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Monitoring of β-Amyloid Dynamics after Human Traumatic Brain Injury

Niklas Marklund,[1] Nina Farrokhnia,[1] Anders Hånell,[1] Eugeen Vanmechelen,[2] Per Enblad,[1] Henrik Zetterberg,[3,4] Kaj Blennow,[3] and Lars Hillered[1]

Abstract

Epidemiological evidence links severe or repeated traumatic brain injury (TBI) to the development of Alzheimer's disease (AD). Accumulation of amyloid precursor protein (APP) occurs with high frequency after TBI, particularly in injured axons, and APP may be cleaved to amyloid-β (Aβ) peptides playing key pathophysiological roles in AD. We used cerebral microdialysis (MD) to test the hypothesis that interstitial Aβ levels are altered following TBI and are related to the injury type, cerebral energy metabolism, age of the patient, and level of consciousness. In the present report, we evaluated 10 mechanically ventilated patients (7 male, 3 female, ages 18–76 years) with a severe TBI, who had intracranial pressure and MD monitoring. Each MD sample was analyzed for hourly routine energy metabolic biomarkers (MD-lactate, MD-pyruvate, MD-glucose, and MD-lactate/pyruvate ratio), cellular distress biomarkers (MD-glutamate, MD-glycerol), and MD-urea. The remaining MD samples were analyzed for Aβ1–40 (Aβ40; $n = 765$ samples) and Aβ1–42 (Aβ42; $n = 765$ samples) in pooled 2 h fractions up to 14 days post-injury, using the Luminex xMAP technique, allowing detection with high temporal resolution of the key Aβ peptides Aβ40 and Aβ42. Data are presented using medians and 25th and 75th percentiles. Both Aβ40 and Aβ42 were consistently higher in patients with predominately diffuse axonal injury compared with patients with focal TBI at days 1–6 post- injury, Aβ42 being significantly increased at 113–116 h post-injury ($p < 0.05$). The Aβ levels did not correlate with the interstitial energy metabolic situation, age of the patient, or the level of consciousness. These results support that interstitial generation of potentially toxic Aβ species may occur following human TBI, particularly related to axonal injury.

Key words: Aβ; APP; axonal injury; cerebral MD; energy metabolism; TBI

Introduction

Traumatic brain injury (TBI) frequently causes long-term sequelae such as cognitive impairment, personality changes, and depression leading to social problems and a reduced quality of life.[1,2] The chronic disabilities associated with apparently mild TBI, occurring from some blast injuries sustained in the military setting or during sport activities, are also increasingly recognized.[3,4] Epidemiological evidence links severe or repeated TBI to the development of Alzheimer's disease (AD).[5–10] Amyloid-β (Aβ) plaques, a hallmark pathological finding observed in the brains of AD patients, have been observed in a subset of TBI patients within hours post-injury.[5,11,12] The membrane glycoprotein amyloid precursor protein (APP) is synthesized in the neuronal cytoplasm, and accumulates within injured axons following TBI.[5,13–16] APP may be proteolytically cleaved by different enzymes including α-secretase, preventing the generation of Aβ peptides, or the β-site APP cleaving enzyme 1 (BACE1). BACE1-mediated APP cleavage, combined with γ-secretase-mediated cleavage of the remaining APP stub, leads to the generation of amyloid-β (Aβ) peptides of various lengths, including the 40 and 42 amino acid long Aβ peptides.[17–20] BACE1 and presenilin, important for Aβ generation, have also been found to accumulate in injured and swollen axons post-injury.[21,22] Although normal neuronal activity can produce Aβ via APP processing,[23] these findings make the formation of Aβ peptides a likely consequence of axonal injury. Importantly, inhibition of APP β- or γ-secretases improves outcome in experimental TBI.[24] As β- and γ-secretase-generated Aβ forms are released into the interstitial fluid, cerebral microdialysis (MD) is a preferred sampling method for these biomarkers.[25,26]

[1]Division of Neurosurgery, Department of Neuroscience, Uppsala University, Uppsala, Sweden.
[2]ADx NeuroSciences, Technologiepark 4, Gent, Belgium.
[3]Institute of Neuroscience and Physiology, Department of Psychiatry and Neurochemistry, the Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden.
[4]University College London, Institute of Neurology, Queen Square, London, United Kingdom.

