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Temporal Window of Metabolic Brain Vulnerability to Concussion: A Pilot ^{1}H-Magnetic Resonance Spectroscopic Study in Concussed Athletes—Part III

OBJECTIVE: In the present study, the occurrence of the temporal window of brain vulnerability was evaluated in concussed athletes by measuring *N*-acetylaspartate (NAA) using proton magnetic resonance (^{1}H-MR) spectroscopy.

METHODS: Thirteen nonprofessional athletes who had a sport-related concussive head injury were examined for NAA determination by means of ^{1}H-MR spectroscopy at 3, 15, and 30 days postinjury. All athletes but three suspended their physical activity. Those who continued their training had a second concussive event and underwent further examination at 45 days from the initial injury. The single case of one professional boxer, who was studied before the match and 4, 7, 15, and 30 days after a knockout, is also presented. Before each magnetic resonance examination, patients were asked for symptoms of mild traumatic brain injury, including physical, cognitive, emotional, and sleep disturbances. Data for ^{1}H-MR spectroscopy recorded in five normal, age-matched, control volunteers, who were previously screened to exclude previous head injuries, were used for comparison. Semiquantitative analysis of NAA relative to creatine (Cr)- and choline (Cho)-containing compounds was performed from proton spectra obtained with a 3-T magnetic resonance system.

RESULTS: Regarding the values of the NAA-to-Cr ratio (2.21 $\pm$ 0.11) recorded in control patients, singly concussed athletes, at 3 days after the concussion, showed a decrease of 18.5% (1.80 $\pm$ 0.04; $P < 0.001$). Only a modest 3% recovery was observed at 15 days (1.88 $\pm$ 0.1; $P < 0.001$); at 30 days postinjury, the NAA-to-Cr ratio was 2.15 $\pm$ 0.1, revealing full metabolic recovery with values not significantly different from those of control patients. These patients declared complete resolution of symptoms at the time of the 3-day study. The three patients who had a second concussive injury before the 15-day study showed an identical decrease of the NAA-to-Cr ratio at 3 days (1.78 $\pm$ 0.08); however, at 15 days after the second injury, a further diminution of the NAA-to-Cr ratio occurred (1.72 $\pm$ 0.07; $P < 0.05$ with respect to singly concussed athletes). At 30 days, the NAA-to-Cr ratio was 1.82 $\pm$ 0.1, and at 45 days postinjury, the NAA-to-Cr ratio showed complete recovery (2.07 $\pm$ 0.1; not significant with respect to control patients). This group of patients declared a complete resolution of symptoms at the time of the 30-day study.

CONCLUSION: Results of this pilot study carried out in a cohort of singly and doubly concussed athletes, examined by ^{1}H-MR spectroscopy for their NAA cerebral content at different time points after concussive events, demonstrate that also in humans, concussion opens a temporal window of brain metabolic imbalance, the closure of which does not coincide with resolution of clinical symptoms. The recovery of brain metabolism is not linearly related to time. A second concussive event prolonged the time of NAA normalization by 15 days. Although needing confirmation in a larger group of patients, these results show that NAA measurement by ^{1}H-MR spectroscopy is a valid tool in assessing the full cerebral metabolic recovery after concussion, thereby suggesting its use in helping to decide when to allow athletes to return to play after a mild traumatic brain injury.

KEY WORDS: Brain vulnerability, Concussion, ^{1}H-Magnetic resonance spectroscopy, *N*-Acetylaspartate, Second impact syndrome

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Despite hundreds of studies and decades of research, there is currently no universally accepted definition of mild traumatic brain injury (mTBI) or concussion (3, 12, 15, 17, 18, 46, 53), even though it is often described as a traumatically induced alteration in mental status, not necessarily with loss of consciousness. As a direct consequence, diagnosis and management of mTBI represent a dilemma of inestimable proportion, considering that more than 75% of all TBIs are suspected to be mild and the majority of these are going unreported and unassessed by medical professionals. The situation is even more confused in sport-related concussions for which the fundamental question of when an athlete can return to play after an episode of mTBI is one of the most challenging endeavors for sports medicine practitioners (34, 41, 60, 61). In addition, many team physicians' decisions are often based on limited observations and sideline evaluations and may frequently be made under intense pressure from coaches, fans, sponsors, and other players, who are all eager to see the injured athlete return to play as quickly as possible.

In the attempt to provide guidance, more than 20 management guidelines have been published. In these, concussion management strategies were based mostly on the opinion of groups of experts rather than on empirical findings (9, 12, 33, 34, 57, 60, 61). However, none of these guidelines is sufficiently supported by data from research studies to substantiate any particular return-to-play protocol. At present, in addition to the subjective indication of the patient, cognitive neuropsychological tests are widely used to assess the condition of mildly injured athletes. This type of monitoring has been considered one of the cornerstones for return to play after a concussion (48, 49, 50, 65), even though concerns have been raised, including the question of when they should be used in the management and assessment of concussion (22, 31, 62). Therefore, the need to find objective parameters to evaluate the extent of and recovery from concussion-induced cerebral damage has been stressed recently (15).

The concussive insult is responsible for a temporal neuronal dysfunction that often resolves completely with minimal or absent structural damage to tissues or cell death (30, 37, 38). It is also very clear that a complex biochemical derangement, triggered by the traumatic insult, adversely affects fundamental metabolic pathways of the cells that survive the insult (energy supply-related pathways, oxidative and nitrosative stress pathways, and others). In fact, markers indicating the mitochondrial phosphorylating capacity, *N*-acetylaspartate (NAA) homeostasis and the overall oxidoreductive state, are altered for days after injury (27, 43, 69, 70, 77). Several authors and recent studies from our laboratories demonstrated that, although clinically "mild," a concussive injury is capable of inducing an extremely dangerous state of brain vulnerability to the point that, if a second concussion occurs within a certain time period, the additional damage can disproportionately lead to a condition similar to that of severe TBI (42, 45, 59, 76, 78). Furthermore, bench data demonstrated either that a well-defined temporal window of such vulnerability indeed exists, ending when the aforementioned neuronal biochemical disturbances are overcome, or that NAA clearly mirrors variations of the cerebral energy state (79). It is worth recalling that NAA is present in such a high concentration within neurons (~10 mmol/l brain water) that it is easily visible by proton magnetic resonance (^{1}H-MR) spectroscopy. This technique is based on the ability to localize the magnetic resonance signal into a specific volume of tissue, thus providing a real-time "image" of the brain neurochemistry (10, 26, 39). In a typical proton spectrum of the human brain, NAA represents one of the largest peaks and is considered a neuron-specific metabolite. A large body of clinical and experimental evidence suggests that NAA is a highly sensitive biomarker of neuronal vitality (36) and that TBI severity can be biochemically graded not only by measuring important parameters of energy metabolism (e.g., ATP, ATP-to-ADP ratio, and NAD^+) but also by determining cerebral NAA (1, 68, 69, 73).

