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Extracellular *N*-acetylaspartate depletion in traumatic brain injury

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Abstract

N-Acetylaspartate (NAA) is almost exclusively localized in neurons in the adult brain and is present in high concentration in the CNS. It can be measured by proton magnetic resonance spectroscopy and is seen as a marker of neuronal damage and death. NMR spectroscopy and animal models have shown NAA depletion to occur in various types of chronic and acute brain injury. We investigated 19 patients with traumatic brain injury (TBI). Microdialysis was utilized to recover NAA, lactate, pyruvate, glycerol and glutamate, at 12-h intervals. These markers were correlated with survival and a 6-month Glasgow Outcome Score. Eleven patients died and eight survived. A linear mixed model analysis showed a significant effect of outcome and of the interaction between time of injury and outcome on NAA levels ($p = 0.009$ and $p = 0.004$, respectively). Overall, extracellular NAA was 34% lower in non-survivors. A significant non-recoverable fall was observed in this group from day 4 onwards, with a concomitant rise in lactate–pyruvate ratio and glycerol. These results suggest that mitochondrial dysfunction is a significant contributor to poor outcome following TBI and propose extracellular NAA as a potential marker for monitoring interventions aimed at preserving mitochondrial function.

Keywords: microdialysis, mitochondria, *N*-acetylaspartate, traumatic brain injury.

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N-Acetylaspartate (NAA) is present at a high concentration in the central nervous system. It is synthesized in the mitochondrion of neurons from L-aspartic acid and acetyl-CoA by the enzyme L-aspartate *N*-acetyl transferase and is then transported to the cytoplasm where it is hydrolysed to aspartate and acetate by an amino acylase.

NAA is almost exclusively localized in neurons in the adult brain and is only second to glutamate in terms of brain concentration of free amino-acids (Birken and Oldendorf 1989). Even although the debate on the precise biological role of NAA is still open, evidence has been produced indicating that NAA is involved in the maintenance of water homeostasis within the cerebral tissue by acting as a 'molecular water pump' (Baslow 2002). This compound has attracted much interest as it can be measured non-invasively by means of magnetic resonance spectroscopy (1H-MRS). Reduced intensity of the NAA peak on 1H-MRS has been demonstrated in a variety of neurological conditions, including stroke (Gideon *et al*. 1992), epilepsy (Cendes

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Abbreviations used: Br-PCO_2, brain PCO_2; Br-PO_2, brain PO_2; Br-Temp, brain temperature; CPP, cerebral perfusion pressure; CT, computerized tomography; eNAA, NAA extracellular concentration; GCS, Glasgow Coma Scale; GOS, Glasgow Outcome Score; ICP, intracranial pressure; LMM, linear mixed model; LPR, lactate : pyruvate ratio; MD, microdialysis; MRS, magnetic resonance spectroscopy; NAA, *N*-Acetylaspartate; NICU, Neuro-intensive Care Unit; TBI, traumatic brain injury.

et al. 1994), perinatal hypoxia–ischaemia (Peden *et al*. 1993), Alzheimer's disease (Shonk *et al*. 1995), multiple sclerosis (Davie *et al*. 1997), neuropsychiatric disorders (Tsai and Coyle 1995), dementia (Shiino *et al*. 1993) and Huntington's disease (Harms *et al*. 1997). More recently, reduced ratios between NAA and other metabolites have been demonstrated in 1H-MRS human and animal studies following traumatic brain injury (TBI) (Choe *et al*. 1995; Cecil *et al*. 1998; Smith *et al*. 1998). These ratios have also been found to have a positive correlation with measures of outcome following TBI (Choe *et al*. 1995; Holshouser *et al*. 1997; Cecil *et al*. 1998; Ross *et al*. 1998; Barkovich *et al*. 1999; Brooks *et al*. 1999; Friedman *et al*. 1999; Brooks *et al*. 2000; Garnett *et al*. 2000; Brooks *et al*. 2001). Although the processes behind this fall in NAA on 1H-MRS following brain injury are unclear, this compound is accepted as a marker of neuronal injury, death or metabolic depression. Histological studies have challenged the initial tenet of NAA reduction as a pure indicator of neuronal depletion. Significant falls in NAA concentration have been demonstrated to occur in areas remote from the focus of the injury and showing disproportionately low neuronal loss in TBI models (Gasparovic *et al*. 2001); conversely, reduced but disproportionately high NAA concentration has been shown in areas of complete neuronal loss in ischaemic models (Demougeot *et al*. 2001). A gradual restoration of NAA levels can occur in patients with acute demyelinating lesions, stroke, epilepsy and carotid revascularization, in concomitance with the improvement of neurological deficits (De Stefano *et al*. 1995; Uno *et al*. 1996; Cendes *et al*. 1997; Kalra *et al*. 1998). In experimental models of diffuse axonal injury, NAA has been shown to recover from an initial drop over a period of days (Rango *et al*. 1995) and also in direct correlation with the brain energy state, as well as in inverse proportion to the severity of the insult (Signoretti *et al*. 2001; Tavazzi *et al*. 2005). The time course of NAA following TBI has also been studied with microdialysis (MD) in animal models (Alessandri *et al*. 2000; Al-Samsam *et al*. 2000) but, to our knowledge, no human MD studies have been reported so far.

Cerebral microdialysis is a well-established laboratory tool that is increasingly used as a bedside monitor to provide continuous *in vivo* analysis of brain tissue biochemistry after TBI (Hillered *et al*. 2005). Microdialysis measures biochemical changes in brain extracellular fluid that are used as surrogate markers of tissue damage and therefore has the potential to monitor the processes of secondary injury after TBI. In this prospective longitudinal study we have analysed the time course of NAA extracellular concentration (eNAA) in 19 patients with traumatic brain injury admitted to the neuro-intensive care unit (NICU) of a university teaching hospital. The first objective of the study was to demonstrate the feasibility of applying cerebral MD to recover and measure serial NAA levels in humans and in a clinical setting. Secondly, we tested the hypothesis, suggested by previous animal and 1H-MRS studies, that patients with worse outcome would show a more pronounced progressive loss of NAA. Finally, in order to elucidate the significance of eNAA fluctuations, we report the correlations between this biomarker and other MD and physiological variables, such as the lactate : pyruvate ratio (LPR), glycerol, glutamate, brain tissue PO_2 (Br-PO_2), PCO_2 (Br-PCO_2), pH (Br-pH) and temperature (Br-Temp).

