischemic events followed by reperfusion independently of the outcome and can thus be considered as an indicator of recent transient ischemia. On the other hand, a reduction of the score obtained by summing the concentrations of N-acetyl aspartate, glutamate, and taurine was a good predictor of an irreversible lesion as early as 3 hours after ischemia.

Conclusions—We identified biomarkers of reversible and irreversible ischemic damage, which can be used in an early predictive evaluation of stroke outcome. (Stroke. 2011;42:799-805.)

Key Words: biological markers ■ magnetic resonance spectroscopy ■ middle cerebral artery occlusion ■ transient ischemic attack ■ stroke
after reperfusion when neither T2 hyperintensities nor abnormal water diffusion are detectable by MRI.

**Methods**

All animal experiments were conducted in accordance with the guidelines of and with approval of the cantonal veterinary office.

**Transient Middle Cerebral Artery Occlusion in the Mouse**

For MR studies, 29 male ICR-CD1 mice (20 to 33 g; Charles River, L’Arbresle, France) underwent transient middle cerebral artery occlusion (tMCAO) as described previously. Briefly, mice were anesthetized and maintained under 1.5% to 2% isoflurane in 30% oxygen and 70% nitrous oxide using a face mask. At 0 hour, ischemia was induced by inserting a silicone-coated nylon filament (diameter: 0.17 mm; Doccol Co, Redlands, CA) through the left common carotid artery into the internal carotid artery. The filament was withdrawn after 10 minutes allowing reperfusion. Regional cerebral blood flow was measured throughout the entire operation by a laser-Doppler flowmetry (Periflux 5000; Perimed) with a flexible probe fixed on the skull, 2 mm posteriorly and 6 mm laterally from the bregma. The average regional cerebral blood flow of included mice was 15%±5% (no lesion) and 14%±4% (lesion) of the baseline level during ischemia and 74%±16% (no lesion) and 74%±20% (lesion) of the baseline after reperfusion. Throughout surgery and until awaking, the rectal temperature was maintained at 37±0.5°C with a temperature control unit (FHC Inc).

Mice were euthanized 30 hours after ischemia. Among these mice, 4 were excluded due to unsatisfactory ischemia or spectral quality (2

![Figure 1](image.png)

**Figure 1.** The 2 10-minute middle cerebral artery occlusion (MCAO) subgroups have different MR spectra at all measured time points. Evolution of MR spectra over time and T2-weighted images at 24 hours. Each column corresponds to a representative mouse from each group (sham, TIA, minor stroke). Each row shows typical measurements of ¹H-MR spectra at 3 hours (A), 8 hours (B), and 24 hours (C) or T2-weighted images at 24 hours after ischemia (D) of the same mouse. All spectra were scaled relative to the heights of the phosphocreatine+ creatine and macromolecule peaks in the spectrum of the sham operated group at 3 hours (A). Arrows show the apparent changes in the spectra from both 10-minute tMCAO groups compared with sham-operated animals. Cr indicates creatine; Gln, glutamine; Glu, glutamate; GPC, glycerophosphocholine; Lac, lactate; Mac, macromolecules; myo-Ins, myo-inositol; PCr, phosphocreatine; Tau, taurine; T2WI, T2-weighted images.

![Figure 2](image.png)

**Figure 2.** Evolution over time of some metabolites involved in the developing lesion after ischemia. The moderate stroke group is described in a recent publication. (A) Glutamine (Gln), (B) glutamate (Glu), (C) taurine (Tau), (D) N-acetyl aspartate+glutamate+taurine (NAA+Glu+Tau). Error bars reflect SD.
The design of the present study was to compare animals below and above the threshold for irreversible ischemic
lesions to find biomarkers for outcome prediction. We noticed that in our model, 10 minutes of ischemia produced small striatal lesions in half the mice only, indicating that this ischemia duration lies at this threshold. These mice were divided into 2 groups depending on the presence (n=7) or absence (n=10) of a lesion on T2-weighted MR images at 24 hours. These mice were compared with sham-operated and 30-minute tMCAO mice. All animals were euthanized 30 hours after ischemia onset for histology and lesion size determination (Supplemental Figure I, available at http://stroke.ahajournals.org). Small lesions were detected 30 hours after 10 minutes tMCAO (3.3±2.9 mm$^3$) in the lesion 10-minute tMCAO group consistent with the lesion size measured at 24 hours on MR images of 8.3±3.5 mm$^3$. The slight difference in lesion size between MRI and Nissl staining (P=0.026, 2-tailed Mann-Whitney test) probably results from a small overestimation of the lesion size by MRI due to edema or to the 6-hour difference between these measurements. It is consistent with similar previously observed differences.12