On the basis of this background, the objective of the present study was to investigate concussive biochemical neuronal damage in mildly brain-injured athletes by the ^{1}H-MR spectroscopic quantification of NAA. The robust experimental evidence collected to date prompted us to undertake this research in a cohort of concussed athletes studied at different time points in the attempt to "biochemically" characterize the concussion, to estimate the coincidence in the time-related evolution of clinical symptoms and brain metabolic recovery, and to verify the existence of a window of metabolic brain vulnerability in humans.

PATIENTS AND METHODS

Patient Selection

After having provided informed consent according to institutional procedures, 13 nonprofessional athletes of different sport disciplines who had a sport-related concussive head injury (defined as a traumatically induced alteration in mental status, not necessarily with loss of consciousness) were enrolled in the study. Athletes underwent a magnetic resonance imaging (MRI) and a proton spectroscopy examination at 3 days postinjury, followed by two additional ^{1}H-MR spectroscopic examinations at 15 and 30 days postinjury. During this observation period, patients were advised to refrain from any further physical activity. However, three patients did not suspend their training and sustained a second concussive injury during the follow-up period, at precisely 10, 12, and 13 days after the first injury. This subgroup of athletes underwent a further examination at 45 days from the initial injury. The single case of one professional boxer, who was studied before the match and 4, 7, 15, and 30 days after a knockout characterized by the clinical signs of concussion, is also presented. Results collected from all patients were compared with those observed in five normal, age-matched, control volunteers who were screened to exclude previous head injuries.

Any intracranial lesion observed on the first MRI scan automatically excluded the candidate from the study. Before each magnetic resonance examination, patients were asked for symptoms of mTBI, including physical, cognitive, emotional, and sleep disturbances.

MRI and ^{1}H-MR Spectroscopy Acquisition Techniques

Semiquantitative analysis of NAA was performed after proton spectra were obtained by use of a 3-T system (Philips Intera Achieva; Philips Medical Systems, Eindhoven, The Netherlands). Relative NAA levels were calculated as the ratio of the peak area of NAA with that of

either creatine (Cr)- or choline (Cho)-containing compounds. For conventional MRI studies, T1- and T2-weighted turbo-spin-echo images were acquired in axial, coronal, and sagittal planes, and, to rule out even the smallest amount of intracerebral blood, fast field echo T2* sequences were used. A multichannel coil (eight channels) sense head with 4-mm slice thickness, 1-mm gap, and a field of view of 230 mm was used for all MRI sequences. After localized shimming and water suppression, the spectroscopic examination was performed using a point-resolved spectroscopy pulse sequence, with the following settings: echo time, 144 milliseconds; repetition time, 2000 milliseconds; spectral bandwidth, 2000; and 128 acquisition cycles. MRI scans acquired on axial, coronal, and sagittal planes to facilitate optimal three-dimensional placement of the voxel, adjacent to the cortical-subcortical junction to include only the white matter of the frontal lobes bilaterally. This location was chosen to obtain the most homogeneous data as possible. Thus, a spectrum from a single voxel customized to sample a volume of interest of 3.375 cm^3 (1.5 × 1.5 × 1.5 cm) was finally obtained (acquisition time about 5 min for each voxel). In follow-up studies, exact repositioning of the voxel on the same acquisition planes obtained in the previous MRI study was achieved by using dedicated software (SameScan; Philips Medical Systems). Metabolite intensity ratios (NAA-to-Cr and Cho-to-Cr) were automatically calculated at the end of each acquisition using dedicated software (SpectroView; Philips Medical Systems), by which gaussian-fitted peak areas relative to a baseline computed from a moving average of the noise regions of each spectrum were determined.

Statistical analysis

Differences between controls and all concussed athletes were assessed by one-way analysis of variance followed by Fisher's protected least-significant difference post hoc test. Differences among the three time points in the group of singly concussed athletes and the four time points of doubly concussed athletes were assessed by two-way analysis of variance followed by Fisher's protected least-significant difference post hoc test. The values at 3, 15, and 30 days recorded in singly versus doubly concussed athletes were compared by the Wilcoxon rank-sum test. A P value less than 0.05 was considered significant.

RESULTS

Study Population

A total of 52 single voxel ^{1}H-MR spectroscopic studies (five volunteers, 10 patients studied at three time points, three patients who had second concussion studied at four time points, and one professional boxer studied at five time points) were performed, for a total of 104 brain spectra acquired successfully.

The duration of each study averaged 16 ± 1 minute, with no complications. All patients were admitted as outpatients and discharged 1 hour after the study. Demographic data are reported in *Table 1*. The mean age of the entire study population was 27 ± 4.8 years, ranging from 20 to 35 years.