Materials and methods

Patient population and management

Following institutional ethics committee approval and written agreement from the next of kin, 19 patients admitted to NICU of the National Hospital for Neurology and Neurosurgery (NHNN) were recruited into the study. Inclusion criteria were: (i) traumatic brain injury, (ii) age ≥ 16 years, (iii) absence of documented hypotensive (systolic blood pressure < 90 mmHg) or hypoxic (peripheral oximetry saturation < 90%) prior to monitoring.

On NICU, all patients were sedated with propofol and fentanyl and were mechanically ventilated to maintain $PaCO_2$ between 4.5 and 5.0 kPa and PaO_2 > 13.5 kPa. Routine monitoring requirements included electrocardiogram, non-invasive oxygen saturation, invasive arterial blood pressure, temperature and end-tidal CO_2 and intracranial pressure (ICP). Eleven patients underwent Br-PO_2, Br-PCO_2, Br-pH and Br-Temp monitoring with a Neurotrend device (Codman, Johnson and Johnson, Randolph, MA, USA). All patients received local protocol-guided therapy, based on the Joint Section of Neurotrauma and Critical Care of the American Association of Neurological Surgeons (Brain Trauma Foundation 1996) and the European Brain Injury Consortium guidelines (Maas *et al*. 1997), to limit rises in ICP and maintain cerebral perfusion pressure (CPP) > 60 mmHg. Blood glucose was maintained between 4.0 and 6.0 mmol/L with an insulin infusion sliding scale. The management of the patients was not affected by involvement in the study. For the purpose of the analysis, two outcome endpoints were considered: survival at discharge from the National Hospital for Neurology and Neurosurgery and Glasgow Outcome Score (GOS) at 5–9 months (median 6) after injury (GOS: 5 = good recovery; 4 = moderate disability, 3 = severe disability; 2 = vegetative state; 1 = death). GOS was dichotomized into 'favourable' (GOS 4–5) and 'unfavourable' (GOS 1–3).

Microdialysis interventions

Cerebral microdialysis was monitored in all patients using a CMA 600 bedside analyser (CMA Microdialysis, Stockholm, Sweden) and a gold-tipped microdialysis catheter (CMA 70; CMA Microdialysis) that has a membrane length of 10 mm, a diameter of 0.52 mm and a molecular mass cut-off of 20 kDa.

The catheters were inserted either intraoperatively, if the patient was undergoing surgery, or via a skull-fixed triple lumen bolt (Technicam Ltd, Newton Abbot, UK). In all cases, the position of the probe was verified on subsequent computerized tomography (CT) scans. For the purpose of this study, only one catheter was

used and this was placed in the 'penumbra' surrounding a mass lesion (within 2 cm) or in the right frontal region in those with a diffuse injury. Placement of the catheter was verified on post-insertion CT scans in all cases. An isotonic (NaCl 147 mmol/L, KCl 2.7 mmol/L, $CaCl_2$ 1.2 mmol/L, $MgCl_2$ 0.85 mmol/L) proprietary perfusion fluid (Perfusion Fluid, CMA/Microdialysis, Solna, Sweden) was perfused at a rate of 0.3 μL/min. In order to minimize the influence of implantation injury on the results, the first sample was collected for 3 h and then discarded. MD samples were subsequently collected for periods of 12 consecutive hours; at the end of each collection period, the dialysate was analysed for lactate, pyruvate, glycerol and glutamate using a bedside CMA 600 Analyser (CMA Microdialysis) that utilizes an enzymatic reagent and colorimetric technique. For each analysis, the LPR was calculated. MD values were not corrected for *in vivo* recovery. After collections and bedside analysis, the samples were immediately labelled and frozen at −80°C for subsequent NAA measurement.

Sample preparation and NAA assay

Aliquots of MD samples (15–30 μL) were properly diluted with doubly distilled water to a final volume of 250 μL. Diluted MD samples were then filtered through a 0.45-mm HV Millipore filter and loaded (200 μL) onto a Kromasil C-18, 250 × 4.6 mm, 5-μm particle size column, provided with its own guard column (Eka Chemicals AB, Bohus, Sweden) and connected to an HPLC apparatus consisting of a SpectraSystem P2000 pump system (ThermoElectron Italia, Milan, Italy) and a highly sensitive UV6000LP diode array detector (ThermoElectron Italia) equipped with a 5-cm light path flow cell and set up between 200 and 300 nm wavelength. Data acquisition and analysis were performed by a PC using the ChromQuest® software package provided by the HPLC manufacturer.

NAA was determined according to the ion-pairing isocratic separation described by Tavazzi *et al*. (2000) by using tetrabutylammonium hydroxide as the pairing reagent. NAA calculation was carried out at 206 nm wavelength by comparing retention times, absorption spectra and areas of peaks of MD samples with those of freshly prepared ultra-pure standard with known concentration.

Statistical analyses

Data were checked for normal distribution and logarithmically transformed if appropriate (eNAA, LPR, glycerol and glutamate). Non-parametric tests were used for analyses of variables whose normality could not be assumed.

In order to account for repeated observations within subjects, and for missing data, a linear mixed model (LMM) was used to analyse the effect of time from injury and outcome, as well as the interaction between the two, on the following variables of interest: eNAA, LPR, glycerol, glutamate and CPP. Daily eNAA means with standard deviations are reported, in the original scale, as totals and separately for outcome groups. A multivariate ANOVA with contrast for repeated measurements (General Linear Model) was utilized to study changes from day to day in the values of eNAA, LPR, glycerol, glutamate and CPP.

Correlations between eNAA, Br-PO_2, Br-PCO_2, Br-pH, Br-Temp, CPP and ICP, and between MD variables were calculated using the Spearman's test with Bonferroni correction. For this purpose, we used the average values of Br-PO_2, Br-PCO_2, Br-pH, Br-Temp, CPP and ICP recorded during the period of MD monitoring, adjusted for a lag of 20 min to account for the time taken by the microdialysate to reach the collection vial. The Mann–Whitney *U*-test was utilized to compare Glasgow Coma Scale (GCS) and age between outcome groups. A p-value < 0.05 (2-tailed) was considered significant.

