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Assessment of metabolic brain damage and recovery following mild traumatic brain injury: a multicentre, proton magnetic resonance spectroscopic study in concussed patients

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Concussive head injury opens a temporary window of brain vulnerability due to the impairment of cellular energetic metabolism. As experimentally demonstrated, a second mild injury occurring during this period can lead to severe brain damage, a condition clinically described as the second impact syndrome. To corroborate the validity of proton magnetic resonance spectroscopy in monitoring cerebral metabolic changes following mild traumatic brain injury, apart from the magnetic field strength (1.5 or 3.0 T) and mode of acquisition, we undertook a multicentre prospective study in which a cohort of 40 athletes suffering from concussion and a group of 30 control healthy subjects were admitted. Athletes (aged 16–35 years) were recruited and examined at three different institutions between September 2007 and June 2009. They underwent assessment of brain metabolism at 3, 15, 22 and 30 days post-injury through proton magnetic resonance spectroscopy for the determination of *N*-acetylaspartate, creatine and choline-containing compounds. Values of these representative brain metabolites were compared with those observed in the group of non-injured controls. Comparison of spectroscopic data, obtained in controls using different field strength and/or mode of acquisition, did not show any difference in the brain metabolite ratios. Athletes with concussion exhibited the most significant alteration of metabolite ratios at Day 3 post-injury (*N*-acetylaspartate/creatine: −17.6%, *N*-acetylaspartate/choline: −21.4%; $P < 0.001$ with respect to controls). On average, metabolic disturbance gradually recovered,

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initially in a slow fashion and, following Day 15, more rapidly. At 30 days post-injury, all athletes showed complete recovery, having metabolite ratios returned to values detected in controls. Athletes self-declared symptom clearance between 3 and 15 days after concussion. Results indicate that *N*-acetylaspartate determination by proton magnetic resonance spectroscopy represents a non-invasive tool to accurately measure changes in cerebral energy metabolism occurring in mild traumatic brain injury. In particular, this metabolic evaluation may significantly improve, along with other clinical assessments, the management of athletes suffering from concussion. Further studies to verify the effects of a second concussive event occurring at different time points of the recovery curve of brain metabolism are needed.

Keywords: mild traumatic brain injury; ^{1}H-magnetic resonance spectroscopy; *N*-acetylaspartate; metabolic brain vulnerability; sport-related concussion

Abbreviations: Cho = choline-containing compounds; Cr = creatine-containing compounds; ^{1}H-MRS = proton magnetic resonance spectroscopy; NAA = *N*-acetylaspartate; PTA = post-traumatic amnesia; TBI = traumatic brain injury

Introduction

Mild traumatic brain injury (TBI) is a neglected pathological state, involving the overwhelming majority of the head-injured population treated in emergency departments in Europe and the USA (Engberg and Teasdale, 2001; Gerberding, 2003). It has been calculated that the ratio in the occurrence of mild TBI to severe TBI is ~22:1, with mild TBI accounting for at least 75% of patients who survive after TBI each year (Tagliaferri *et al.*, 2006). Since most of the mild TBI-injured patients are asymptomatic, the larger proportion receives no medical attention and remains unreported. Of mild TBIs, at least 20% are sports-related injuries (concussions), of which 30–45% receive no medical care (McCrea *et al.*, 2004). Athletes, therefore, represent a population at great risk of occurrence of concussive episodes and are, for several reasons, the population of choice with which to undertake the trials to study the pathobiology of mild TBI (Meehan and Bachur, 2009).

Despite recent efforts, a unanimous definition of concussion has not yet been widely accepted (Cantu, 2007). It is possible to define concussion as a traumatic insult capable of provoking an acceleration–deceleration phenomenon within the skull (Barth *et al.*, 2001). Clinically, concussion is not necessarily accompanied by loss of consciousness and is associated with various physical (headache, equilibrium, vision disturbances, etc.), cognitive (memory, concentration, etc.), emotional (behaviour) and sleep alterations (Gosselin *et al.*, 2009; Randolph *et al.*, 2009; Hunt and Asplund, 2010). These symptoms comprise the so-called post-concussive syndrome and can affect, to various degrees, everyday life, resolving spontaneously within 7–10 days post-injury in the majority of cases. It is generally believed that the pathobiology of mild TBI in concussed subjects cannot be delineated by classical imaging techniques such as CT scan and MRI (Kurca *et al.*, 2006). This fact, coupled with the faintness and variability of symptoms, mainly assessed by the patient's self-evaluation, is not of help in diagnosing and monitoring concussed patients.

It is well established that after the first traumatic episode, the probability of recurrence of concussion in athletes increases 3-fold (Cantu, 2003) and that currently there is no agreement as to how many concussions are too many (Guskiewicz *et al.*, 2003; Pellman *et al.*, 2004), nor is there a unanimously approved diagnostic approach to monitor concussed athletes (Delaney *et al.*, 2005; Kissick and Johnstone, 2005; Ponsford, 2005). Consequently, the criteria to assess a safe return of concussed athletes to play remain unclear (Lovell *et al.*, 2004; Guskiewicz *et al.*, 2006; McClincy *et al.*, 2006).

An increasing number of studies on mild TBI have focused attention on concussion-induced changes in brain metabolism (Bergsneider *et al.*, 2000; Giza and Hovda, 2001; Praticò *et al.*, 2002), including those related to cerebral energy state (Vagnozzi *et al.*, 1999). Hovda and colleagues (1993, 1999) first suggested the concept of metabolic vulnerability occurring in brain tissue after any concussive episode. During this transient period of altered brain metabolism and function, a second concussive episode of even modest entity may cause significant addition and/or dramatic brain damage (Longhi *et al.*, 2005), thereby underlying the so-called second impact syndrome, encountered occasionally in sports medicine (Saunders and Harbaugh, 1984). By using a rodent model of closed diffuse mild head injury (Foda and Marmarou, 1994; Marmarou *et al.*, 1994), data from our laboratories confirmed the concept of metabolic vulnerability (Tavazzi *et al.*, 2007; Vagnozzi *et al.*, 2007) and have also produced solid experimental evidence linking the severity of brain injury and recovery with the extent of ATP and *N*-acetylaspartate (NAA) decrease and recovery (Vagnozzi *et al.*, 2005, 2007; Tavazzi *et al.*, 2007).