One predominant goal of neurocritical care (NCC) is the detection and avoidance of cerebral ischemia. Ischemic brain damage has long been considered a major secondary injury mechanism in TBI.[27] In modern NCC, MD may be used to detect patterns suggestive of ischemia such as an elevated MD lactate/pyruvate ratio (LPR)[28–30] combined with a reduced interstitial glucose (MD-glc[31,32]). In a recent study on 223 TBI patients, MD-LPR and MD-glc were found to be independent predictors of poor outcome.[33] Experimental cerebral ischemia leads to increased APP production and upregulation of BACE activity.[34–36] Additionally, cerebral ischemia has been associated with increased production of Aβ from APP, both in experimental models[37,38] and in humans.[39–41] These reports suggest a link between energy metabolic disturbance and Aβ peptide formation.

Experimentally, Aβ may also have direct neurotoxic properties,[42–44] and, therefore, be important for the pathophysiology of TBI. In three recent publications,[25,26,45] MD was used to detect interstitial Aβ formation early following severe TBI in humans. Using an Aβ(1-x) enzyme-linked immunosorbent assay (ELISA) not directly measuring Aβ40 or Aβ42, MD- Aβ levels were found to correlate with the level of consciousness in neurovascular and TBI patients in an NCC setting.[26] In another report, the temporal patterns of Aβ peptides could be studied in 12 h fractions using a sandwich ELISA.[25] The results of this pilot study showed that Aβ42 was consistently higher in patients with diffuse TBI, calling for additional studies of this potentially useful biomarker of diffuse axonal injury (DAI). However, the sensitivity of the assay was limited. In the present report, we used the Luminex technique to improve the analytical sensitivity to enhance the temporal resolution of Aβ peptide dynamics in 10 patients with moderate to severe TBI. Our main hypotheses were that Aβ peptide levels would correlate with the presence of diffuse brain injury, indicating axonal injury, cerebral energy metabolic perturbation, patient age, and the level of consciousness.

Methods

All research procedures described herein were approved by the Regional Research Ethics Committee at Uppsala University and informed consent was obtained from the patient's closest relative. This single center study was performed in a university hospital NCC setting.

Patient population and neurocritical care management

We included 10 patients (7 male, 3 female) conveniently recruited with a severe TBI, defined as a post-resuscitation Glasgow Coma Scale (GCS) score ≤ 8 at the primary hospital. On arrival in our unit, all patients were intubated and the motor component of the GCS (the Glasgow Motor Scale [GMS]) is presented in Table 1. No patient had a previous history of a neurodegenerative disease or Down's syndrome. All patients required treatment in a NCC setting, including intubation and mechanical ventilation, and were managed according to a standardized brain injury protocol aiming to keep intracranial pressure (ICP) at ≤ 20 and cerebral perfusion pressure (CPP) at ≥ 60 mm Hg.[46,47] ICP was monitored in all patients, either with an intraparenchymal ICP monitor (Codman, Johnson & Johnson, six patients), an external ventricular drain (three patients) or both (one patient). We used a sedation protocol in which patients were sedated using continuous intravenous propofol infusion (1–4 mg/kg/h Propofol-Lipuro; B. Braun Melsungen AG, Melsungen, Germany) combined with intermittent intravenous morphine (1–3 mg Morfin Meda; Meda, Sollentuna, Sweden). When ICP elevations could not be controlled using standard therapy, continuous sodium pentobarbital infusion therapy was initiated in one patient. Volume substitution and/or inotropic agents (Dobutamine or norepinephrine) were administered when needed. Plasma glucose was frequently evaluated and maintained at 5–10 mmol/L where insulin was administered when plasma glucose levels reached > 10 mmol/L. A neurological examination was performed regularly, up to six times daily, using the neurological wake-up test to register the motor component of the GCS (the GMS) and focal neurological signs on computerized observation charts.[46] For presentation of data, the patients were classified into conscious (GMS = 6) or not (GMS < 6) during the time for Aβ sampling (described subsequently). Additional injuries were scored according to the New Injury Severity Score (NISS), ranging from 1 to 75.[48] At ~6 months post-injury, patient outcome was assessed using the extended Glasgow Outcome Scale (eGOS[49]).