Results

Patient details

A summary of patients' demographic data, injury mechanism and clinical interventions is shown in Table 1.

Eleven patients died on NICU (58%) and a further patient was dead at 6 months. This figure is comparable with the overall TBI mortality on NICU in the same period (52%). The two groups of survivors and non-survivors were similarly distributed in terms of age (survivors' mean 37.7 years SD 20.7, median 32.5; non-survivors 44.2 SD 19.2, median 45; $p = 0.51$) and GCS after initial resuscitation (survivors' mean 8.3 SD 4.2, median 7.5; non-survivors 7.5 SD 3.3, median 7; $p = 0.73$). Survivors were monitored for an average of 6.1 days (SD 3.3, median 6) from the time of injury, non-survivors for 5.1 (SD 2.2, median 5); there was no significant difference between the two groups in this respect ($p = 0.31$).

No infection, dislodgment or haematoma were observed after MD insertion; however, one patient developed an intracerebral haematoma, requiring surgical evacuation, after removal of the MD catheter (case 10).

Time course of eNAA and outcome following TBI

The time course of NAA in survivors and non-survivors is represented in Fig. 1(a). As illustrated in Table 2, survivors had significantly higher eNAA values than non-survivors ($p = 0.009$). Significant difference was also found when the dichotomized 6-month outcome was considered ($p = 0.04$).

The LMM analysis also revealed that, whilst the effect of time from injury on eNAA was not statistically significant when all patients where considered together, there was a significant effect of interaction between time from injury and outcome ($p = 0.004$), indicating a different time course of this marker between survivors and non-survivors. Moreover, the contrasted ANOVA showed a sharp non-recoverable fall in non-survivors from day 4 onwards ($p = 0.01$), whereas no significant variations occur from day to day in survivors.

LPR, glycerol, glutamate and CPP

The analysis showed a significant effect of the interaction between time from injury and outcome for LPR ($p = 0.001$), with non-survivors displaying significantly higher levels than survivors, again from day 4 onwards (Fig. 1b). A similar effect was observed for glycerol ($p = 0.0001$); in

Table 1 Patients' demographics and interventions

Case no.	Main finding on CT scan	Surgical intervention	GCS[a]	GOS[b]	GOS[c]	MD	Age
1	ICH, SAH, hydrocephalus	EVD	11	1		2	60
2	Small EDH, hydrocephalus	EVD	9	1		3	25
3	ASDH, SAH	Evacuation and lobectomy	13	2	1	2	58
4	ICH, contusions	None	11	4	5	1	22
5	Contusions	None	3	3	3	3	25
6	Small EDH, contusions	Decompressive craniectomy, lobectomy	5	4	4	3	28
7	ASDH	Evacuation	6	1		1	69
8	GSW	None	4	1		1	45
9	Traumatic SAH from stabbing	EVD	5	1		3	21
10	ICH, ASDH and EDH	Evacuation	8	3	4	4	37
11	Acute or chronic SDH	Evacuation	15	5	5	3	78
12	Contusion, ASDH	Evacuation	5	1		2	61
13	Diffuse axonal injury	None	7	4	4	1	38
14	Contusions	None	7	1		4	29
15	ASDH	Evacuation	8	1		4	18
16	Traumatic SAH	Decompressive craniectomy, EVD	15	1		3	35
17	ASDH, bilateral contusions	Evacuation, decompressive craniectomy	4	1		3	57
18	Bilateral contusions	None	8	1		3	66
19	Diffuse axonal injury	Decompressive craniectomy, lobectomy	5	3	4	2	16

ASDH, acute subdural haematoma; EDH, extradural haematoma; EVD, external ventricular drain; GSW, gunshot wound; ICH, intracerebral haematoma; MD, days from injury when MD monitoring started; SAH, subarachnoid haemorrhage; SDH, subdural haematoma. [a]Glasgow Coma Scale after initial resuscitation; [b]Glasgow Outcome Scale at discharge (scores: 1 = death, 2 = persistent vegetative state, 3 = severe disability, 4 = moderate disability, 5 = good recovery); [c]Glasgow Outcome Scale at follow-up.

non-survivors there was a marked elevation of this marker on day 4 ($p = 0.001$), followed by a steady decline over several days (Fig. 1c). No significant effect was found for glutamate (Fig. 1d).

We found a significant difference in CPP values between survivors and non-survivors (71.3 mmHg SD 7.9, and 62.9 SD 12.5, respectively; $p = 0.012$). The LMM analysis also showed that the interaction between outcome and time of injury was significant for this parameter ($p = 0.0001$) and the contrasted model indicated a pronounced CPP fall in non-survivors on day 2 and day 4 ($p = 0.0001$), as shown in Fig. 1(e). Although there was no significant difference in ICP means between the two groups, there was a significant interaction between time from injury and outcome on this parameter ($p = 0.0001$). The analysis showed a significant sharp ICP rise in the non-survivors' group between day 3 and day 4, from 13.6 mmHg SD 4.7 to 27.8 SD 2.8 ($p = 0.002$), followed by a sharp fall over the next 24 h.

The significance of these differences was maintained when the dichotomized 6-month outcome was substituted for survival in the analysis.

Extracellular biomarkers and brain tissue monitoring variables

Extracellular *N*-acetylaspartate was found to have a positive correlation with Br-pH ($r = 0.48$, $p = 0.007$), but not with ICP, Br-PO_2, Br-PCO_2, Br-Temp or any other MD variable. There was correlation between LPR and glycerol ($r = 0.4$, $p < 0.001$), LPR and glutamate ($r = 0.61$, $p = 0.001$), and glycerol and glutamate ($r = 0.13$, $p = 0.001$).

Similar correlations were seen when the two groups were analysed separately (data not shown), except for the finding that in survivors eNAA had an inverse correlation with Br-pH ($r = –0.52$, $p = 0.04$), whilst in non-survivors the correlation was reversed ($r = 0.55$, $p = 0.032$).