Although NAA is the most abundant neuronal-specific *N*-acetylated amino acid in cerebral tissue, its biological function remains poorly understood (Baslow, 2003*a*, *b*). The biosynthesis of NAA is uniquely linked to the mitochondrial phosphorylating capacity, the cell energy state and ATP level, and to acetyl-CoA availability. Due to indirect high-energy expenditure, NAA can be synthesized under conditions of energy surplus, i.e. high ATP and acetyl-CoA concentrations. These observations led to the suggestion that NAA measurement may be a valid indirect biomarker of the brain energy state (Vagnozzi *et al.*, 2005, 2007; Tavazzi *et al.*, 2007). Coupled with its non-invasive *in vivo* detectability by proton magnetic resonance spectroscopy (^{1}H-MRS) (Moreno *et al.*, 2001; Sinson *et al.*, 2001), NAA appears to be a useful index in monitoring metabolic brain changes following concussion in humans. Alterations of central energy metabolism might well represent a reasonable explanation of the striking and

unpredictable discordance often observed between the minimal findings on conventional imaging and the extent of neurocognitive deficits exhibited by patients with mild TBI (Cohen *et al.*, 2007; Rigotti *et al.*, 2007).

Since none of the currently available diagnostic tests (McCrea *et al.*, 2003; Schatz *et al.*, 2006; Broglio *et al.*, 2007; Register-Mihalik *et al.*, 2008) are capable of measuring this unique, transient and potentially dangerous state of metabolic vulnerability of brain tissue, we recently characterized NAA alterations in a restricted number of concussed amateur athletes using ^{1}H-MRS (Vagnozzi *et al.*, 2008). The results of this pilot study indicated that NAA cerebral levels are decreased for weeks after concussion, even after the resolution of all concussion-associated clinical symptoms, with normalization to control values occurring only 30 days post-injury.

Prompted by the current lack of a validated, objective biochemical marker reflecting brain metabolism in mild TBI patients and the need to measure the mild TBI-mediated temporal window of metabolic brain vulnerability, we carried out the present multicentre study in a reasonably large group of professional and amateur athletes from different sport disciplines who had sustained a concussive brain injury. In order to compare different techniques of spectral data acquisition, the ^{1}H-MRS determination of NAA was performed using different magnetic fields in either the single-voxel modality (1.5 and 3.0 T) or the multivoxel mode (3.0 T). The potential importance of biochemical monitoring of the mild traumatically injured brain, as well as the possibility of incorporating NAA measurements using ^{1}H-MRS as a qualified diagnostic tool to evaluate the return of concussed athletes to play, is discussed.

Material and methods

Patient selection and clinical protocol

Sample size was determined assuming a 15% reduction in the normal values of NAA/creatine-containing compounds (NAA/Cr) and NAA/choline-containing compounds (NAA/Cho) as significantly pathological (Nakabayashi *et al.*, 2007; Signoretti *et al.*, 2008; Vagnozzi *et al.*, 2008; Sarmento *et al.*, 2009). With an alpha value of 5% and with 30 participants for each sample (controls and patients with mild TBI), a 94% power was achieved.

After obtaining informed consent according to the Declaration of Helsinki and to the institutional procedures, 40 patients who had sustained a concussive head injury and 30 healthy volunteers were enrolled in this study. All procedures were approved by the Ethical Committee of each of the three centres involved. More specifically, patients and controls were recruited by the Department of Diagnostic Imaging and Interventional Radiology and the Department of Neurosciences, University of Rome 'Tor Vergata', Rome, Italy (patients with mild TBI = 14; uninjured controls = 10); by the Department of Neurosurgery, Ospedale Maggiore di Verona 'Borgo Trento', Verona, Italy (patients with mild TBI = 22; uninjured controls = 15) and by the Department of Neurosciences, Division of Neuroradiology, University of Parma, Italy (patients with mild TBI = 4; uninjured controls = 5).

Patient selection was characterized by strict inclusion criteria: (i) concussive head injury; (ii) Glasgow Coma Scale $\geq$14; (iii) normal neurological objective examination at the time of enrolment; (iv) an age ranging between 16 and 35 years; (v) the requirement to refrain from further physical activity for 30 days; and (vi) no change in the Cho/Cr ratio registered throughout the observational period. Patients were initially referred to our centres either by on-field personnel or, in the case of hospital admission, by emergency room doctors. The final selection of a patient candidate to be enrolled in the study was carried out by the investigators on the basis of the presence of post-concussive symptoms, either at the time of the first MRI or in case of symptom clearance, in anamnesis. Patients not fulfilling the aforementioned entry criteria, with positive MRI for post-traumatic anatomical lesions (the presence of blood, etc.), suffering from polytrauma or presenting with risk factors for subsequent complications (coagulopathy, epilepsy, former neurosurgical interventions, alcohol or drug abuse, disabilities), were excluded from the study.

The selected cohort included five professional and 35 non-professional athletes from different sport disciplines, who had suffered from a sport-related concussion, defined as a traumatically induced transient alteration in mental status, not necessarily accompanied with loss of consciousness. The first clinical evaluation, MRI and ^{1}H-MRS were carried out at 3 days post-injury; follow-up MRI and ^{1}H-MRS analysis, as well as clinical evaluation of concussion-associated symptoms, were performed at 15, 22 and 30 days post-injury in all cases.

None of the enrolled subjects were taking medications for recovery or treatment of the post-concussive syndromes at the time of the study.