Radiological analysis

CT scans were frequently performed as part of the monitoring strategy. When analyzing each CT scan, the ''worst'' CT scan (i.e.,

Table 1. Clinical Characteristics of Each Patient Included in this Study

Case no.	*Group*	*Age (yrs), sex*	*Cause of injury*	*NISS/associated conditions*[a]	*GMS scores on arrival*	*Pupils*	*MD duration (h)*[c]	*LOS/ extubation (days)*	*GMS score on departure*	*eGOS*
1	Focal/mixed	76, M	Fall	51	6	Normal	8–214	21/8	6	4
2	DAI	42, M	MVA	50	2	Bilat small	17–346	17/7	3	3
3	DAI	52, F	MVA	[a]	3	Rt dilated	19–145	8/6	6	4
4	Focal/mixed	19, M	MVA	57	5	Normal	24–150	18/14	6	8
5	DAI	25, F	MVA	38	5	Normal	14–164	7/6	6	8
6	Focal/mixed	31, M	MVA	29	3	Rt dilated	7–147	10/9	5	5
7	Focal/mixed	43, M	Tire explosion	30	4	Normal	13–58	6/2	6	7
8	DAI	35, F	Fall	19	5	Rt dilated	7–188	11/8	6	3
9	Focal/mixed	55, M	Fall	21[a]	3	Bilat dilated unresponsive	13–273	20/17	4	4
10	DAI	18, M	MVA	29	4	Rt dilated	75–311	17/9	4	5

[a]Isolated brain injury, NISS cannot be applied.
[b]Alcohol indicates a high serum ethanol on arrival in the primary hospital.
[c]Number of hours post-injury at which the microdialysis was initiated and stopped; the interval between the numbers represents the total duration of microdialysis.

DAI, diffuse axonal injury; eGOS, extended Glasgow Outcome Scale at 6 months post-injury; GMS, motor component of the Glasgow Coma Scale; NISS, New Injury Severity Score; LOS, length of stay; MD, microdialysis; MVA, motor vehicle accident.

the CT scan with the most intracranial lesions and/or mass effect during the course of the disease) was selected and analyzed for each of the 10 patients.[50] We used the Marshall classification[51] and divided the patients into having either a DAI or a focal/mixed TBI.[25] Radiological characteristics of focal/mixed TBI included focal lesions such as epi- and subdural hemorrhages and lobar contusions. Criteria for DAI were diffuse brain swelling and/or small petechial hemorrhages located at the gray/white matter junction, corpus callosum, and/or brainstem. Compression of basal cisterns was determined by the following scoring system: 0 = normal, 1 = compressed yet visible, 2 = compressed. The midline shift was calculated at the level of the thalami. The volume in milliliters of each mass lesion was calculated using the formula length × width × height/2.

MD procedure

The MD probe was inserted in conjunction with implantation of the ICP monitoring device, typically in the nondominant frontal lobe 1–2 cm anterior to the coronal suture ($n = 7$) or at the craniotomy site after evacuation of a focal mass lesion ($n = 3$). Care was taken to insert the MD catheter obliquely into the cortex using a non-traumatic technique.[25]