Discussion

The results of our study indicate differences in the time course and concentration of eNAA in outcome groups of our TBI patients. The cohorts were comparable in terms of age, GCS after initial resuscitation, ICP values and range of CT abnormalities, although non-survivors included two patients with penetrating brain injury (no. 9 and no. 10). However, a censored analysis excluding the latter two cases yielded similar results to those presented above. Microdialysis was implanted in the penumbra region to reflect changes occurring in a vulnerable part of the brain, except in patients with diffuse injury (no. 13 and no. 19). Again, if these two cases were excluded from the analysis the results would not alter significantly.

We do not have reference values of eNAA in healthy controls, as it would be unethical to perform invasive

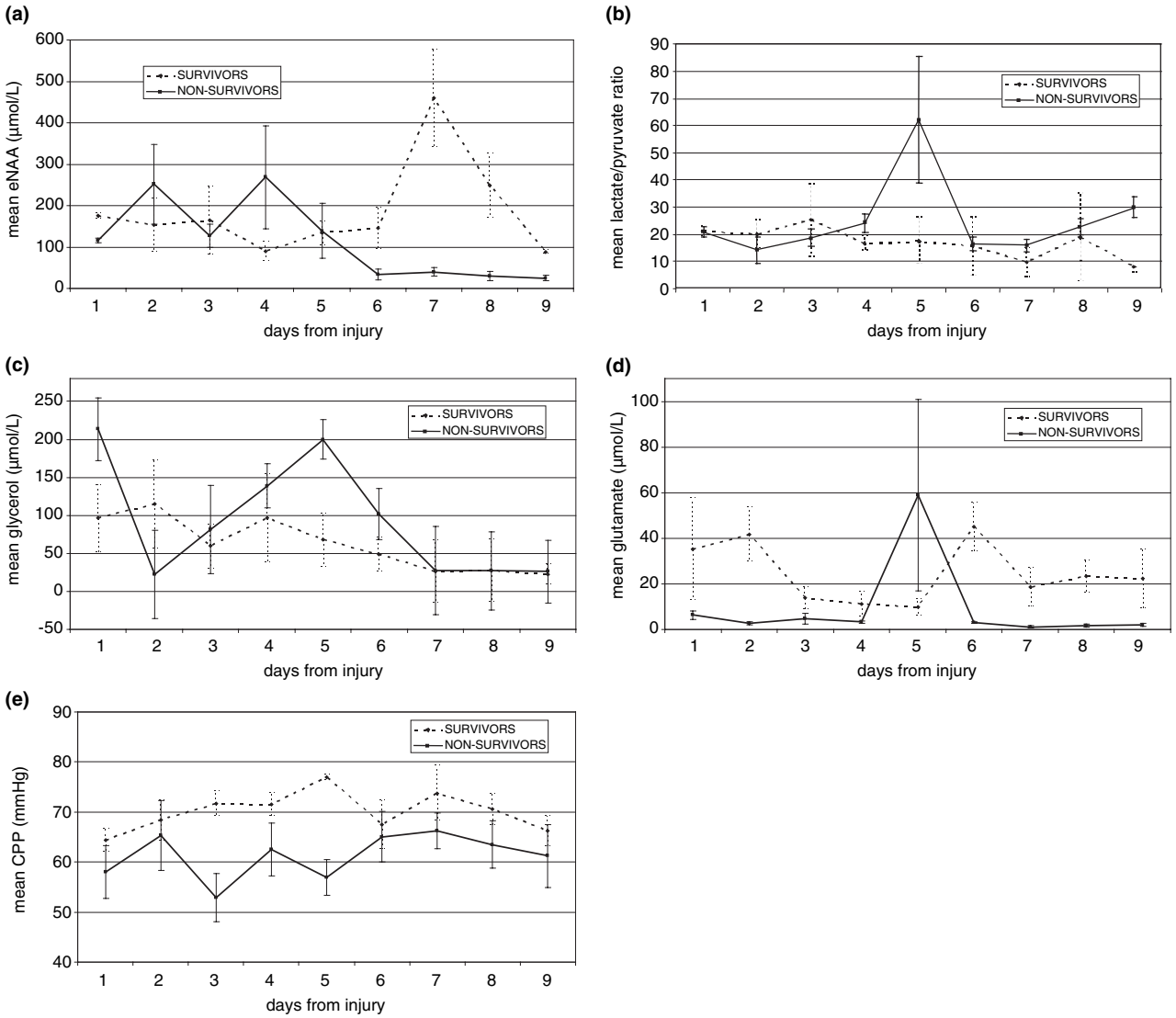

Fig. 1 Time course of eNAA (a), LPR (b), glycerol (c), glutamate (d) and CPP (e) in survivors (dashed line) and non-survivors (continuous line). Only survivors are present after day 9, so comparative data are only shown up to this time. Note a steady fall of eNAA levels in non-survivors starting from day 4.

Table 2 Mean daily values of eNAA (μmol/L), with SD and *n*-values, for different outcome groups

		Days from injury									
		1	2	3	4	5	6	7	8	9	Total
Survivors	eNAA	174.9	153.4	164.2	89.7	134.3	145.8	460.1	247.9	88.4	175.27
	SD	14.3	126.2	163.3	44	58	101.1	236.8	156.2	8.7	149.55
	n	2	6	11	6	8	5	5	5	5	53
Non-survivors	eNAA	115.4	251.5	127.1	268.3	138.8	32.9	40	29.6	25.2	115.7
	SD	11	193	56.3	248.6	132.7	26	21.6	23.6	12.8	141.75
	n	2	6	7	8	11	8	6	8	4	60
Total	eNAA	145.2	200.3	154.1	188.9	137.1	75.3	250	111.5	63.1	145.48
	SD	35.9	151.5	140	201.3	106.8	82	274.9	141.6	35.7	147.7
	n	4	12	18	14	19	13	11	13	9	113

microdialysis procedures in such individuals. However, we observed initial high eNAA followed by a dramatic fall in patients who died. This is an interesting finding that is in line with the above mentioned TBI study by Signoretti *et al.* (2001), and also with unreported data from a recent 1H-MRS study by the same group showing an actual increase in NAA in the first 72 h after trauma in patients with diffuse TBI, with a subsequent decline evident at 10 days post-injury (Signoretti *et al*., personal communication). Animal models have also shown that, when a hypotensive–hypoxic insult is added to TBI, the late NAA fall is even more pronounced (Al-Samsam *et al*. 2000; Signoretti *et al*. 2001; Tavazzi *et al*. 2005).