Magnetic resonance imaging and magnetic resonance spectroscopy acquisition techniques

In patients recruited at the University of Rome, the semi-quantitative analysis of NAA relative to creatine- and choline-containing compounds was performed in the single-voxel mode, after obtaining proton spectra using a 3.0 T system (Philips, Intera Achieva). For conventional MRI studies, T_1- and T_2-weighted turbo spin echo images were acquired in axial, coronal and sagittal planes and, in order to rule out even the smallest amount of intra-cerebral blood, fast field echo T_2* sequences were used. A multichannel coil (8 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. Following localized shimming and water suppression, the spectroscopic examination was carried out using a point-resolved spectroscopy sequence pulse, with the following settings: echo time = 144 ms; time to repetition = 2000 ms; spectral bandwidth = 2000 Hz; acquisition cycles = 128. The optimal positioning of the voxel was determined using the MRIs acquired on axial, coronal and sagittal planes to facilitate its 3D placement (adjacent to the cortical–subcortical junction in order to include only the white matter of the frontal lobes), bilaterally, and the choice of this location as the region of interest was made to obtain the most homogeneous data possible. To this end, a spectrum from a single voxel customized to sample a volume of interest of 3.375 cm^3 ($1.5 \times 1.5 \times 1.5$ cm) was obtained (acquisition time about 5 min for each voxel). In follow-up studies, the exact repositioning of the voxel on the same acquisition plane obtained in the previous MRI study was achieved by using dedicated software (SameScan, Philips Medical Systems).

Patients recruited at the University of Parma were studied with a 1.5 T system (Philips Intera Achieva) using MRI procedures identical to those applied in Rome. Briefly, the point-resolved spectroscopy sequence with 144/2000 ms time to repetition/echo time, spectral bandwidth = 1000 Hz and 128 acquisition cycles was used to obtain

a single voxel from a 3.375 cm^3 volume of interest, optimally positioned in the same areas of the frontal lobes, following the same criteria utilized at the University of Rome. Precise voxel repositioning in follow-up studies was again achieved by using the same dedicated software (SameScan, Philips Medical Systems).

At the University of Verona, the study was performed by using a 3.0 T system (Siemens Allegra, Erlanger, Germany). Conventional MRI again included T_1- and T_2-weighted turbo spin echo images, and fast field echo T_2* sequences acquired using a multichannel birdcage head-coil (four channels, Sense-IPAT acquisition) with 4-mm slice thickness, 1 mm gap and a field of view of 230 mm. Using the same 3D localization criteria as the previously described single voxel studies, a larger region of tissue was examined employing the multivoxel technique (chemical shift imaging). To this end, a specific standard slice of the frontal lobes adjacent to the superior aspect of the corpus callosum was selected, carefully avoiding the lateral ventricles, with potentially consequential partial volume effects of the cerebrospinal fluid. Following the standard procedure of shimming and water suppression, the spectroscopic examination was carried out using a stimulated echo acquisition mode, single slice, chemical shift imaging pulse sequence, with the following parameters: echo time = 135 ms; time to repetition = 2000 ms; spectral bandwidth = 2000 Hz; number of excitations = 3; 240×240 mm^2 field of view; slice thickness = 1.5 cm; phase encoding = 16×16; data points = 1024. Acquisition time was 8 min for the complete magnetic resonance spectroscopy (MRS) evaluation. In follow-up studies, the exact volume of interest repositioning on the same acquisition plans was re-achieved using dedicated software (Spectroscopy Card, Siemens).

Analysis of spectroscopic data

In single-voxel studies, post-processing of spectral data allowed us to calculate the area under the peaks of NAA, Cho and Cr, using common criteria for peak integration. In the case of a single, well-defined peak (typically the NAA peak), a valley-to-valley integration was performed to obtain the area under the peaks. In the case of not fully resolved peaks (frequently the Cho and Cr peaks), a horizontal baseline between the start of the first peak to the end of the second peak was selected; the grouped peaks were then split by a vertical line, drawn from the median point of the common valley between peaks to the horizontal baseline and the area under the peaks calculated. These values were used to determine the metabolite ratios NAA/Cho, NAA/Cr and Cho/Cr.

In multivoxel studies, phase encoding in 2D and Fourier transformation yielded chemical shift imaging data to be examined as single spectra related to individual voxels and as spectral maps, by utilizing a 2D grid over a transverse localizer. The grid was 12×12 with each of the 144 voxels measuring 3.375 cm^3 for a total volume of brain sample of 486 cm^3. To acquire data comparable with single-voxel studies performed in the other centres, an identical volume of interest was selected within the white matter of the frontal lobes, including 8 voxels, and the final matrix available for calculation of metabolite intensity was a grid of 2×4 voxels (volume of interest = 27 cm^3) for each hemisphere. Metabolite ratios NAA/Cr, NAA/Cho and Cho/Cr were automatically calculated at the end of each acquisition using an appropriate scanner software (Spectroscopy Card, Siemens). To compare differences in left and right frontal lobe damage, NAA/Cho, NAA/Cr and Cho/Cr ratios of all selected voxels were then averaged and presented as mean ratios, respectively. Using this method (Nakabayashi *et al.*, 2007), it was possible to quantify the neurochemical damage in a single discrete volume of interest of the frontal lobes, similar to that we obtained in single-voxel studies.

Both single-voxel and chemical shift imaging spectroscopic results collected from the patients were compared with those recorded in the control group of healthy volunteers, previously screened to exclude prior concussive head injuries during the preceding year, and matched for sex and age to the group of patients.

Statistical analysis

All data analyses and calculations of sample size were performed using the Statistical Package for the Social Sciences Windows version 13.0 (SPSS, Chicago, IL, USA). Descriptive statistics for quantitative continuous variables were presented as mean ± standard deviation (SD). Assumptions of normality were demonstrated using the Kolmogorov–Smirnov test. The homogeneity of the variance was evaluated with Levene's test. ANOVA for repeated measures, corrected by Bonferroni, was used to evaluate significant differences among groups. Differences were considered to be statistically significant when $P < 0.05$.

Results

Study population

No concussed patient was excluded from the study. In the 40 patients (concussed athletes), 160 ^{1}H-MRS analyses were performed, while 30 parallel studies were performed in control volunteers, for a total number of 190 proton spectroscopic acquisitions. Single-voxel studies were obtained in 18 brain-injured patients and 15 volunteers, while the remaining 22 brain-injured patients and 15 normal volunteers were studied with the multivoxel technique. In brief, 87 bilateral single-voxel examinations (15 volunteers, 18 initial studies in concussed athletes with 54 follow-up studies) and 103 chemical shift imaging acquisitions (15 volunteers, 22 initial studies in concussed athletes and 66 follow-up studies) were successfully performed.