We used 71 High Cut-Off Brain MD catheters with a membrane length of 10 mm and a 100 kDa nominal molecular weight cut-off polyarylethersulfone (PAES) membrane (M Dialysis AB, Solna, Sweden). The outflow hydrostatic pressure of the perfusion system was set at the zero midcranial reference level by taping the collecting vials at the bandage on the patient's head to avoid additional hydrostatic effects on fluid recovery of the catheter. Perfusion of the catheters was performed using artificial cerebrospinal fluid (CSF) (Perfusion Fluid CNS, M Dialysis), containing NaCl 147 mM, KCl 2.7 mM, $CaCl_2$ 1.2 mM, and $MgCl_2$ 0.85 mM with the addition of 1.5% human serum albumin,[26] at a rate of 0.3 μL/min using a 106 MD pump (M Dialysis). At least 2 h passed after insertion of the MD catheter and start of sampling to allow for normalization of changes caused by catheter insertion. MD vials ($\sim$18 μL samples) were changed hourly. Samples were analyzed at the bedside using CMA microdialysis analyzers (CMA600 and ISCUSflex; M Dialysis) for concentrations of glucose (MD-glc), lactate (MD-lac), and pyruvate (MD-pyr), and the LPR was calculated. Glutamate (MD-glut) and glycerol (MD-glyc) were analyzed later (CMA600). Urea was monitored to control the MD catheter performance.[52] The remaining samples were stored at -70°C until further analyzed for Aβ peptides (described subsequently). The CMA 600 Analyser was automatically calibrated when started, as well as every 6th h using standard calibration solutions from the manufacturer (M Dialysis). Quality controls at two different concentrations for each substance were performed every weekday (CMA600 and ISCUSflex). The correlation between CMA600 and ISCUSflex data from separate runs with the same control sample was found to be excellent (for all analytes used here r was ≥ 0.99) allowing for direct comparison of patient data from both instruments. Imprecision values for between assay coefficient of variation was < 10% for all analytes. The location of the MD catheter was evaluated on follow-up CT scans in all patients and checked for hemorrhages. The presence of any hemorrhage visible on TBI within 3 cm of the MD probe was analyzed on all CT scans and noted in Table 2.

Based on previous data,[53,54] the following values for the routine biomarkers were considered critical: MD-glc < 1 mmol/L; LPR > 30; MD-lac > 3.8mmol/L; MD-pyr < 120 μmol/L; MD-glut > 15 μmol/L; MD-glyc > 100 μmol/L.

Analysis of Aβ peptides

After bedside analysis, the remaining hourly microdialysis samples ($\sim$14 μL) were pooled into 2 h fractions. Aβ42 and Aβ40 levels were measured using the INNO-BIA Aβ forms assay (Innogenetics, Ghent, Belgium), which is a multiplex microsphere-based Luminex xMAP technique. In this assay, the monoclonal antibodies 21F12 and 2G3, which specifically bind Aβ peptides ending at Ala42 and Val40, respectively, were used as capture antibodies, and the monoclonal antibody 3D6, which specifically binds Aβ peptides starting at Asp1, was used as detector antibody.[55] All samples were analyzed in the presence of a mild detergent in the kit, which makes most of the Aβ accessible to the antibodies, although without using denaturing extraction protocols. Because of the small sample volume available, all samples were diluted 15-fold before analysis. The lowest standard point was 15 pg/mL for Aβ40 and 5 pg/mL for Aβ42. The lowest reported levels were set to half the value of the lowest standard point, which after correction for dilution gives a cutoff of 112 pg/mL for Aβ40 and 37 pg/mL for Aβ42. All analyses were performed by experienced and board-certified laboratory technicians. Serial samples from the same patient were analyszd on the same plate. Intra- and inter-plate coefficients of variation were below 10%.

Table 2. Radiological Analysis of the Worst CT Scan during the Course of the Disease (See Text and Servadei et al.[50] for Details)

Case no.	*Group*	*Marshall classification*	*Lesion type*	*Hematoma vol (mL)*	*tSAH*[a]	*Midline shift (mm)*	*IVH*	*Basal Cisterns*[b]	*Hematoma location/MD probe location*
1	Focal/mixed	EML	ICH & SDH	51.4	+	7.5	+	2	RF/RF cranio[c]
2	DAI	DI II	Cps	3.5	SF + + +	None	None	0	RFP/RF
3	DAI	DI II	Cps	< 0.1	BP + + +	None	+ +	0	LBG/RF
4	Focal/mixed	EML	EDH	24.9	None	4.5	None	1	RFP/RF cranio[c]
5	DAI	DI III	Cps	< 0.1	LTP +	None	+ +	1	LF/RF
6	Focal/mixed	non-EML	Cps	10.0	BFP + + +	None	None	1	LF/RF
7	Focal/mixed	EML	EDH	54.9	BSF +	2.0	None	2	LT/RF cranio
8	DAI	DI III	Cps	0.3	SF +	None	+	1	LBG/RF[c]
9	Focal/mixed	non-EML	ICH & SDH	17.8	Tent. + + +	None	None	0	Bilat/F
10	DAI	DI II	Cps	0.6	Occ, +	none	+ +	0	RT/RF*