Higher NAA levels were found in patients with better outcome at a median time of 41 days from TBI in a 1H-MRS human study (Sinson *et al*. 2001). Although 1H-MRS and brain tissue studies measure both intracellular and extracellular NAA, our results suggest that eNAA is in itself related to outcome. NAA is characterized by high tissue to extracellular concentration ratio under normal conditions (Taylor *et al*. 1994; Sager *et al*. 1997). Whilst the mechanism of its release into the extracellular fluid is unclear, it would be difficult to attribute this to microdialysis implantation in our study, as dialysate NAA levels have been found to be low and stable within a few hours of catheter insertion (Obrenovitch *et al*. 1993; Sager *et al*. 1997). It has been ascertained that, under physiological conditions, NAA changes cellular compartment from neurons (where it is synthesized) to oligodendrocytes (where it is hydrolysed) with transient permanence in the extracellular space (Bhakoo *et al*. 2001; Chakraborty *et al*. 2001; Baslow 2003; Lu *et al*. 2004). The central role of NAA in water extrusion from neurons into the blood stream (with active involvement of astrocytes), in a glucose consumption-associated process, has been proposed in experimental studies (Baslow 2002, 2003). NAA decrease in pathological states has been proposed to occur via a transient membrane microporation (Taylor *et al*. 1994), although this phenomenon has also been attributed to neuronal dysfunction or death (Giroud *et al*. 1996; Demougeot *et al*. 2001; Gasparovic *et al*. 2001). Our results show a steady fall of extracellular NAA in non-survivors from day 4 after injury. As this phenomenon was not observed in survivors, it can be hypothesized that in non-survivors the late pronounced fall is, at least in part, as a result of sustained metabolic dysfunction in the neuronal population, reflected in impaired NAA mitochondrial biosynthesis abnormal release, extracellular degradation, reuptake or clearance of this compound. Previous studies have furnished evidence that NAA reduction may be an indicator of mitochondrial dysfunction (Saragea *et al*. 1965; Knizley 1967; Goldstein 1969; Patel and Clark 1979; Brenner *et al*. 1993; Heales *et al*. 1995; Clark 1998; Signoretti *et al*. 2001). NAA depletion and recovery following TBI has been found to be mirrored by similar changes in ATP levels (Signoretti *et al*. 2001; Tavazzi *et al*. 2005). Mitochondrial impairment in TBI has been demonstrated in human (Verweij *et al*. 1997, 2000), animal (Xiong *et al*. 1997; Sullivan *et al*. 1998; Xiong *et al*. 1998; Vagnozzi *et al*. 1999; Signoretti *et al*. 2004) and *in vitro* studies (Ahmed *et al*. 2000).

Taylor *et al*. (1995) have postulated that eNAA may serve as on osmoregulator and may protect neurons against marked ionic changes and Gotoh *et al*. (1997) have demonstrated a massive efflux of NAA into the extracellular compartment in response to tissue acidosis. It is of note that, in our study, eNAA had an inverse correlation with Br-pH in survivors and the opposite in non-survivors, suggesting that this protective mechanism may be impaired in the cohort of a patient with worse outcome.

It has been shown that cerebral ischaemia and reperfusion cause a transient increase in eNAA, rather than depletion (Sager *et al*. 1997). In our cohort we found no significant overall correlation between eNAA and LPR that is thought to be a marker of tissue ischaemia (Hillered *et al*. 2005). If energy failure persists, cell damage ensues, a phenomenon that in our cohort is probably indicated by the rise in glycerol, a marker of cell membrane breakdown (Paschen *et al*. 1986; Marklund *et al*. 1997), observed in non-survivors. Although the role of MD glutamate in monitoring excitotoxicity and $Ca2^{+}$-mediated cell damage has been challenged (Obrenovitch and Urenjak 1997a,b; Obrenovitch 1999), prolonged elevations of this marker in dialysate have been described in human TBI studies (Koura *et al*. 1998; Obrenovitch 1999; Hutchinson *et al*. 2000; Reinert *et al*. 2000). We could not confirm the finding by previous studies (Koura *et al*. 1998; Yamamoto *et al*. 1999; Hutchinson *et al*. 2000) that sustained or late rises in dialysate glutamate are associated with poorer outcome.

Between day 3 and day 5 we observed the most pronounced changes in ICP, CPP, eNAA, LPR and glycerol values in the non-survivors' groups. Our data do not allow us to elucidate whether poor cerebral perfusion triggered a metabolic crisis or whether the latter induced cell swelling and consequent intracranial hypertension. However, it is of note that in non-survivors eNAA levels remain 65–78% below the mean value for this group beyond day 5, despite a normalization of glycerol, CPP and, temporarily, LPR. If NAA depletion were solely attributable to neuronal loss, we would have expected a simultaneous rise in glycerol in this phase, suggesting that the metabolism of NAA is somewhat compromised even in viable neurons. Moreover, follow-up CT scans, performed at various stages after insertion of microdialysis in all patients, did not reveal areas of necrosis or infarct around the catheter in any of the cases. Ideally, neuronal loss in the vicinity of the catheter should be verified histologically, e.g. when contused areas are removed or lobectomies were performed to control ICP, but this was not available in this study.

As NAA depletion can be a reversible phenomenon, this compound should be seen as both a functional and structural marker of cell integrity. This makes eNAA a candidate marker for monitoring therapeutic strategies aimed at preserving mitochondrial function.

We believe this to be the first study to demonstrate that eNAA can be recovered using microdialysis in patients with head injury. We have also shown that levels of this marker in the extracellular fluid correlate with outcome following traumatic brain injury, in line with magnetic resonance spectroscopy studies. Unlike 1H-MRS, NAA measurement by microdialysis only provides information on a very limited area of the brain, but it has the advantage of allowing frequent sampling, with minimal intervention after implantation of the catheter. Further research is warranted to evaluate the usefulness and robustness of eNAA as a marker in therapeutic studies.