The neuroscore determined at 30 hours was not significantly different between no lesion 10-minute tMCAO (0 [0 to 0.5]) and lesion 10-minute tMCAO (0 [0 to 1]) but between both of them and 30-minute tMCAO (1 [0.5 to 2]; P<0.01 and P<0.05, respectively, using Kruskal-Wallis followed by Dunn multiple comparison test). There was complete recovery of the neurological deficit at 30 hours in almost all animals in our "no lesion" tMCAO group and no lesion was detected by MRI. The short ischemia in this group therefore corresponds to TIAs according to the new definition proposed by Easton et al.21 We thus called it the "TIA group." Similarly, we called the 10-minute tMCAO group with lesion the "minor stroke" group and the 30-minute tMCAO "moderate stroke" group.

**MR Assessments of Metabolic Evolution After Ischemia**

At 3 hours after transient ischemia, there was no detectable signal change in any group on either T2-weighted images or on ADC maps (6.82±0.19×10$^{-5}$ mm$^2$/s in ipsilateral striatum versus 6.85±0.24×10$^{-4}$ mm$^2$/s in contralateral striatum with P=0.80 by paired Student $t$ test). On the contrary, several changes were apparent in the spectra of both TIA and minor stroke groups (Figure 1) as early as 3 hours after ischemia: increased glutamine was observed in both groups, which was accompanied by a decrease in glutamate only in the minor stroke group. The metabolic profile of the sham-operated mice did not vary. In mice that were developing a lesion, additional metabolite changes appeared at 8 hours (increased lactate) and 24 hours (decreased NAA and myo-inositol), whereas the neurochemical profile of the TIA group almost normalized at 24 hours.

**Neurochemical Changes Can Be Used to Characterize the Severity of Transient Ischemic Insults**

The visually noticeable MR spectra changes were confirmed by quantitative measurements of the neurochemical profiles (Figures 2 and 3; Supplemental Figure II), which further revealed additional changes. The increase in glutamine 3 hours after ischemia was a common hallmark of all transient ischemic episodes regardless of the duration. It returned to basal levels more quickly in the absence of a lesion (Figure 2A; Supplemental Figure II).12 On the other hand, glutamate, NAA, and taurine were significantly decreased as early as 3 hours after the insult only in animals with irreversible damage (Figures 2B–C and 3B; Supplemental Figure II). We summed the concentrations of these 3 metabolites and noted that this composite score separated mice that will develop a lesion or not (Figure 2D), whereas lactate or NAA concentrations were unable to separate them before 8 hours (Figure 3A–B).
Scatterplotting the NAA+glutamate+taurine score against the glutamine concentration at 3 hours (Figure 4) allowed excellent separation of the 4 conditions with nearly no overlap, especially between the TIA and stroke groups. After 3 hours of permanent ischemia (Supplemental Figure III), the metabolism is different, showing a decrease of glutamine instead of an increase. However, the composite score of NAA+glutamate+taurine is also decreased, indicating that a lesion will develop. It is thus also a good predictor in the absence of reperfusion.

### Neurochemical Changes Predict Ischemic Lesion Size

The correlations between lactate or NAA at 3 hours and MRI lesions at 24 hours \((R=0.73\) and \(R=0.77\), respectively) were found to be less precise than the correlation between NAA+glutamate+taurine at 3 hours and the resulting lesion size at 24 hours \((R=0.90\); Figure 5A). Even if, at later time points, the prediction of lesion size with lactate \((R=0.76\) at 8 hours and \(R=0.78\) at 24 hours; Figure 5D) and NAA \((R=0.76\) at 8 hours and \(R=0.95\) at 24 hours; Figure 5D) becomes more accurate, the sum of NAA+glutamate+taurine remains the best predictor up to 8 hours \((R=0.87\); Figure 5D) and is similar to NAA at 24 hours \((R=0.97\); Figure 5D).