^{1}H-MR Spectroscopic Analysis of NAA in Normal Volunteers

After having obtained informed consent, we obtained a bilateral single voxel ^{1}H-MR spectroscopic study in five age-matched volunteers (ranging in age from 22 to 34 yr). *Figure 1*

TABLE 1. Demographic data, sport activity, type of concussion, mechanisms of concussion and clinical symptoms of 13 nonprofessional athletes and one professional athlete who had single (n = 11) or double (n = 3) concussion

Patient no.	Age (yr)/sex	Sport practiced	Concussion	Mechanisms of concussion	Symptoms
1	21/M	Alpine skiing	Single	Contracoup injury caused by low back impact	Headache
2	24/M	Soccer	Single	Knee to head impact	Headache, nausea, retrograde amnesia
3	32/F	Kickboxing (light contact)	Single	Foot to head impact	Headache, sleeping more than usual
4	27/F	Kickboxing (light contact)	Single	Foot to head impact	Headache, troubling falling asleep
5	20/M	Soccer	Single	Head to head impact	Headache, "foggy" vision, troubling falling asleep
6	27/M	Boxing (amateur)	Single	Fist to chin impact	Headache, anterograde amnesia
7	22/F	Kickboxing (light contact)	Single	Foot to head impact	Headache, fatigue, nervousness
8	24/F	Kickboxing (light contact)	Single	Fist to face impact	Headache, irritability
9	27/M	Rugby	Single	Head to trunk impact	Headache, retrograde amnesia
10	28/F	Kickboxing (light contact)	Single	Foot to head impact	Headache, difficulty concentrating
11	29/M	Boxing (amateur)	Double	Fist to chin impact	Headache, sleeping more than usual
12	30/M	Boxing (amateur)	Double	Fist to chin impact	Headache, difficulty concentrating, irritability
13	25/M	Kickboxing (full contact)	Double	Foot to face impact	Headache, feeling "foggy," irritability
14	35/M	Boxing (professional)	Single	Fist to chin impact	Headache, anterograde amnesia, dizziness, tingling

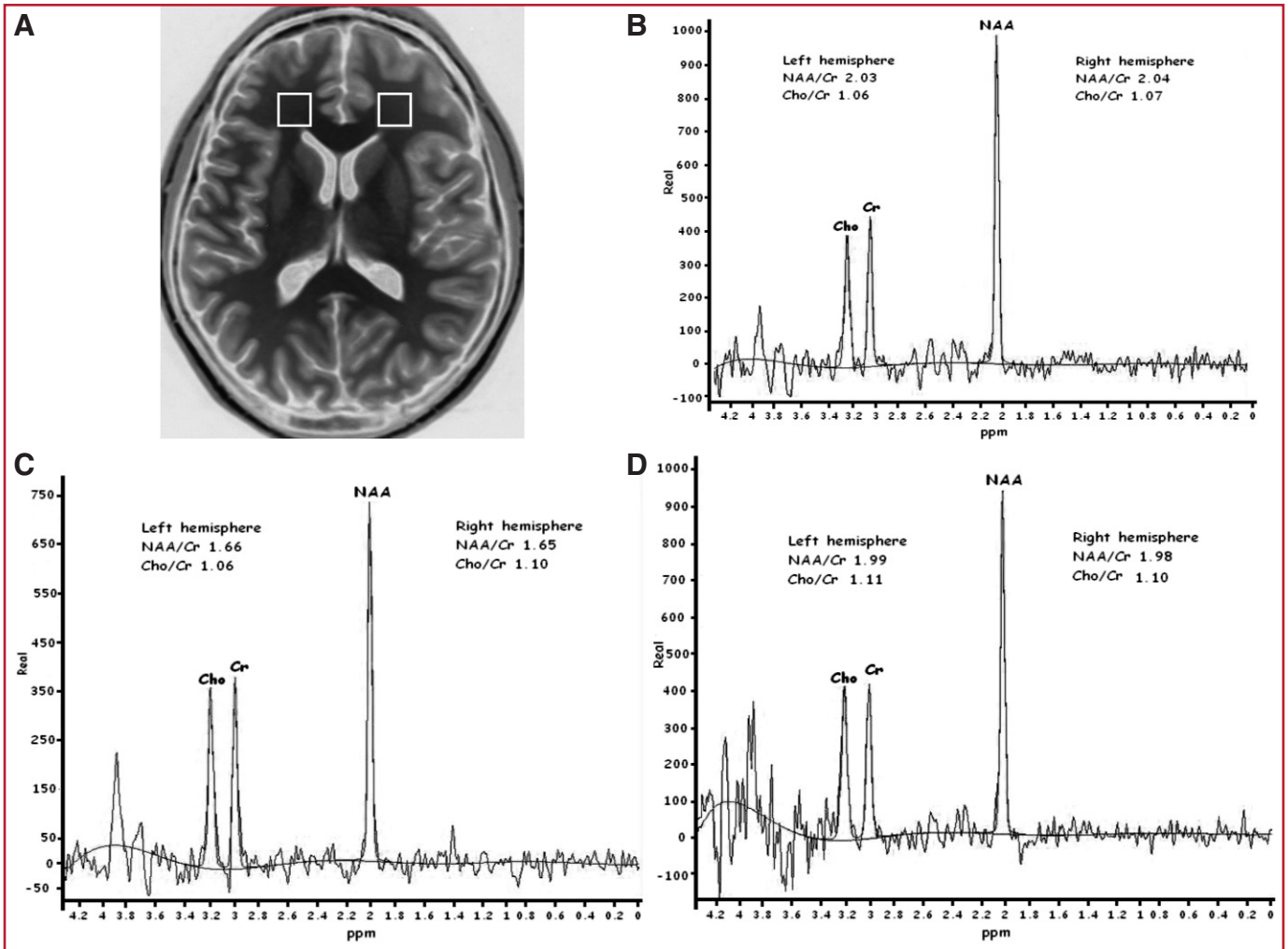

FIGURE 1. **A**, *axial proton magnetic resonance (^{1}H-MR) spectroscopic scout image of a normal volunteer showing the volume-of-interest single voxel located adjacent to the cortical-subcortical junction to include the white matter only of the frontal lobes bilaterally.* **B**, *proton spectrum of a control subject showing the peaks corresponding to the metabolites of interest, N-acetylaspartate (NAA) and creatine (Cr) and choline (Cho)-containing compounds, including calculated NAA-to-Cr and Cho-to-Cr ratios of the two hemispheres.* **C**, *proton spectrum of a singly concussed athlete (Patient 4) obtained 3 days postinjury. Note the different NAA-to-Cr ratio with respect to the value of the control subject.* **D**, *proton spectrum of a singly concussed athlete (Patient 4) obtained 30 days postinjury. Note that the NAA-to-Cr ratio was now similar to that of the control subject.*

shows the axial MRI acquisition used to identify the spectroscopic voxel location, along with the corresponding proton spectrum. The tallest peak on the right represents NAA, the middle peak represents Cr-containing compounds and the leftmost peak represents Cho-containing compounds. The integral values of NAA, Cr, and Cho peak areas were calculated from the spectra in both left and right hemispheres. NAA-to-Cr and Cho-to-Cr ratios were 2.17 ± 0.12 and 1.21 ± 0.07, respectively, in the right hemisphere and 2.25 ± 0.11 and 1.15 ± 0.05, respectively, in the left hemisphere. There were no significant differences in the NAA-to-Cr and Cho-to-Cr values between the two hemispheres.