The control group had a mean age of 27.6 ± 3.58 years and was composed of 7 females and 23 males, whilst the group of concussed athletes had a mean age of 26.5 ± 5.53 years and was composed of 9 females and 31 males. The sports practiced by concussed athletes were soccer ($n = 11$), rugby ($n = 5$), horse riding ($n = 4$), boxing ($n = 4$), basketball ($n = 3$), kick-boxing ($n = 6$), alpine skiing ($n = 3$) and bike riding ($n = 4$). Soccer was observed to be the sport having the highest number of injuries (27.5%). These data may be explained by taking into account that in Italy, soccer is practiced weekly by more than a million people at either amateur or professional level.

Table 1 presents the clinical features of the 40 patients enrolled in the study. Among them 19 (48%) reported a loss of consciousness at the time of impact, with an estimated duration varying from 15 to 90 s. This higher percentage of loss of consciousness than that commonly reported in the literature (~10%) may be due to the fact that in Italy, unfortunately, sport-related concussions are still recognized only in the presence of gross clinical evidence. Among the different symptoms described, post-traumatic amnesia (PTA) was the most frequent (78%), this symptom being of variable duration and generally resolving within 15 min from injury (PTA < 5 min, 8/31 = 25.6%; PTA 5–10 min,

5/31 = 16.1%; PTA 10–15 min, 9/31 = 29%; PTA > 15 min, 9/31 = 29%).

Magnetic resonance spectroscopy in controls

Since one of the main aims of the present study was to characterize and validate the method of assessing metabolism in the post-concussed brain by determining NAA using ^{1}H-MRS, the study was purposely carried out at three centres, each one utilizing a different MRS apparatus with its own technical and acquisition parameters.

Figure 1 shows an axial MRI used to identify the location of the spectroscopic voxel and the corresponding proton spectrum, whilst an example of the chemical shift imaging technique application is illustrated in Fig. 2. Although the initial volume of brain sampled was much larger, the use of the chemical shift imaging technique allowed the investigators to select, within the standard slice, a new specific volume of interest included in a 2×4 voxel array for each frontal lobe and to calculate the relative brain metabolite values within the identical region selected for the single-voxel studies.

Comparison of the results from the three centres (Table 2) showed no differences in the values of the metabolite ratios recorded with different magnetic resonance devices and acquisition modes. Furthermore, the statistical analysis revealed that the probabilistic distribution of these parameters was clearly Gaussian. These observations allowed us to group data recorded at the three centres in both controls and concussed athletes, treating them as a single homogeneous data set for subsequent statistical comparison.

Table 1 Evaluation of the clinical features of the 40 patients enrolled in the study

Clinical symptoms	Number of patients	Total (%)
Post-traumatic amnesia	31	78
State of confusion	29	73
Sleep/asleep alterations	25	62
Loss of consciousness	19	48
Headache	17	43
Behavioural alterations	8	20
Nausea	2	5

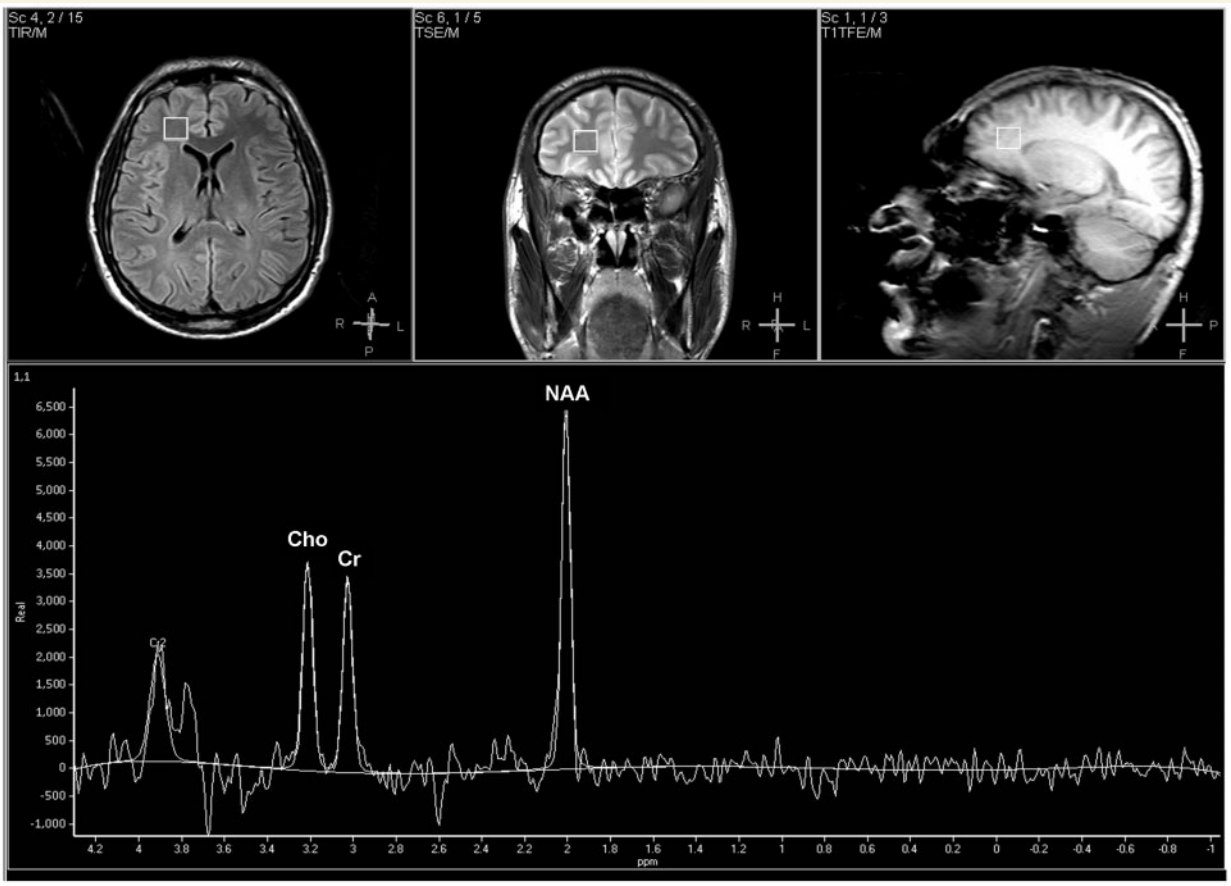

Figure 1 Axial MRI of a normal volunteer showing the single volume of interest (single voxel) located in the right frontal lobe, along with the corresponding proton spectrum. The tallest peak on the right represents *N*-acetylaspartate (NAA), the middle peak creatine-containing compounds (Cr) and leftmost choline-containing compounds (Cho).