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References

Ahmed S. M., Rzigalinski B. A., Willoughby K. A., Sitterding H. A. and Ellis E. F. (2000) Stretch-induced injury alters mitochondrial membrane potential and cellular ATP in cultured astrocytes and neurons. *J. Neurochem.* **74**, 1951–1960.

Alessandri B., al-Samsam R., Corwin F., Fatouros P., Young H. F. and Bullock R. M. (2000) Acute and late changes in *N*-acetyl-aspartate following diffuse axonal injury in rats: an MRI spectroscopy and microdialysis study. *Neurol. Res.* **22**, 705–712.

Al-Samsam R. H., Alessandri B. and Bullock R. (2000) Extracellular *N*-acetyl-aspartate as a biochemical marker of the severity of neuronal damage following experimental acute traumatic brain injury. *J. Neurotrauma* **17**, 31–39.

Barkovich A. J., Baranski K., Vigneron D., Partridge J. C., Hallam D. K., Hajnal B. L. and Ferriero D. M. (1999) Proton MR spectroscopy for the evaluation of brain injury in asphyxiated, term neonates. *Am. J. Neuroradiol.* **20**, 1399–1405.

Baslow M. H. (2002) Evidence supporting a role for *N*-acetyl-L-aspartate as a molecular water pump in myelinated neurons in the central nervous system. An analytical review. *Neurochem. Int.* **40**, 295–300.

Baslow M. H. (2003) *N*-acetylaspartate in the vertebrate brain: metabolism and function. *Neurochem. Res.* **28**, 941–953.

Bhakoo K. K., Craig T. J. and Styles P. (2001) Developmental and regional distribution of aspartoacylase in rat brain tissue. *J. Neurochem.* **79**, 211–220.

Birken D. L. and Oldendorf W. H. (1989) *N*-acetyl-L-aspartic acid: a literature review of a compound prominent in 1H-NMR spectroscopic studies of brain. *Neurosci. Biobehav Rev.* **13**, 23–31.

Brain Trauma Foundation (1996) Guidelines for the management of severe head injury. Brain Trauma Foundation, American Association of Neurological Surgeons, Joint Section on Neurotrauma and Critical Care. *J. Neurotrauma* **13**, 641–734.

Brenner R. E., Munro P. M., Williams S. C., Bell J. D., Barker G. J., Hawkins C. P., Landon D. N. and McDonald W. I. (1993) The proton NMR spectrum in acute EAE: the significance of the change in the Cho: Cr ratio. *Magn. Reson. Med.* **29**, 737–745.

Brooks W. M., Jung R. E., Ford C. C., Greinel E. J. and Sibbitt W. L. Jr (1999) Relationship between neurometabolite derangement and neurocognitive dysfunction in systemic lupus erythematosus. *J. Rheumatol.* **26**, 81–85.

Brooks W. M., Stidley C. A., Petropoulos H., Jung R. E., Weers D. C., Friedman S. D., Barlow M. A., Sibbitt W. L. Jr and Yeo R. A. (2000) Metabolic and cognitive response to human traumatic brain injury: a quantitative proton magnetic resonance study. *J. Neurotrauma* **17**, 629–640.

Brooks W. M., Friedman S. D. and Gasparovic C. (2001) Magnetic resonance spectroscopy in traumatic brain injury. *J. Head Trauma Rehabil.* **16**, 149–164.

Cecil K. M., Hills E. C., Sandel M. E., Smith D. H., McIntosh T. K., Mannon L. J., Sinson G. P., Bagley L. J., Grossman R. I. and Lenkinski R. E. (1998) Proton magnetic resonance spectroscopy for detection of axonal injury in the splenium of the corpus callosum of brain-injured patients. *J. Neurosurg.* **88**, 795–801.

Cendes F., Andermann F., Preul M. C. and Arnold D. L. (1994) Lateralization of temporal lobe epilepsy based on regional metabolic abnormalities in proton magnetic resonance spectroscopic images. *Ann. Neurol.* **35**, 211–216.

Cendes F., Andermann F., Dubeau F., Matthews P. M. and Arnold D. L. (1997) Normalization of neuronal metabolic dysfunction after surgery for temporal lobe epilepsy. Evidence from proton MR spectroscopic imaging. *Neurology* **49**, 1525–1533.

Chakraborty G., Mekala P., Yahya D., Wu G. and Ledeen R. W. (2001) Intraneuronal *N*-acetylaspartate supplies acetyl groups for myelin lipid synthesis: evidence for myelin-associated aspartoacylase. *J. Neurochem.* **78**, 736–745.

Choe B. Y., Suh T. S., Choi K. H., Shinn K. S., Park C. K. and Kang J. K. (1995) Neuronal dysfunction in patients with closed head injury evaluated by *in vivo* 1H magnetic resonance spectroscopy. *Invest. Radiol.* **30**, 502–506.

Clark J. B. (1998) *N*-acetyl aspartate: a marker for neuronal loss or mitochondrial dysfunction. *Dev. Neurosci.* **20**, 271–276.

Davie C. A., Barker G. J., Thompson A. J., Tofts P. S., McDonald W. I. and Miller D. H. (1997) 1H magnetic resonance spectroscopy of chronic cerebral white matter lesions and normal appearing white matter in multiple sclerosis. *J. Neurol. Neurosurg. Psychiatry* **63**, 736–742.

De Stefano N., Matthews P. M. and Arnold D. L. (1995) Reversible decreases in *N*-acetylaspartate after acute brain injury. *Magn. Reson. Med.* **34**, 721–727.

Demougeot C., Garnier P., Mossiat C., Bertrand N., Giroud M., Beley A. and Marie C. (2001) *N*-Acetylaspartate, a marker of both cellular dysfunction and neuronal loss: its relevance to studies of acute brain injury. *J. Neurochem.* **77**, 408–415.

Friedman S. D., Brooks W. M., Jung R. E., Chiulli S. J., Sloan J. H., Montoya B. T., Hart B. L. and Yeo R. A. (1999) Quantitative proton MRS predicts outcome after traumatic brain injury. *Neurology* **52**, 1384–1391.

Garnett M. R., Blamire A. M., Corkill R. G., Cadoux-Hudson T. A., Rajagopalan B. and Styles P. (2000) Early proton magnetic resonance spectroscopy in normal-appearing brain correlates with outcome in patients following traumatic brain injury. *Brain* **123**, 2046–2054.