^{1}H-MR Spectroscopic Analysis of NAA in Concussed Patients

Figure 2 shows the mean values of NAA-to-Cr and Cho-to-Cr ratios for 10 head-injured patients at different time points. At 3 days after concussion, the NAA-to-Cr ratio decreased to 18.5% (1.80 ± 0.04; $P < 0.001$ with respect to that of control subjects), showing only a modest 3% recovery (1.88 ± 0.1) at 15 days. Although this value was significantly higher than that observed at 3 days ($P < 0.02$), it was still significantly lower than that of control subjects (−15%; $P < 0.001$). At 30 days postinjury, the NAA-to-Cr ratio was 2.15 ± 0.1, revealing full recovery (not significant compared with that of normal control subjects). The

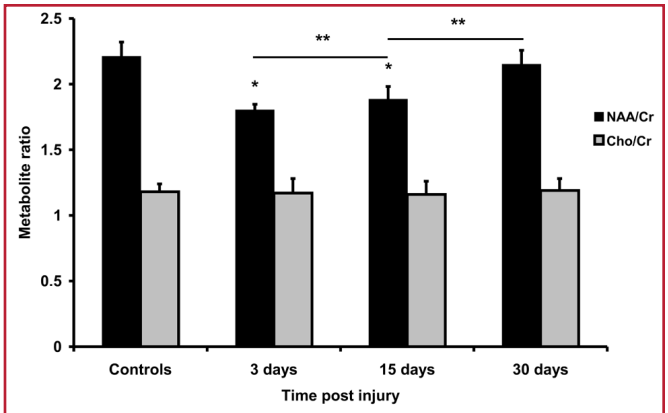

FIGURE 2. *Bar graph showing the NAA-to-Cr and Cho-to-Cr ratios determined by ^{1}H-MR spectroscopy in healthy control volunteers ($n = 5$) and in singly concussed athletes ($n = 10$). Time course of NAA variations indicated a minimum at 3 days postinjury, followed by a gradual, nonlinear, recovery with a slow phase until the 15th day postinjury and a fast phase (up to complete recovery) within the 30th day postinjury. Values are the mean of 10 single voxel spectra in controls (five left and five right hemispheres) and 20 single voxel spectra for each time point in singly concussed athletes (10 left and 10 right hemispheres). Standard deviations are represented by* vertical bars. Single asterisk, *significantly different from controls ($P < 0.05$).* Double asterisk, *significantly different from immediately preceding time point ($P < 0.05$).*

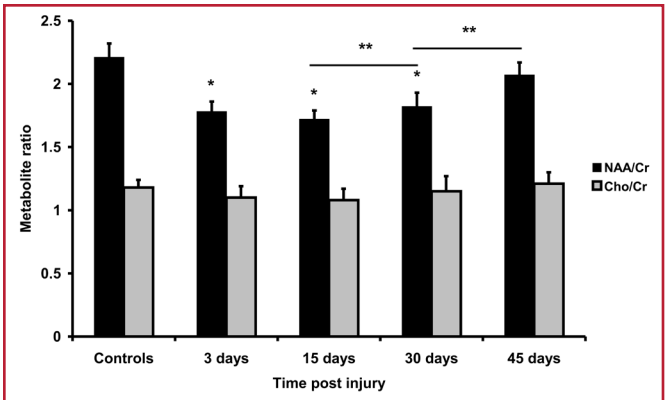

FIGURE 3. *Bar graph showing the NAA-to-Cr and Cho-to-Cr ratios determined by ^{1}H-MR spectroscopy in healthy control volunteers ($n = 5$) and in doubly concussed athletes ($n = 3$) in whom the second concussion occurred between the 10th and the 13th day after the first insult. Time course of NAA variations indicated a minimum at 15 days postinjury, i.e. after the occurrence of the second concussion. A subsequent gradual, nonlinear, recovery with a slow phase until the 30th day postinjury and a fast phase (up to complete recovery) within the 45th day postinjury was observed. Values are the mean of 10 single voxel spectra in controls (5 left and 5 right hemispheres) and 6 single voxel spectra for each time point in doubly concussed athletes (3 left and 3 right hemispheres). Standard deviations are represented by vertical bars.* Single asterisk, *significantly different from controls ($P < 0.05$).* Double asterisk, *significantly different from immediately preceding time point ($P < 0.05$).*

Cho-to-Cr ratio did not exhibit any change compared with that of control subjects at any time point, measuring 1.17 ± 0.1, 1.16 ± 0.1, and 1.19 ± 0.09 at 3, 15, and 30 days, respectively. All patients declared a complete resolution of symptoms at the time of the 3-day study.

In *Figure 3*, the metabolite ratios in the group of patients who had a second concussive injury before the 15-day study are presented. At 3 days, the NAA-to-Cr ratio was 1.78 ± 0.08, a reduction identical to that measured in singly concussed athletes ($P < 0.001$ with respect to control subjects). However, the second injury (occurring at 10, 12, and 13 d from the first one) caused a further decrease of the NAA-to-Cr ratio at 15 days (1.72 ± 0.07; not significant with respect to the 3-d study; $P < 0.0001$ with respect to control subjects). At 30 days, a slight but significant recovery of the NAA-to-Cr ratio was evident (1.82 ± 0.1; $P < 0.05$ with respect to 15 d), but this ratio was still lower than that of control subjects ($P < 0.0001$). Full restoration of the NAA-to-Cr ratio was recorded only at 45 days postinjury (2.07 ± 0.1; $P < 0.001$ with respect to 30 d; not significant with respect to control subjects). Again, no significant changes in the Cho-to-Cr ratio were noticed at any time points (1.10 ± 0.09, 1.08 ± 0.09, 1.15 ± 0.12, and 1.11 ± 0.09 at 3, 15, 30, and 45 d, respectively). These patients declared a complete resolution of symptoms caused by the second concussive event at the time of the 30-day study.