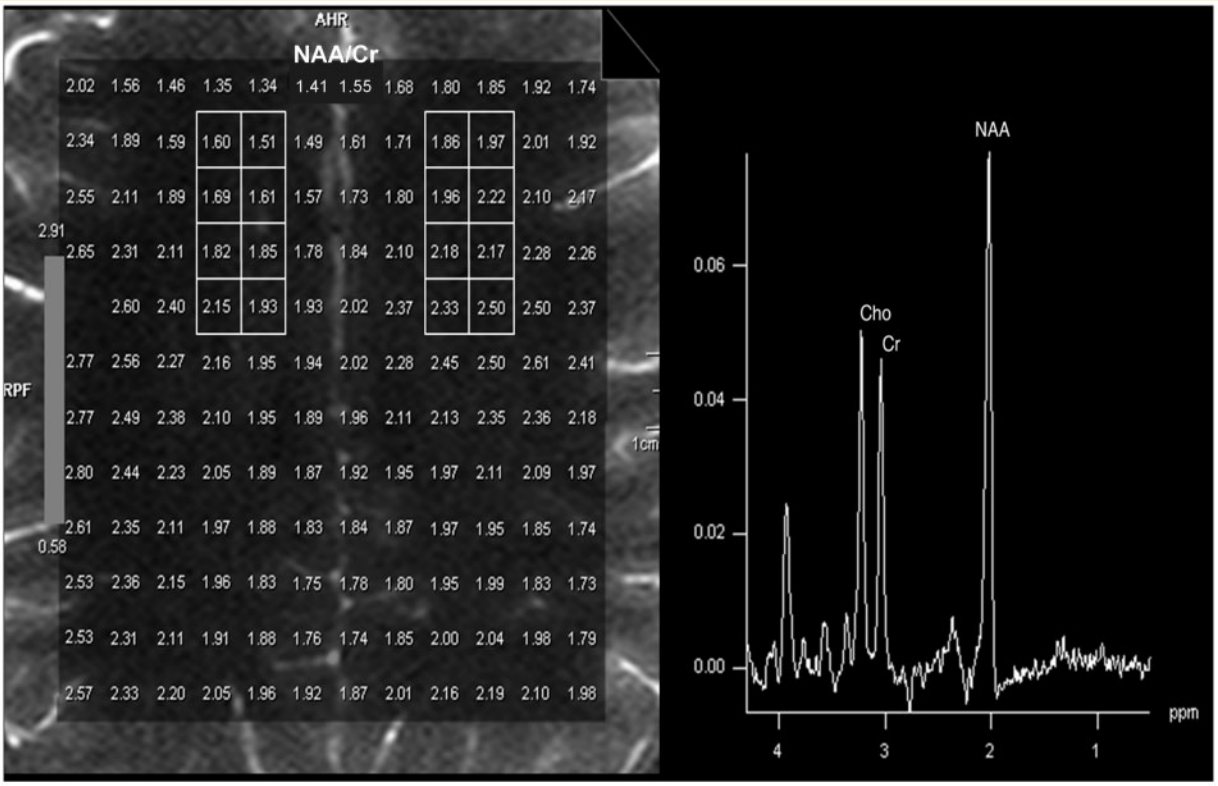

Figure 2 Chemical shift imaging of a selected slice of brain just above the corpus callosum to avoid the lateral ventricles and CSF partial volume effect. Each depicted number represents the metabolite ratio (*N*-acetylaspartate/creatine-containing compounds) of the 'mini-voxel'. Within the initial volume of interest of 144 mini-voxels, a region of interest of 2×4 grid including 8 mini-voxels was selected by the investigator. This selection allowed the acquisition of an averaged spectrum and the calculation of the relative brain metabolite values within the identical brain region selected for the single-voxel studies.

Table 2 Comparison of the metabolite ratios recorded in controls by ^{1}H-MRS in the three neuroradiological centres, using different MRS devices and mode of acquisition

Centre of analysis	NAA/Cr	NAA/Cho	Cho/Cr
Parma ($n=5$)[a]	2.25 ± 0.11[b]	1.90 ± 0.10	1.18 ± 0.10
Rome ($n=10$)	2.19 ± 0.14[c]	1.89 ± 0.16	1.16 ± 0.13
Verona ($n=15$)	2.22 ± 0.15	1.96 ± 0.12	1.13 ± 0.11

a n = the number of control subjects evaluated at each centre.
b Values are expressed as means ± standard deviations.
c No significant differences were observed when comparing values collected at the three centres.

Magnetic resonance spectroscopy following mild TBI

The data illustrated in Fig. 3 show time-course changes in the mean values of the metabolite ratios (NAA/Cr, NAA/Cho and Cho/Cr) assessed in the 40 concussed athletes. We observed that the Cho/Cr ratio did not change following the concussion and was not significantly different, at any time point, from the values observed in the uninjured control group. This allowed us to use the area under the peaks of either Cr or Cho to calculate the actual variation of the NAA peak relative to the two other invariant metabolites. At the time of the first ^{1}H-MRS performed at 3 days post-injury, a significant alteration in both NAA/Cr and NAA/Cho ratios (−17.6% and −21.4%, respectively) was observed in the 40 concussed athletes ($P<0.001$ with respect to controls). By 15 or 22 days post-concussion, both NAA/Cr and NAA/Cho ratios remained significantly different from controls ($P<0.001$). Values for these ratios were significantly higher at both 15 and 22 days post-injury than the corresponding values recorded at 3 days ($P<0.001$), while values at 22 days were significantly higher than those recorded at 15 days ($P<0.001$). It is important to emphasize that athletes had self-declared to have spontaneously recovered from any clinical post-concussive symptom between 3 and 15 days after concussion, indicating a profound discrepancy between clinical self-observational return to 'normal' and complete metabolic recovery. This latter was in fact observed at only 30 days post-injury, when the metabolite ratios had returned to values detected in control healthy subjects.