Gasparovic C., Arfai N., Smid N. and Feeney D. M. (2001) Decrease and recovery of *N*-acetylaspartate/creatine in rat brain remote from focal injury. *J. Neurotrauma* **18**, 241–246.

Gideon P., Henriksen O., Sperling B., Christiansen P., Olsen T. S., Jorgensen H. S. and Arlien-Soborg P. (1992) Early time course of *N*-acetylaspartate, creatine and phosphocreatine, and compounds containing choline in the brain after acute stroke. A proton magnetic resonance spectroscopy study. *Stroke* **23**, 1566–1572.

Giroud M., Walker P., Bernard D., Lemesle M., Martin D., Baudouin N., Brunotte F. and Dumas R. (1996) Reduced brain *N*-acetyl-aspartate in frontal lobes suggests neuronal loss in patients with amyotrophic lateral sclerosis. *Neurol. Res.* **18**, 241–243.

Goldstein F. B. (1969) The enzymatic synthesis of *N*-acetyl-L-aspartic acid by subcellular preparations of rat brain. *J. Biol. Chem.* **244**, 4257–4260.

Gotoh M., Davies S. E. and Obrenovitch T. P. (1997) Brain tissue acidosis: effects on the extracellular concentration of *N*-acetylaspartate. *J. Neurochem.* **69**, 655–661.

Harms L., Meierkord H., Timm G., Pfeiffer L. and Ludolph A. C. (1997) Decreased *N*-acetyl-aspartate/choline ratio and increased lactate in the frontal lobe of patients with Huntington's disease: a proton magnetic resonance spectroscopy study. *J. Neurol. Neurosurg. Psychiatry* **62**, 27–30.

Heales S. J., Davies S. E., Bates T. E. and Clark J. B. (1995) Depletion of brain glutathione is accompanied by impaired mitochondrial function and decreased *N*-acetyl aspartate concentration. *Neurochem. Res.* **20**, 31–38.

Hillered L., Vespa P. M. and Hovda D. A. (2005) Translational neurochemical research in acute human brain injury: the current status and potential future for cerebral microdialysis. *J. Neurotrauma* **22**, 3–41.

Holshouser B. A., Ashwal S., Luh G. Y., Shu S., Kahlon S., Auld K. L., Tomasi L. G., Perkin R. M. and Hinshaw D. B. Jr (1997) Proton MR spectroscopy after acute central nervous system injury: outcome prediction in neonates, infants, and children. *Radiology* **202**, 487–496.

Hutchinson P. J., al-Rawi P. G., O'Connell M. T., Gupta A. K., Maskell L. B., Hutchinson D. B., Pickard J. D. and Kirkpatrick P. J. (2000) Head injury monitoring using cerebral microdialysis and Paratrend multiparameter sensors. *Zentralbl. Neurochir.* **61**, 88–94.

Kalra S., Cashman N. R., Genge A. and Arnold D. L. (1998) Recovery of *N*-acetylaspartate in corticomotor neurons of patients with ALS after riluzole therapy. *Neuroreport* **9**, 1757–1761.

Knizley H. Jr (1967) The enzymatic synthesis of *N*-acetyl-L-aspartic acid by a water-insoluble preparation of a cat brain acetone powder. *J. Biol. Chem.* **242**, 4619–4622.

Koura S. S., Doppenberg E. M., Marmarou A., Choi S., Young H. F. and Bullock R. (1998) Relationship between excitatory amino acid release and outcome after severe human head injury. *Acta Neurochir. Suppl.* **71**, 244–246.

Lu Z. H., Chakraborty G., Ledeen R. W., Yahya D. and Wu G. (2004) *N*-Acetylaspartate synthase is bimodally expressed in microsomes and mitochondria of brain. *Brain Res. Mol. Brain Res.* **122**, 71–78.

Maas A. I., Dearden M., Teasdale G. M. *et al.* (1997) EBIC-guidelines for management of severe head injury in adults. European Brain Injury Consortium. *Acta Neurochir. (Wien)* **139**, 286–294.

Marklund N., Salci K., Lewen A. and Hillered L. (1997) Glycerol as a marker for post-traumatic membrane phospholipid degradation in rat brain. *Neuroreport* **8**, 1457–1461.

Obrenovitch T. P. (1999) High extracellular glutamate and neuronal death in neurological disorders. Cause, contribution or consequence? *Ann. NY Acad. Sci.* **890**, 273–286.

Obrenovitch T. P. and Urenjak J. (1997a) Is high extracellular glutamate the key to excitotoxicity in traumatic brain injury? *J. Neurotrauma* **14**, 677–698.

Obrenovitch T. P. and Urenjak J. (1997b) Altered glutamatergic transmission in neurological disorders: from high extracellular glutamate to excessive synaptic efficacy. *Prog. Neurobiol.* **51**, 39–87.

Obrenovitch T. P., Richards D. A., Sarna G. S. and Symon L. (1993) Combined intracerebral microdialysis and electrophysiological recording: methodology and applications. *J. Neurosci. Meth.* **47**, 139–145.

Paschen W., van den Kerchhoff W. and Hossmann K. A. (1986) Glycerol as an indicator of lipid degradation in bicuculline-induced seizures and experimental cerebral ischemia. *Metab. Brain Dis.* **1**, 37–44.

Patel T. B. and Clark J. B. (1979) Synthesis of *N*-acetyl-L-aspartate by rat brain mitochondria and its involvement in mitochondrial/cytosolic carbon transport. *Biochem. J.* **184**, 539–546.

Peden C. J., Rutherford M. A., Sargentoni J., Cox I. J., Bryant D. J. and Dubowitz L. M. (1993) Proton spectroscopy of the neonatal brain following hypoxic-ischaemic injury. *Dev. Med. Child Neurol.* **35**, 502–510.

Rango M., Spagnoli D., Tomei G., Bamonti F., Scarlato G. and Zetta L. (1995) Central nervous system trans-synaptic effects of acute axonal injury: a 1H magnetic resonance spectroscopy study. *Magn. Reson. Med.* **33**, 595–600.

Reinert M., Hoelper B., Doppenberg E., Zauner A. and Bullock R. (2000) Substrate delivery and ionic balance disturbance after severe human head injury. *Acta Neurochir. Suppl.* **76**, 439–444.