### Discussion

By measuring 19 neurochemical constituents in the mouse striatum 3 and 8 hours after cerebral ischemia of varying intensity, we have shown that \(^1\)H MRS highlights metabolic changes reflecting a recent transient ischemia and predicts the outcome at 24 hours. Ischemia triggered a transient increase in glutamine after reperfusion independent of its severity, whereas irreversibly damaged tissue was characterized by a significant decrease in NAA, glutamate, and taurine independently of reperfusion. To our knowledge, this is the first time that predictive biomarkers of cerebral ischemia have been described at an early time point when no abnormality can be detected with standard and widely used clinical MRI techniques.\(^6,22\)

It is remarkable to see that the only metabolic changes common to all ischemic conditions after reperfusion is increased glutamine, which we previously hypothesized to result from the transformation of excitotoxic glutamate by astrocytes after restoration of energy supplies.\(^12\) Our observation of reduced glutamine levels 3 hours after permanent ischemia (Supplemental Figure III) supports this hypothesis. Thus, the elevated glutamine can be used as a biomarker of reperfused ischemia at an early time point.

Of the more established metabolic changes after ischemia, it is well known that the NAA concentration correlates well with observed ischemic lesion size and extent of neuronal death.\(^8–10,23,24\) However, this cannot be reliably correlated earlier than 8 to 24 hours after ischemia (Figure 4) probably because neuronal death is a gradual process after mild to moderate ischemia. In addition to NAA, increased lactate is a hallmark of ischemia\(^23,25,26\) that has been described in patients with TIAs.\(^27,28\) However, the early measurements in the present study (showing no change in lactate concentration after 10 minutes middle cerebral artery occlusion) suggest that lactate may not reliably identify ischemic episodes or predict ischemic outcome. The difference in lactate concentrations between patients with TIA and our 10-minute ischemic mice might be due to the time of \(^1\)H MRS measurements, which were performed >1 day after the attack in the human studies. Moreover, the lactate profile is complex with 2 episodes of lactate accumulation,\(^29\) and probably lactate content at early stages does not reliably predict the outcome.

In addition, 2 further metabolites mainly linked to neuronal metabolism and strongly involved in ischemia,\(^8–10,23,24,30\) glutamate and taurine, decreased in the minor stroke group. The glutamate decrease is consistent with the well-known glutamate release from neurons during ischemia, which is then taken up by astrocytes and either transformed to glutamine or oxidized after reperfusion.\(^31\) On the other hand, taurine is thought to be neuroprotective by both acting on osmoregulation\(^32\) and neuromodulation.\(^33\) Recently, Saran-

sari and Oja\(^10\) have shown that taurine release in ischemia is modulated by glutamate receptors, which may explain the correlation between taurine decrease and severity of ischemia. Importantly, the final ischemic lesion stands in excellent correlation with our cumulative score of NAA+glutamate+taurine not only at 24 hours, but also as early as 3 hours and 8 hours (Figure 5). Therefore, this combination score is well adapted for the prediction of ischemia severity.

The highly predictive power of these biomarkers in animal models now needs testing in human patients with TIA and...
stroke. Indeed, our goal is to test if MRS biomarkers could be used for early diagnosis for treatment optimization. The distinction between TIA and stroke is an important but difficult question based on the definition of TIA. The biomarkers described in this study could help distinguish between TIA and stroke only if we use the new definition of TIA. In any case, both patients with TIA and those with stroke have to be observed in the hospital. The importance of the biomarkers will be to help determine if a patient with an acute neurological deficit will later develop a lesion and thus if neuroprotective treatment is needed. Moreover, the observation of glutamine increase in patients with TIA can help determine which areas had a lack of perfusion. The advantage of MRS compared with routinely used imaging techniques is its capacity to identify recent ischemic events and predict outcome in the first hours after reperfusion, at a time when none of the other techniques detect any abnormality (Figure 6) and where diagnostic blood biomarkers are less precise.