Figure 4 illustrates the box plot of the NAA/Cr values determined in controls and in the 40 concussed athletes at different time points following concussion. Pearson's correlation analysis of the data demonstrates that the exponential model had $r^2=0.639$, whilst the linear model had $r^2=0.608$. Therefore, the exponential model is the best to fit the trend in the kinetics of the NAA/Cr recovery following concussion, with high statistical

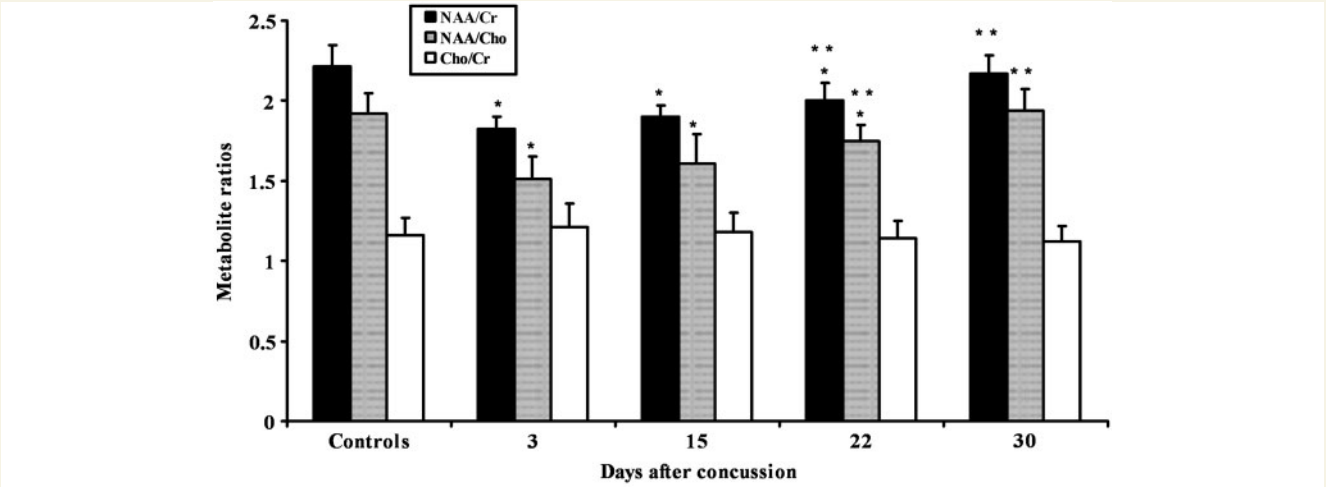

Figure 3 Bar graph showing the metabolite ratios of *N*-acetylaspartate/choline-containing compounds, *N*-acetylaspartate/creatine-containing compounds and choline-containing compounds/creatine-containing compounds in controls and concussed patients. Each histogram is the mean value determined in 30 healthy controls and 40 concussed athletes. Standard deviations are represented by vertical bars. At 3 days *N*-acetylaspartate/creatine-containing compounds decreased by 17.6%, *N*-acetylaspartate/choline-containing compounds by 21.4% and both gradually recovered to complete normalization at 30 days. The choline-containing compounds/creatine-containing compounds ratio did not show any significant variation. $*P<0.01$ with respect to controls; $**P<0.01$ with respect to values determined at the previous time points.

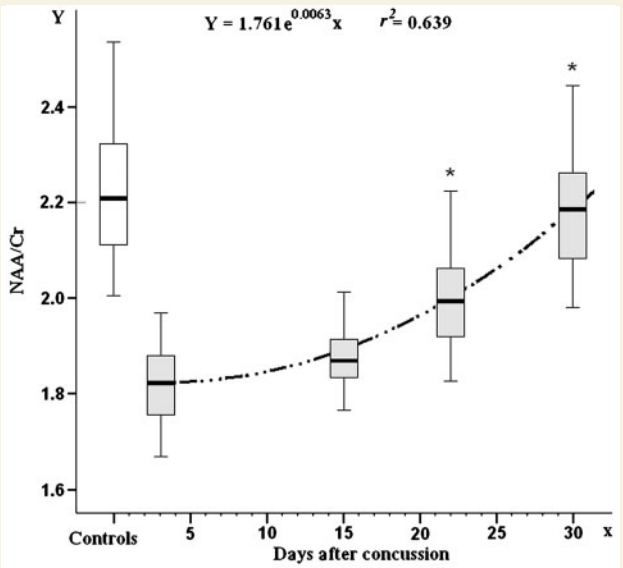

Figure 4 A box plot showing the recovery of the *N*-acetylaspartate/creatine-containing compounds ratio occurring in 40 athletes following concussion and reporting the equation and the best fit curve to the data (indicated by a dash dotted line). Each box is the mean value determined in 30 healthy controls and 40 concussed athletes. Confidence intervals (95%) are represented by vertical bars. $*P<0.01$ with respect to 3 days.

significance ($P<0.001$). These data support previous findings (Vagnozzi *et al*., 2008), suggesting that the metabolite normalization process after a concussive episode is an exponential phenomenon, rather slow in the first 2 weeks, when a daily increase of ~0.35% is observed, and faster between 22 and 30 days, when the daily increase of the NAA/Cr ratio is ~1% (ANOVA for repeated measures, $P<0.01$).