[a]Semiquantitative scale; + = minimal, localized SAH; + + + = marked, widespread SAH.
[b]Scores defined as follows: 0 = normal, 1 = compressed yet visible, 2 = compressed.
[c]MD probe placed within 3 cm of a CT-verified hemorrhage/contusion.

DAI, diffuse axonal injury; Cps, parasagittal contusions; cranio, craniotomy; DI II/III, diffuse injury[51]; EML, evacuated mass lesion; IVH, intraventricular hemorrhage; bilat., bilateral, BG, left basal ganglia; F, frontal; T, temporal; Tent, tentorium; FP, frontoparietal; Occ, occipital; tSAH, traumatic subarachnoid hemorrhage.

Statistical analysis

The microdialysis data was analyzed using the one sample Kolmogorov–Smirnov test and was found not to meet the assumption of normal distribution ($p < 0.05$ for all analytes). Therefore, pairwise comparisons between groups were analyzed using the Mann–Whitney *U* test (Statistica software; StatSoft, Tulsa, OK). Nonparametric data are presented as medians and 75th percentile or individual values, whereas parametric data are presented using the means ± standard deviations (SD). Correlation analysis was performed on the first 48 h MD samples using Spearman's rank correlation. For area under the curve (AUC) calculations, we focused our analysis on the initial 19–146 h (day 1–6) post-injury. A p value < 0.05 was considered statistically significant.

Results

Patient characteristics and radiology

The mean age of the patients was 38.5 ± 18 years (range 18–76 years). The median GMS score on arrival in our NCC unit was 4 (range 2–6). Additional clinical characteristics including the NISS score are presented in Table 1. Based on the Marshall classification, the patients were divided into two groups: focal/mixed and DAI (Table 2). Five patients were categorized as having a DAI and five were categorized as having a focal/mixed injury. Radiological characteristics of each patient are presented in Table 2. Focal mass lesions were surgically evacuated in three patients (1, 4, and 7; Table 2).

Microdialysis procedure

The mean duration from time of accident to start of MD sampling was 19.7 h (range 7–75 h), and the mean duration was 179.9 ± 81 h (range 45–329 h; Table 1). Probe function was monitored using MD-urea levels, which were found to be stable, thus indicating adequate probe function, with a gradually increasing trend during the course of the monitoring period as previously shown.[52] Less than one percent of urea samples showed deviating levels from the expected trend, leading to exclusion of all MD data during that hour. No signs of hemorrhage around the MD probe were seen on any of the control CT scans.

Interstitial Aβ40 and Aβ42 levels

We were able to detect Aβ40 and Aβ42 in 2 h MD samples for all 10 patients using the Luminex method.[55] In total, 1530 MD samples were analyzed for both markers ($n = 765$ samples for each marker).

All together, the interstitial Aβ40 and Aβ42 levels were only slightly variable, with minor fluctuations during the course of the disease (Fig. 1A and B). We hypothesized that the Aβ levels were related to injury type, energy metabolic situation, age of the patient, and/or the level of consciousness.

First, we compared the Aβ peptide levels in relation to injury type (Fig. 1A–D).