Comparison of NAA in Singly and Doubly Concussed Patients

As shown in *Figure 4*, the NAA-to-Cr ratio determined at 15 days in singly or doubly concussed athletes showed significant differences (1.88 ± 0.1 and 1.72 ± 0.07, respectively; $P < 0.001$). This difference was even more evident at 30 days when the NAA-to-Cr ratio recovered fully in singly concussed athletes, whereas in the doubly injured group the metabolite ratio was still below the physiological value (2.15 ± 0.1 and 1.82 ± 0.1, respectively; $P < 0.001$). Interestingly, there was no statistical difference between values of the NAA-to-Cr ratio in doubly concussed athletes at 30 days and singly concussed athletes at 15 days (1.82 ± 0.1 and 1.88 ± 0.1, respectively) or between doubly concussed athletes at 45 days and singly concussed athletes at 30 days (2.07 ±0.1 and 2.15 ± 0.1, respectively).

Case Report

The time course of the brain metabolic alteration of a 35-year-old professional boxer who experienced a knockout injury is summarized in *Table 2*. A previous ^{1}H-MR spectroscopic examination obtained before the match showed asymmetrical values of the NAA-to-Cr ratio, with values of 1.84 and 2.10 in the right and left hemisphere, respectively. The first postinjury examination was performed 4 days after the knockout; at that time the athlete reported headache and sleep disturbances, and the NAA-to-Cr ratio was 1.43 in the right hemisphere and 1.61 in the left. The ^{1}H-MR spectroscopic examination at 7 days from injury showed a right hemisphere NAA-to-Cr ratio of 1.36 and a contralateral value of 1.70. It is noteworthy to say that at the time of the second ^{1}H-MR spectroscopic examination, the athlete reported complete clinical recovery and showed no signs of concussion. A third ^{1}H-MR spectroscopic examination obtained at 15 days postinjury showed no change in the NAA-to-Cr ratio in the right hemisphere (1.35) and further recovery in the left (1.75). At 30 days, the right hemisphere revealed an increase

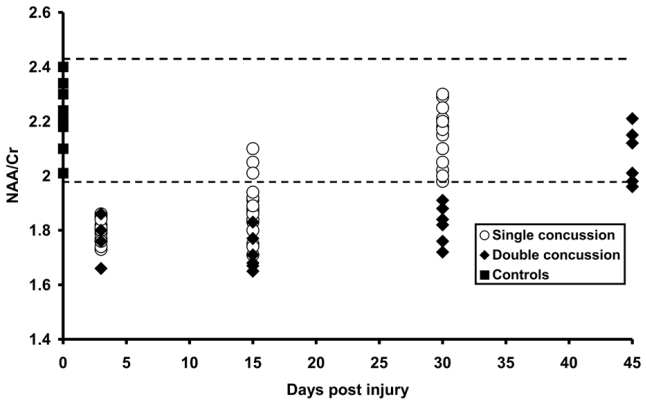

FIGURE 4. *Scatterplot demonstrating the different time course of NAA recovery in athletes having single or double concussive injury, as expressed by the NAA-to-Cr ratio determined by ^{1}H-MR spectroscopy. The second concussion (occurring between the 10th and the 13th d after the first insult) either provoked a further slight NAA decrease or significantly delayed the process of NAA restoration, which was completed at 45 days instead of at 30 days postinjury.*

in the NAA-to-Cr ratio of 1.80, whereas the left hemisphere showed complete recovery (2.03). No variations of the Cho-to-Cr ratio were reported.

TABLE 2. Time course of the brain metabolic alteration in a 35-year-old professional boxer (Patient 14 of *Table 1*)[a]

Timing of ^{1}H-MR spectroscopic examination	Right hemisphere		Left hemisphere	
	NAA-to-Cr ratio	Cho-to-Cr ratio	NAA-to-Cr ratio	Cho-to-Cr ratio
Preimpact	1.84	1.02	2.10	1.15
4 days postimpact	1.43	0.97	1.61	0.98
7 days postimpact	1.36	0.93	1.70	1.14
15 days postimpact	1.35	0.97	1.75	1.07
30 days postimpact	1.80	1.01	2.03	1.13

[a] ^{1}H-MR, proton magnetic resonance; NAA, N-acetylaspartate; Cr, creatine-containing compounds; Cho, choline-containing compounds.

DISCUSSION

The results of the present study show that after a concussion, despite normal radiological appearance and complete resolution of clinical symptoms, substantial neurochemical damage is present in the injured brain and is detectable by measurement of NAA using ^{1}H-MR spectroscopy. Comparison of the NAA-to-Cr ratios of control subjects and concussed athletes (singly and doubly concussed) strongly supports the experimental hypothesis that NAA determination is a valid tool to monitor full recovery of concussion-related metabolic brain damage (78, 79). The gradual process of NAA normalization observed in this study can be inferred in part as the temporal window of the well-known concussive "brain vulnerability" and the resynthesis of NAA as the biochemical proof of complete functional neuronal healing. The present data also corroborate the notion that the NAA decrease is a dynamic process, still detectable 15 days after a concussion: its restoration over time appears not to be linear (slow recovery in the first 2-wk period, followed by fast recovery until normalization in the next week) and can be significantly influenced if a second concussive insult occurs during the recovery process, lengthening the NAA normalization curve.

Use of ^{1}H-MR Spectroscopy in the Evaluation of Concussion

^{1}H-MR spectroscopy has been used to determine NAA in evaluating head injury mainly in patients affected by moderate and severe TBIs (13, 19, 20, 28, 29, 67, 71). Reports focused entirely on magnetic resonance spectroscopic evaluation of the mildly injured brain are uncommon (32, 72). The rationale for investigating the use of this magnetic resonance spectroscopic-based technique in mTBI comes from the experimental evidence that this type of brain injury induces NAA depletion over time with subsequent recovery to preimpact levels (68, 69, 73, 77, 78). Clinical support for this phenomenon was recently reported by Nakabayashi et al. (56) in a series of 30 patients (Glasgow Coma Scale scores of 9–15, including 12 mTBIs), who demonstrated a reduction in the NAA-to-Cr ratio in normal-appearing white matter at 1 week postinjury that spontaneously normalized at 1 month (56). All of the aforementioned authors reached the unanimous conclusion that ^{1}H-MR spectroscopic observation of neurometabolites can give valuable information about the neuronal dysfunction, which is "invisible" with structural neuroimaging modalities. Data in the present study, in accordance with the above-mentioned literature, are distinct in having been obtained from a selected group of patients: a cohort of concussed athletes.