Ross B. D., Ernst T., Kreis R. *et al.* (1998) 1H MRS in acute traumatic brain injury. *J. Magn. Reson. Imaging* **8**, 829–840.

Sager T. N., Fink-Jensen A. and Hansen A. J. (1997) Transient elevation of interstitial *N*-acetylaspartate in reversible global brain ischemia. *J. Neurochem.* **68**, 675–682.

Saragea M., Clopotaru M., Sica M., Vladutiu A., Negru T. and Rotaru N. (1965) Biochemical changes occurring in animals with experimental allergic encephalomyelitis. *Med. Pharmacol. Exp. Int. J. Exp. Med.* **13**, 74–80.

Shiino A., Matsuda M., Morikawa S., Inubushi T., Akiguchi I. and Handa J. (1993) Proton magnetic resonance spectroscopy with dementia. *Surg. Neurol.* **39**, 143–147.

Shonk T. K., Moats R. A., Gifford P., Michaelis T., Mandigo J. C., Izumi J. and Ross B. D. (1995) Probable Alzheimer disease: diagnosis with proton MR spectroscopy. *Radiology* **195**, 65–72.

Signoretti S., Marmarou A., Tavazzi B., Lazzarino G., Beaumont A. and Vagnozzi R. (2001) *N*-Acetylaspartate reduction as a measure of injury severity and mitochondrial dysfunction following diffuse traumatic brain injury. *J. Neurotrauma* **18**, 977–991.

Signoretti S., Marmarou A., Tavazzi B., Dunbar J., Amorini A. M., Lazzarino G. and Vagnozzi R. (2004) The protective effect of cyclosporin A upon *N*-acetylaspartate and mitochondrial dysfunction following experimental diffuse traumatic brain injury. *J. Neurotrauma* **21**, 1154–1167.

Sinson G., Bagley L. J., Cecil K. M., Torchia M., McGowan J. C., Lenkinski R. E., McIntosh T. K. and Grossman R. I. (2001) Magnetization transfer imaging and proton MR spectroscopy in the evaluation of axonal injury: correlation with clinical outcome after traumatic brain injury. *Am. J. Neuroradiol.* **22**, 143–151.

Smith D. H., Cecil K. M., Meaney D. F., Chen X. H., McIntosh T. K., Gennarelli T. A. and Lenkinski R. E. (1998) Magnetic resonance spectroscopy of diffuse brain trauma in the pig. *J. Neurotrauma* **15**, 665–674.

Sullivan P. G., Keller J. N., Mattson M. P. and Scheff S. W. (1998) Traumatic brain injury alters synaptic homeostasis: implications for impaired mitochondrial and transport function. *J. Neurotrauma* **15**, 789–798.

Tavazzi B., Vagnozzi R., Di Pierro D., Amorini A. M., Fazzina G., Signoretti S., Marmarou A., Caruso I. and Lazzarino G. (2000) Ion-pairing high-performance liquid chromatographic method for the detection of *N*-acetylaspartate and *N*-acetylglutamate in cerebral tissue extracts. *Anal Biochem.* **277**, 104–108.

Tavazzi B., Signoretti S., Lazzarino G., Amorini A. M., Delfini R., Cimatti M., Marmarou A. and Vagnozzi R. (2005) Cerebral oxidative stress and depression of energy metabolism correlate with severity of diffuse brain injury in rats. *Neurosurgery* **56**, 582–589.

Taylor D. L., Davies S. E., Obrenovitch T. P., Urenjak J., Richards D. A., Clark J. B. and Symon L. (1994) Extracellular *N*-acetylaspartate in the rat brain: *in vivo* determination of basal levels and changes evoked by high K+. *J. Neurochem.* **62**, 2349–2355.

Taylor D. L., Davies S. E., Obrenovitch T. P., Doheny M. H., Patsalos P. N., Clark J. B. and Symon L. (1995) Investigation into the role of *N*-acetylaspartate in cerebral osmoregulation. *J. Neurochem.* **65**, 275–281.

Tsai G. and Coyle J. T. (1995) *N*-acetylaspartate in neuropsychiatric disorders. *Prog. Neurobiol.* **46**, 531–540.

Uno M., Ueda S., Hondo H., Matsumoto K. and Harada M. (1996) Effectiveness of revascularization surgery evaluated by proton magnetic resonance spectroscopy and single photon emission computed tomography. *Neurol. Med. Chir (Tokyo)* **36**, 560–567.

Vagnozzi R., Marmarou A., Tavazzi B., Signoretti S., Di Pierro D., del Bolgia F., Amorini A. M., Fazzina G., Sherkat S. and Lazzarino G. (1999) Changes of cerebral energy metabolism and lipid peroxidation in rats leading to mitochondrial dysfunction after diffuse brain injury. *J. Neurotrauma* **16**, 903–913.

Verweij B. H., Muizelaar J. P., Vinas F. C., Peterson P. L., Xiong Y. and Lee C. P. (1997) Mitochondrial dysfunction after experimental and human brain injury and its possible reversal with a selective N-type calcium channel antagonist (SNX-111). *Neurol. Res.* **19**, 334–339.

Verweij B. H., Muizelaar J. P., Vinas F. C., Peterson P. L., Xiong Y. and Lee C. P. (2000) Impaired cerebral mitochondrial function after traumatic brain injury in humans. *J. Neurosurg.* **93**, 815–820.

Xiong Y., Gu Q., Peterson P. L., Muizelaar J. P. and Lee C. P. (1997) Mitochondrial dysfunction and calcium perturbation induced by traumatic brain injury. *J. Neurotrauma* **14**, 23–34.

Xiong Y., Peterson P. L., Verweij B. H., Vinas F. C., Muizelaar J. P. and Lee C. P. (1998) Mitochondrial dysfunction after experimental traumatic brain injury: combined efficacy of SNX-111 and U-101033E. *J. Neurotrauma* **15**, 531–544.

Yamamoto T., Rossi S., Stiefel M., Doppenberg E., Zauner A., Bullock R. and Marmarou A. (1999) CSF and ECF glutamate concentrations in head injured patients. *Acta Neurochir. Suppl.* **75**, 17–19.