In conclusion, the ability to identify ischemic damage and predict the outcome (neuronal loss or recovery) based on metabolite concentrations between 3 and 8 hours after ischemia opens exciting perspectives for stroke diagnosis and treatment. Given that the biomarkers described in this study can also be measured in humans using clinical 3-T MR equipment, 1H-MRS could thus provide critical information for the evaluation and diagnosis in patients with stroke and TIA.

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Disclosures

None.

References


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Supplementary Figure 1

Characterization of degrees of ischemic severity in mice.

Histological characterization of the 3 groups of mice (sham, TIA, minor stroke), compared to moderate stroke, 30h after the beginning of ischemia. The moderate stroke group is taken from our recent publication\(^1\).

Immunohistochemistry and Fluoro-Jade B staining were performed as described by Benakis et al.\(^2\), using the following antibodies: mouse anti-GFAP (1/500, MAB 3402 from Millipore, UK), mouse anti-Hsp70 (1/200, \#3096-100 from Biovision, Mountain View, California) or rat anti-cd11b (1/100, MCA74G from AbD Serotec, Oxford, UK) and biotinylated mouse (1/200, BA-2000 from Vector laboratories, Burlingame, California) or rat (1/500, BA-9400 from Vector laboratories) secondary antibody.

A. Nissl staining used to measure lesion size; the squares represent the area where images b-e were taken. B. Fluorojade B staining shows dying neurons in the lesion determined by Nissl staining. C. Glial fibrillary acidic protein (GFAP) immunoreactivity is present in all ischemic conditions. D. Heat shock protein 70 (Hsp70)
immunoreactivity is mainly present in minor stroke group, E. cd11b immunoreactivity is usually not detected after 10min MCAO, but rather after 30min MCAO. Scale bar: 100µm for general images and 25µm for inserts.


Supplementary Figure 2

Neurochemical profiles in the striatum at 3 (A), 8 (B) and 24h (C) after 10min ischemia. Statistical differences between sham and TIA is shown with « * » (*P<0.05, ***P<0.001) and between sham and minor stroke with « $ » ($P<0.05, $$P<0.01, $$$P<0.001). The statistical test used was one way ANOVA followed by the Dunnett’s multiple comparison’s test; n= 7 for sham, n=10 for TIA, n=7 for minor stroke. Abbreviations: alanine (Ala), ascorbate (Asc), apartate (Asp), creatine (Cr), g-aminobutyric acid (GABA), glucose (Glc), glutamine (Gln), glutamate (Glu), glycine (Gly), glycerophosphocholine (GPC), glutathione (GSH), myo-inositol (Ins), lactate (Lac), macromolecules (Mac), N-acetyl-aspartate (NAA), N-acetyl-aspartyl-glutamate (NAAG), phosphocholine (Pcho), phosphocreatine (PCr), phosphoatyletanolamine (PE), taurine (Tau).
Supplementary Figure 3

Comparison between transient stroke and permanent occlusion.

In order to test the validity of our biomarkers in the absence of reperfusion, 6 mice (25-31g) underwent permanent MCAO with the same procedure as tMCAO, but without withdrawing of the filament. They were sacrificed 3h after the beginning of ischemia, just after MRS measurements.

A: One typical MR spectrum at 3h after permanent occlusion (top row, preliminary data), minor stroke (middle row, as in Figure 1) or sham operation (bottom row, as in Figure 1). The glutamine is highlighted in gray.

B: Quantified glutamine (Gln, black bars) and N-acetyl-aspartate + glutamate + taurine (NAA+Glu+Tau, white bars) contents at 3h from sham, stroke (minor and moderate) and permanent occlusion. Gln is decreased in
permanent ischemia compared to sham and becomes significantly decreased in comparison to stroke (p<0.001, Kruskal-Wallis). The combined score obtained by adding the concentrations of NAA+Glu+Tau is significantly decreased in stroke and permanent occlusion compared to sham (p<0.01, Kruskal-Wallis) and appropriately predicts the development of a lesion later on. Error bars are s.d.