One of the most important findings emerging from this research is the fact that at 3 days postinjury, all concussed athletes declared a complete resolution of symptoms, whereas a concomitant 18.5% NAA reduction was undoubtedly detectable. Furthermore, at 15 days after concussion, only a modest recovery was noted, with NAA still being 15% lower than that in control subjects. Full restoration of brain neurochemistry was only achieved at 30 days, but this occurred only if no other traumatic event interfered (*Fig. 2*). In fact, a second concussive injury occurring before the complete NAA restoration, held up the recovery process considerably, stalling it and delaying it for at least another 15 days, when an identical recovery trend was noted (*Fig. 3*). These athletes were completely symptomless 30 days after concussion; however, at that time a 17.5% NAA reduction was still plainly detectable. It is worth mentioning that the voxel was positioned within the white matter only in our magnetic resonance spectroscopic determinations, whereas the majority of mitochondria are in the gray matter. It is possible that the reported changes in NAA might have been underrepresented in this particular study. We

believe these data are valuable in helping to manage the postinjury period, when the sports medicine physician is faced with the challenging decision of when the athlete is safely able to return to play (16, 25, 41, 46, 49), especially considering the facts that the concussive symptoms reported are probably underappreciated, are frequently unrecognized by the athletes, and are difficult to document without standardized, sensitive, objective diagnostic tests (15, 46). Given the importance of accurately detecting concussions in athletes, it has been pointed out that multimodal appraisal is necessary (5). It is, therefore, conceivable that, in combination with a symptom checklist, clinical examination, and neuropsychological test evaluation, NAA measurement by ^{1}H-MR spectroscopy is a discriminating diagnostic instrument to establish when a concussed athlete can safely return to play. Finally, the usefulness of ^{1}H-MR spectroscopy is exemplified in the case report of the professional boxer. The striking finding of pre- and postinjury asymmetrical NAA-to-Cr ratios in the two hemispheres (6) emphasizes the importance of acquiring baseline data for all those athletes who, in the previous season, had more than one concussive injury. This precaution would permit physicians to carefully compare follow-up studies after the occurrence of a new concussive event, an insult that is by definition a "diffuse injury" but, as shown in the case report, can account for different stages and locations of neuronal metabolic dysfunction (*Table 2*).

To our knowledge, this is the first report of evaluation of NAA using ^{1}H-MR spectroscopy in a series of athletes who had concussions.

Significance of Posttraumatic NAA Reduction and Recovery

It is an accepted fact that NAA, which is present in the brain at concentrations 100-fold higher than those in non-nervous tissues, is a brain-specific metabolite (54, 74). NAA metabolism involves different brain compartments, with neuronal mitochondria taking care of its biosynthesis via the activity of aspartate-*N*-acetyltransferase and oligodendrocytes contributing to its degradation via the activity of *N*-acetylaspartate acylase. NAA homeostasis is finely regulated by three different velocities: 1) rate of neuronal biosynthesis, 2) rate of neuronal outflow in the extracellular space, and 3) rate of oligodendrocyte uptake and degradation (7, 8). Severity of TBI can be biochemically graded in terms of changes in energy-related parameters (ATP, the ATP-to-ADP ratio, and acetyl-coenzyme A [-CoA]) and NAA, with NAA clearly mirroring variations of ATP and of the ATP-to-ADP ratio (69, 73, 78). The biosynthesis of NAA requires acetyl-CoA as the acetyl group donor in the aspartate-*N*-acetyltransferase-catalyzed acetylation reaction of aspartate (7, 8). This implies that for the biosynthesis of each NAA molecule, an acetyl-CoA molecule does not enter the Krebs cycle, being consumed by aspartate-*N*-acetyltransferase. This in turn will cause two NADH molecules and one $FADH_2$ molecule, normally generated through the reactions of the Krebs cycle, to be missing. Because the reoxidation of two NADH molecules and one $FADH_2$ molecule through the electron transport chain generates eight ATP molecules, the indirect energy expenditure necessary for NAA biosynthesis is evident. It is, therefore, conceivable to hypothesize that, in metabolic conditions of low ATP availability, all of the pathways and cycles devoted to energy supply operate at their maximal activity with the aim of replenishing ATP levels. Conversely, a state of high ATP availability, occurring in nonpathological conditions, allows the cell to decrease the rate of such pathways and cycles, including the Krebs cycle, thereby allowing the use of acetyl-CoA for NAA biosynthesis. To reinforce this hypothesis, it is well known that high ATP concentrations are able to inhibit the activity of citrate synthase, which is the enzyme of the Krebs cycle using acetyl-CoA to synthesize citric acid (35). According to these concepts, it is evident that if NAA is not recovering after a mild TBI, the concussive metabolic derangement (involving more complex pathways than the sole NAA homeostasis) cannot be considered to be overcome. Under these conditions, the cells, although functional, are still suffering from energetic imbalance and are vulnerable to a second impact.

With these fundamental concepts in mind, we may affirm that NAA embodies a biochemical surrogate marker to monitor overall cerebral metabolic status; the present data demonstrate that ^{1}H-MR spectroscopy represents a rapid, noninvasive, ready-to-use technique for such assessment. This affirmation also implies that monitoring NAA by ^{1}H-MR spectroscopy is an indirect way to evaluate mitochondria-related cerebral energy metabolism.

Second Impact Syndrome: A Concept to Broaden?

Returning an athlete to play before complete recovery may greatly increase the risk of long-term or persistent neurological sequelae. The possibility of having a second concussive injury within a not yet defined period of time from the first (i.e., days or weeks) has been reported to be fatal in some instances (11, 14, 21, 44, 55, 64); this entity is also known as the second impact syndrome (SIS). Notwithstanding these reported cases, concerns still exist about the real occurrence of this peculiar pathological condition (51, 52, 58).