Discussion

The results from the present study show that following a concussive TBI, ^{1}H-MRS is capable of detecting significant neurochemical changes present in the injured brain despite the normal appearance of neuroimaging, absence of symptoms and normal neurological examination. The consistent temporal changes of NAA levels, observed in 40 concussed athletes from various sport disciplines, suggest that ^{1}H-MRS can be used to monitor the depletion and recovery of a metabolite (NAA) that, besides indicating a general alteration in cellular homeostasis, might reflect a condition of energetic imbalance. As long as this energy deficit persists, NAA re-synthesis is significantly impaired (Vagnozzi *et al.*, 2005, 2007; Tavazzi *et al.*, 2007). The results of our research demonstrate the existence of a profound period of bio-energetic depression in the concussed brain corresponding to the well-known state of metabolic 'brain vulnerability', which follows a concussive event (Jenkins *et al.*, 1986, 1989; Andersen and Marmarou, 1992; Hovda *et al.*, 1993, 1999; Vagnozzi *et al.*, 2005, 2007; Tavazzi *et al.*, 2007). In addition, these data show that ^{1}H-MRS determination of NAA is a rapid, non-invasive, reliable and objective tool that may be of significant clinical utility in assessing and monitoring this peculiar sub-clinical event. Moreover, the use of standardized volumes of interest allowed the acquisition of fairly reproducible data so that differences in magnetic resonance scanners should not be considered a source of confounding variability in multicentre studies.

By confirming NAA reduction, we also provide evidence that self-assessed resolution of concussion-associated symptoms is

unreliable and that by this time substantial metabolic alterations are still present (Vagnozzi *et al.*, 2008).

None of the methods most frequently used to follow post-concussed patients is capable of either measuring brain metabolism or evaluating when it has normalized (McCrea *et al.*, 2003; Schatz *et al.*, 2006; Broglio *et al.*, 2007; Register-Mihalik *et al.*, 2008).

This study is the first to document the clinical utility of ^{1}H-MRS to evaluate a biomarker of brain bio-energetic status in patients with mild TBI and suggests that, following a single concussive event, the brain remains in a metabolically vulnerable condition, despite evidence of clinical recovery.

Mild TBI and sport-related concussion

Although all concussions are generally regarded as mild TBIs, it is not possible to confirm that all mild TBIs are 'concussive'. Hence it is generally accepted that the study of concussion includes mild TBI. For many reasons it has become more common to diagnose and follow concussion in athletes than in non-athletes, due to the intrinsic risks inherent in several types of contact and non-contact sports (Meehan and Bachur, 2009). Athletes represent a group of people particularly at risk for concussive brain injury and, in professional athletes, the economic interests are markedly related to the athlete's health and performance (Schleimer, 2002). The most recent Consensus Conference on Sports-Related Concussion (McCrory *et al.*, 2009*a*,*b*) introduced the general concept that the return of athletes to play is possible only when all the concussion-associated symptoms (neurological, behavioural, cognitive) have fully resolved. Furthermore, it was confirmed that no clinical or diagnostic biomarker, providing a prognostic index of the time necessary for a full recovery of athletes after a concussive episode, indeed exists.

Experimental studies reporting metabolic and molecular changes triggered in brain tissue by concussive events (Hovda *et al.*, 1993, 1999; Vagnozzi *et al.*, 2005, 2007; Tavazzi *et al.*, 2007) render even more uncertain how to manage post-concussed athletes for their safe return to play. A distinct period of metabolic imbalance, characterized by altered energy state and decreased NAA concentrations following concussion in rodents has been shown to persist in post-injured brain for a specific number of days. This interval, known as the window of metabolic 'brain vulnerability' (Hovda *et al.*, 1993, 1999; Vagnozzi *et al.*, 2005, 2007; Tavazzi *et al.*, 2007), has been similarly shown to occur in a recent pilot study carried out in post-concussed athletes (Vagnozzi *et al.*, 2008). In this preliminary study, ^{1}H-MRS determination of NAA was reported to be a possible valid tool for monitoring this critical period of time.

The first aim of the present study was to confirm and extend the conclusions obtained in our initial pilot work (Vagnozzi *et al.*, 2008). The number of athletes and controls enrolled in the current study was sufficient to ensure valid statistical evaluation of the data. Our results demonstrated that concussion induces an acute and marked depletion of brain NAA, clearly evident at 3 days post-injury (17.6% decrease with respect to controls). By 15 days post-injury, as a consequence of the initial slow rate of re-synthesis, NAA levels had returned to the minimal value recorded in controls in only 5 of 40 concussed athletes. Brain concentration of NAA remained profoundly depressed in 21 of 40 concussed athletes at 22 days post-injury but had returned to normal (control) values by 30 days post-injury. The relevance of these findings is also related to the role of NAA as an indirect marker of the neuronal energy state (Vagnozzi *et al.*, 2005, 2007; Tavazzi *et al.*, 2007).

The significance of reduced NAA levels and its clinical implication

Although an exact role of NAA remains to be established, this metabolite is present in the brain in concentrations 100-fold higher than in non-nervous system tissue and is therefore considered to be a brain-specific metabolite (Mehta *et al.*, 1995; Moffett *et al.*, 2007). The biochemistry of NAA involves its formation from aspartate and acetyl coenzyme A through the action of L-aspartate-*N*-acetyltransferase in mitochondria of neurons (Madhavarao *et al.*, 2003) and its catabolism in a different cell (mainly oligodendrocytes) compartment (Madhavarao *et al.*, 2004), where NAA is hydrolysed into L-aspartate and acetate by *N*-acetyl-aspartoacylase. Homoeostasis of NAA, however, is not only regulated by the rate of synthesis, efflux from neurons, uptake by oligodendrocytes and rate of degradation, but it is also strictly related to mitochondrial integrity (Signoretti *et al.*, 2001). We have recently demonstrated that post-traumatic energy imbalance, assessed by high-energy phosphate quantification (ATP, ADP, AMP, etc.), is caused mainly by mitochondrial malfunctioning, as indicated by altered mitochondrial phosphorylating capacity (measured by the ATP/ADP ratio) (Tavazzi *et al.*, 2005; Signoretti *et al.*, 2010). Under these conditions, the remarkable decrease in cerebral NAA, which mirrors the changes in brain ATP, may possibly be attributed to the general energy depression consequent to impaired mitochondrial functions (Lifshitz *et al.*, 2003; Robertson *et al.*, 2006). Incorporating the data obtained in preclinical studies on mild TBI, demonstrating decreased ATP concentration for a given period of time post-injury (Vagnozzi *et al.*, 2005, 2007; Tavazzi *et al.*, 2007), we hypothesize that the process of NAA normalization is markedly hindered by an imbalance of neuronal energy metabolism induced by concussion. The indirect energy expenditure for NAA replenishment is linked to the necessity of using acetyl coenzyme A as an acetyl-group donor to guarantee NAA biosynthesis (catalysed by L-aspartate-*N*-acetyltransferase). Therefore, L-aspartate-*N*-acetyltransferase activity indirectly decreases the amount of mitochondrially produced reduced coenzymes (NADH and $FADH_2$), generated through the citric acid cycle when acetyl coenzyme A is used mainly for the activity of citrate synthase in citric acid synthesis. This implies an indirect cost of 12 ATP molecules for each molecule of NAA produced. Recent experimental studies (Signoretti *et al.*, 2010) have shown that spontaneous re-synthesis of NAA occurs only after recovery of mitochondrial dysfunction with consequential return to normal ATP levels; therefore, it appears possible that normalization of NAA concentrations may occur only after the cerebral energy state has fully recovered. These bench data were recently confirmed by clinical ^{1}H-MRS studies in which