Our results indicated a slow rate of NAA recovery (0.2%/d) during the first 15 days postinjury (*Fig. 2*), suggesting that cerebral metabolism was still engaged mainly in restoring the altered metabolic functions indispensable for cell homeostasis and survival. The complex neurotoxic cascade, directly triggered by the traumatic insult and extending over days or weeks includes changes in ionic fluxes (40), hyperglycolysis (80), indiscriminate glutamate release (2, 63), calcium overloading (24, 75), mitochondrial dysfunction (27, 77), and impaired oxidative metabolism (4, 73). Hence, it might be reasonable to hypothesize that, in a sort of "list of biochemical priorities," these changes have intrinsic greater detrimental effects on cell survival than the NAA resynthesis, which will finally be completed only after normalization of these more important metabolic alterations (79). This hypothesis might explain the complete NAA restoration achieved in the second 15 days at a rate fivefold higher (1%/d) than that observed during the first 2 weeks.

In rats, two consecutive mTBIs occurring within the shortest time interval considered (3 d after the first concussion) produced biochemical damages typical of severe TBI (78), in

terms of either NAA depletion or energetic impairment. Most importantly, when the second injury took place after a longer interval (5 d after the first concussive episode), the two traumatic insults acted as independent events (78, 79). Even though our athletes who experienced a second concussion did not have SIS or show signs of severe TBI, they all had a significant delay in both symptom resolution and NAA normalization, i.e., the effects of the second concussion were somehow not proportionate to the entity of the concussive insult. Most likely, the second concussion occurred when the brain cells were completing recovery of metabolic functions and, thus, it only produced a limited cumulative effect with moderate worsening of the expected clinical and biochemical consequences. It is, therefore, conceivable to infer from the results reported here and from data obtained in experimental repeated mTBI in rats (78, 79) that in humans the time interval between the two concussions drives the concussive clinical and metabolic evolution. The apparent discrepancy with the experimental data obtained in rats may also be attributable to differences in the two concussions suffered by the athletes which, in laboratory animals, were indeed the same (450 g dropped from a 1-m height).

Hence, we believe that SIS should not be thought of solely as an "all or none" phenomenon and limited to instances with fatal resolution only (malignant swelling). The concept of SIS should now be extended to include all occurrences in which a disproportion in the severity of the second injury and the concussive clinical features (intensity and/or time of resolution) or cerebral metabolic changes (extent of NAA decrease and/or delay in its normalization) is clearly observed. The degree of this type of SIS will depend on which phase of metabolic recovery the brain is in when the second concussion occurs. The severe brain swelling observed in SIS (11, 14, 21, 44, 55, 64) is probably attributable to the fact that the second insult took place when the cells were still intensely engaged in restoration of their energetic integrity and were unable to withstand further mitochondrial damage, thus experiencing uncontrolled swelling and massive NAA loss (47).

Perspectives for Future Studies and Conclusions

Although corroborated by statistical significance, the present results certainly need to be substantiated in a larger group of athletes, with the following aims: 1) to confirm nonlinear NAA recovery after concussion; 2) to determine the correlation between concussion severity and extent of NAA depletion and/or timing of its recovery; 3) to verify the effects of a second concussive event occurring at different time intervals from the first one; 4) after having here demonstrated the lack of coincidence between clinical recovery and NAA rescue, to compare the timing of neurocognitive normalization (by neuropsychological tests) with that of brain metabolic functions (^{1}H-MR spectroscopic measurement of NAA); and 5) given the acceptance of a broadened concept of SIS, to scale SIS on the basis of the disproportions among the occurrence of the second concussive event, the severity of symptoms, and the extent of the cerebral metabolic damage (NAA depletion).

CONCLUSION

In conclusion, it seems that concussion in humans opens the window to metabolic brain vulnerability. Because the first episode of head trauma may not necessarily have happened on the athletic field (15, 23, 66), we recommend that anytime a concussive event occurs in any individual, the physician (if informed) should advise the individual to behave safely and avoid further injury for a mandatory time until the end of the vulnerability period. Regarding concussions in sports medicine, we believe the use of ^{1}H-MR spectroscopy to measure NAA levels in concussed athletes offers a unique opportunity to monitor the degree and recovery of the posttraumatic neurochemical damage, in consideration of the much higher risk of recurrent concussions in athletes (81). This technique should provide an objective basis for establishing complete resolution of the concussion-mediated cerebral functional alteration and should permit both physicians and athletes to identify a more defined period "away from the field," for a much safer return to play.

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COMMENTS

There are continuing efforts to establish a sensitive and specific objective marker of full and complete recovery after minor traumatic brain injury (TBI) to guide return to full and normal activity, especially as it relates to athletic endeavors. Vagnozzi et al. advocate the use of proton magnetic resonance (^{1}H-MR) spectroscopy to measure *N*-acetylaspartate (NAA) levels, finding 30 days to normalization ("full metabolic recovery") in singly concussed and 45 days to normalization in multiply concussed athletes. The authors hypothesize that a "perfect storm" of the severity of multiple concussions, the time interval between concussions, and the metabolic state of the brain at the time of the second concussion leads to the second impact syndrome and propose using ^{1}H-MR spectroscopy routinely in refining return to play guidelines.

Although this may eventually be a useful tool in selected athletes, the findings derived from 13 athletes reported herein does not justify such a reccommendation. Although the authors have studied this phemenomenon in detail in animal models, there is insufficient data in humans to make such a scientific leap. ^{1}H-MR spectroscopy results may be highly operator-dependant. Mitochondrial dysfunction in the grey matter leads to the metabolic disturbance, resulting in abnormal NAA levels. In this study, all of the data was derived from the while matter. Although the authors hypothesize that the NAA levels would have been even higher if measured in the grey matter, such is only speculation. The authors are to be commended for pursuing this line of investigation in this important area. Hopefully, they will continue their research to provide sufficiently compelling data to justify clinical application.

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The authors should be congratulated on performing this careful ^{1}H-MR spectroscopy study in athletes with concussion. Although the number of patients studied is only 13, the data is robust, and the changes seen are quite large (i.e., almost 20% change in the NAA/creatine [Cr] ratio). It is surprising that such large magnitude changes were seen; these are comparable to those reported for severe TBI in some studies.