it was clearly evident that alterations in cerebral energy and neurotransmitter metabolism were fundamental abnormalities in mild TBI (Vagnozzi *et al.*, 2008; Gasparovic *et al.*, 2009; Henry *et al.*, 2010).

From the clinical point of view, these novel observations have practical consequences with respect to the establishment of guidelines to regulate the extent of physical activity of athletes during the post-concussion recovery period, on the basis of the ^{1}H-MRS evaluation of their cerebral metabolic state. For example, if upon first examination, NAA is determined to be below control values, i.e. still altered energy metabolism, the patients should be recommended to rest with no physical activity (approximate post-concussion time interval of 1–15 days); if, at the second examination, evidence suggests an initiation of the process of NAA recovery (i.e. quasi-normal energy metabolism), it is advisable that the athlete begin physical activity of increasing intensity (approximate post-concussion time interval of 16–22 days); if, at the third examination, NAA replenishment is observed (i.e. normalized energy metabolism), then physical activity might be intensified to a 'return to play' level of conditions (approximate post-concussion time interval of 23–30 days); if, at the fourth examination, normal NAA, i.e. normal energy metabolism, has been determined, it is suitable that athletes be permitted to return to play (approximate post-concussion time interval $\geq$30 days). In the case of non-athletes suffering from concussion, the 'return to play' guidelines should be translated into recommendations based on personal lifestyle during the recovery of NAA post-concussion: (i) NAA below control values (prolonged altered energy metabolism) would recommend rest, with no physical activity and sedentary lifestyle (approximate post-concussion time interval of 1–15 days); (ii) signs of initiation of NAA recovery (i.e. quasi-normal energy metabolism) would suggest normal working activity and moderate physical activity (approximate post-concussion time interval of 16–22 days); and (iii) normal NAA observed (i.e. normal energy metabolism re-established) would implicate return to full normal lifestyle (approximate post-concussion time interval of 23–30 days).

The results of this study definitively demonstrate the existence of a state of energetic depression, corresponding to the period of metabolic brain vulnerability following a concussive event, and corroborate the notion that NAA decrease and recovery is an extremely variable and dynamic process, evolving over time. If, during this period of time, a second concussive insult should occur, it could cause a further metabolism imbalance with catastrophic consequences (if the second concussion falls in the period of profound metabolism depression, i.e. when NAA is still slowly recovering) or it could simply lengthen the normalization curve (if the second concussion falls in the period of metabolism normalization, i.e. when NAA is rapidly recovering).

According to the data presented, which show an expected subject-to-subject variation, it is obvious that the aforementioned length of time of each period, in both athletes and non-athletes, should be dictated by the rate of NAA normalization following concussion. We suggest, therefore, that data on cerebral metabolic state should be used uniquely to determine the extent of allowable post-injury activity to minimize the likelihood of the individual risks of sustaining a second impact during the critical period of energy metabolism depression. In fact, persisting low NAA after recovery of symptoms may suggest either that the concussive-induced decreases in NAA follow a threshold effect above which symptoms clear or that the areas of the brain measured by the spectroscopy were not responsible for the functions whose disruptions would cause the post-concussive deficits.

To address this topic, future studies comparing metabolic regional data with neuropsychological evaluations should be conducted.

The importance of ^{1}H-MRS in monitoring mild TBI

At the time of the last Consensus Conference on Sports-Related Concussion held in Zurich in 2008, the use of ^{1}H-MRS as a diagnostic tool to monitor post-concussed athletes was considered by the conference committee to be insufficiently supported by clinical data (McCrory *et al.*, 2009*a*,*b*). This statement was again reaffirmed by a recent review in which different diagnostic tools to evaluate sport-related concussions, including MRS, were compared (Davis *et al.*, 2009). These novel results argue that this scepticism is unfounded. The large number of controls and concussed subjects enrolled in our multicentre study, as well as the comparison of three MRS apparatuses and modes of acquisition, allowed us to validate the utility of NAA determination to assess the temporal metabolic recovery of the post-concussed brain using ^{1}H-MRS.

Conclusion

To our knowledge, NAA determination by ^{1}H-MRS currently represents the only non-invasive tool to accurately measure the transient changes in energy metabolism occurring in real time in the post-concussed brain. Moreover, we demonstrated that this phenomenon can be detected using a single-voxel acquisition mode with a 1.5 T MRS apparatus, a single-voxel acquisition mode with a 3.0 T MRS apparatus or a multivoxel acquisition mode with a 3.0 T MRS apparatus, i.e. the phenomenon is independent of the type of MRS apparatus and mode of acquisition. This should permit the possibility of applying this technique in all neuroradiological centres for monitoring patients with mild TBI (Benedetti *et al.*, 2007). Regarding the safe return of concussed athletes to play, our results demonstrate that NAA determination by ^{1}H-MRS can serve as an objective biochemical marker of brain metabolism and brain vulnerability following a concussive insult, the normalization of which should markedly enhance the ability of physicians and trainers to decide the return to play schedule of concussed players. Further studies are warranted to evaluate the utility of this novel, non-invasive diagnostic tool in determining metabolic effects of multiple concussions, as well as in comparing metabolic regional data with neuropsychological evaluation, or in combining longitudinal ^{1}H-MRS and diffusion tensor imaging studies to correlate metabolic alteration with possible white matter tract damage.

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