Although several groups are working in the area of magnetic resonance imaging in mild TBI, the result from this study seem to be particularly robust. It is salient that the symptoms of concussion in these athletes had disappeared by Day 3, yet their ^{1}H-MR spectroscopy abnormalities persisted until Day 30. This suggests that the subtle biochemical abnormality that is seemingly detected by the NAA/Cr ratio changes is sufficiently mild that brain functioning is not affected, even though the spectra were measured in the dorsal lateral frontal lobes. The major finding of the study and the reason this is such an important study, is that the study showed a doubling of the normalization time for the NAA/Cr ratio in those few athletes that had a second impact after they had undergone magnetic resonance imaging for the first concussion. Even though only three patients were in this second impact group, their NAA/Cr ratios took 15 days longer to normalize; the authors have speculated that this provides a tangible bio-

chemical organic basis for the "second impact syndrome." Although this is an important finding and does require further study both in the lab and in patients, it is perhaps overreaching to claim that this finding denotes "substantial biochemical damage" as the authors state in the discussion. It is hard to know how significant an apparent 18% reduction in NAA/Cr ratio actually is because these patients were functioning normally. As the authors indicate, it would be valuable to cross-correlate the psychometric testing with this ^{1}H-MR spectroscopy finding. Similarly, in my view, it is not yet appropriate for the authors to claim that ^{1}H-MR spectroscopy is a future screening tool for use in athletes because, as they state in the discussion, each patient must be studied with a baseline ^{1}H-MR spectroscopy evaluation, and it is the difference between the post-concussion value and the baseline that is interesting here. It is, therefore, not yet possible to propose a normal range for this ratio, outside of which an athlete could be kept back from play in my view. More validation is needed before that claim can be made. Similarly, for the authors to claim that "concussion opens a window of metabolic vulnerability" based upon the ^{1}H-MR spectroscopy data shown here is overreaching. What the authors have very importantly shown is that ^{1}H-MR spectroscopy may provide a tangible marker of concussion and that this finding needs to be validated by other groups and laboratory investigations. The excellent list of future studies provided in the discussion section is absolutely relevant and should provide fertile ground for future studies not only by this group but by others active in this important field of mild TBI.

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This study by Vagnozzi et al. provides important information in terms of mitochondrial dysfunction after human mild TBI. One could question the choice of the region of interest; however, the data does indicate a remarkable degree of consistency between subjects sustaining a cerebral concussion. Two very important findings are reported in this study. The first (although only studied in two patients) is that when a second concussion is sustained during the period of the first injury-induced reduction in the NAA/Cr ratio, the ability of the brain to recover its mitochondrial function is prolonged. The second important finding is that this maker of mitochondrial dysfunction, in terms of a reduction in NAA/Cr, can be present even after classical neuropsychological and/or neurological recovery. Consequently, the results have important implications regard "return to play" issues. It is with great interest that the field will look forward to this group in their continued work in this area of mild traumatic brain injury.

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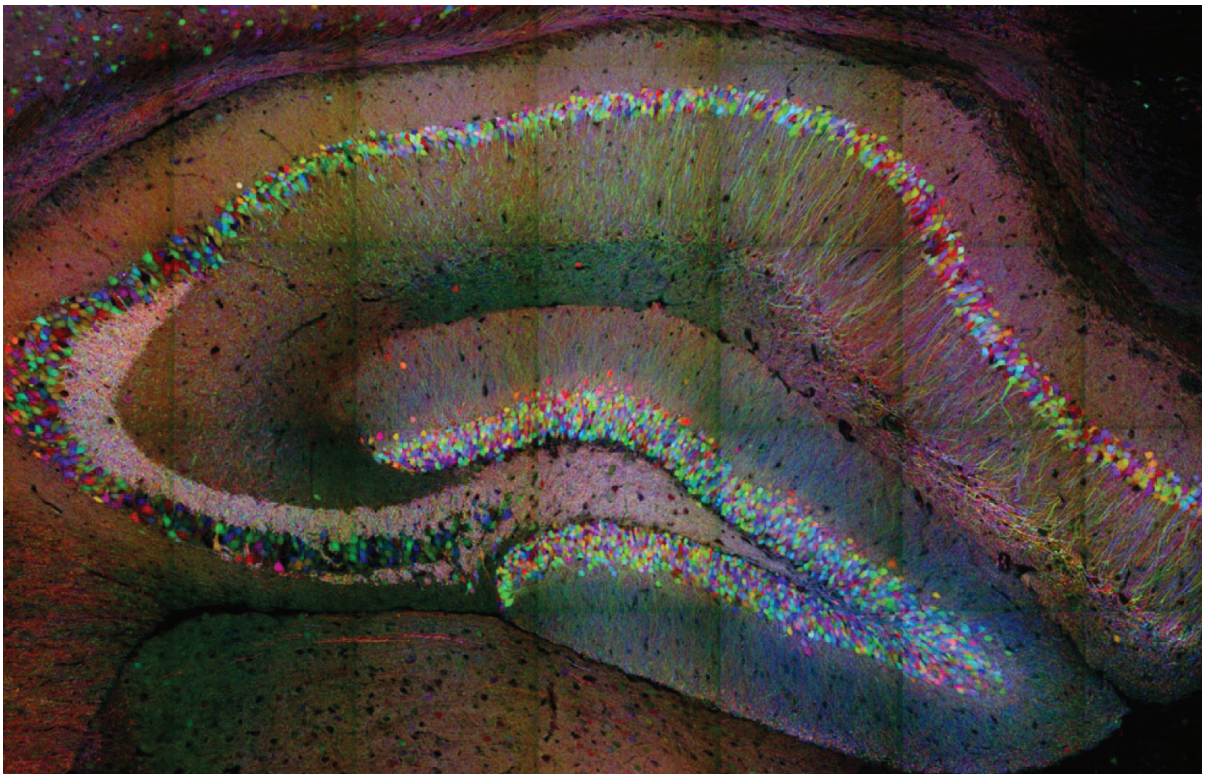

Transgenic method for combinatorial expression of fluorescent proteins. Low magnification view of mouse hippocampus. From, Livet J, Weissman TA, Kang H, Draft RW, Lu J, Bennis RA, Sanes JR, Lichtman JW: Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. **Nature** 450:56–62, 2007. See Elder, pp 1358–1359.