Including all MD samples, the median interstitial Aß40 levels were 782 pg/mL (range 457.0–971.3 pg/mL) and 436 pg/mL (range 281.3–766.5 pg/mL) in the DAI and focal TBI groups, respectively. Aβ42 levels were 106 pg/mL (range 62–127.5 pg/mL) and 60 pg/mL (range 46–90 pg/mL) in the DAI and focal TBI group, respectively. The Aβ peptide levels were consistently higher in the patients with DAI than in patients with a focal TBI, being statistically significant for Aβ42 at 113–116 h post-injury ($p < 0.05$; Fig. 1A and B). Area under the curve (AUC), calculated from 19–146 h post-injury, was also compared (Fig. 1C and D). The AUC in the DAI group tended to be higher than the total AUC in the focal injury group (Fig. 1C and D), although without reaching statistical significance. For all samples, there was a strong correlation between interstitial Aß40 and Aβ42 levels ($r = 0.987$; Fig. 1E) and no association of the Aβ42/Aβ40 ratio with any of the outcome parameters was observed (data not shown).

We hypothesized that the energy metabolic situation could influence Aβ levels, and we performed a correlation analysis between the low molecular weight (LMW) biomarkers and Aß-peptide levels (Fig. 2 A–D). However, Aβ levels only weakly correlated with the evaluated biomarkers for cerebral ischemia (shown for the LPR ($r = 0.40$ and $r = 0.41$ for Aß40 and Aβ42, respectively; $p < 0.05$) in Fig. 2 A and B, MD-glc ($r = 0.76$ and $r = 0.77$ for Aß40 and Aβ42, respectively; $p < 0.05$) in Fig. 2C and D and for MD-pyr ($r = 0.13$ for both Aß40 and Aβ42, $p =$ n.s, data not shown; not shown for MD-glut, MD-glyc and MD-lac). MD-glut ($r = 0.755$ and $r = 0.735$, respectively) and MD-glyc ($r = 0.45$ and $r = 0.84$) was weakly correlated to interstitial Aß40 and Aβ42 levels (data not shown). The LPR was frequently higher early and MD-glyc was higher late following the injury in patients with a focal TBI than in patients with diffuse TBI ($p < 0.05$, Fig. 6A) without other significant differences in energy metabolic markers depending upon injury type or age of the patient. We then evaluated the influence of age on Aβ levels, divided into two groups (< 40 and > 40 years old). Age did not significantly influence our Aβ peptide results (Fig 3A–D). There was no correlation between the clinical outcome evaluated using the extended Glasgow coma outcome scale (eGOS) and the Aβ peptide levels (data not shown).

Five patients never obeyed command during the monitoring time (deemed unconscious) evaluated by the neurological wake-up test, five did (deemed conscious), and we next analyzed the effect of level of consciousness on Aβ peptide levels.[26] The levels of Aβ40 and Aβ42 were not significantly influenced by the level of consciousness (Fig. 4A–D) nor was there a correlation between Aβ and LPR or MD-glc (data not shown). Additionally, the frequent neurological wake-up tests did not markedly alter Aβ40 and Aβ42 levels (for an illustrative patient example see Fig. 5). In most patients, there were only slight fluctuations in Aβ40 and Aβ42 levels. One patient in the DAI group (case 8, Fig. 4) had very high Aβ levels (Fig. 5).

Analyses of low-molecular weight MD markers

LPR. A total of 1582 MD samples could be analyzed for the LPR. LPR levels were significantly higher early post-injury in the focal injury group (Fig. 6A). Age (Fig. 6B) and the level of consciousness (data not shown) did not influence the LPR levels.

MD-glc. A total of 1582 MD samples were analyzed for glucose (MD-glc). Of these, 179 samples (11.3%) had a critical MD-glc value of < 1 mmol/L. Injury type (Fig. 6A), age (Fig. 6B), and level of consciousness (data not shown) did not significantly influence MD-glc levels.

MD-glut. We could analyze 1302 samples for glutamate (MD-glut). The MD-glut values did not markedly differ between the injury groups (Fig. 6A) and was not influenced by the level of consciousness (data not shown). In young patients, MD-glut levels were consistently higher mainly because of one young patient with high MD-glut levels (Fig. 6B).

MD-glyc. We could analyze 1657 samples for glycerol (MD-glyc). When compared with the focal TBI group, patients with diffuse TBI had consistently lower MD-glyc values ($p < 0.05$ in

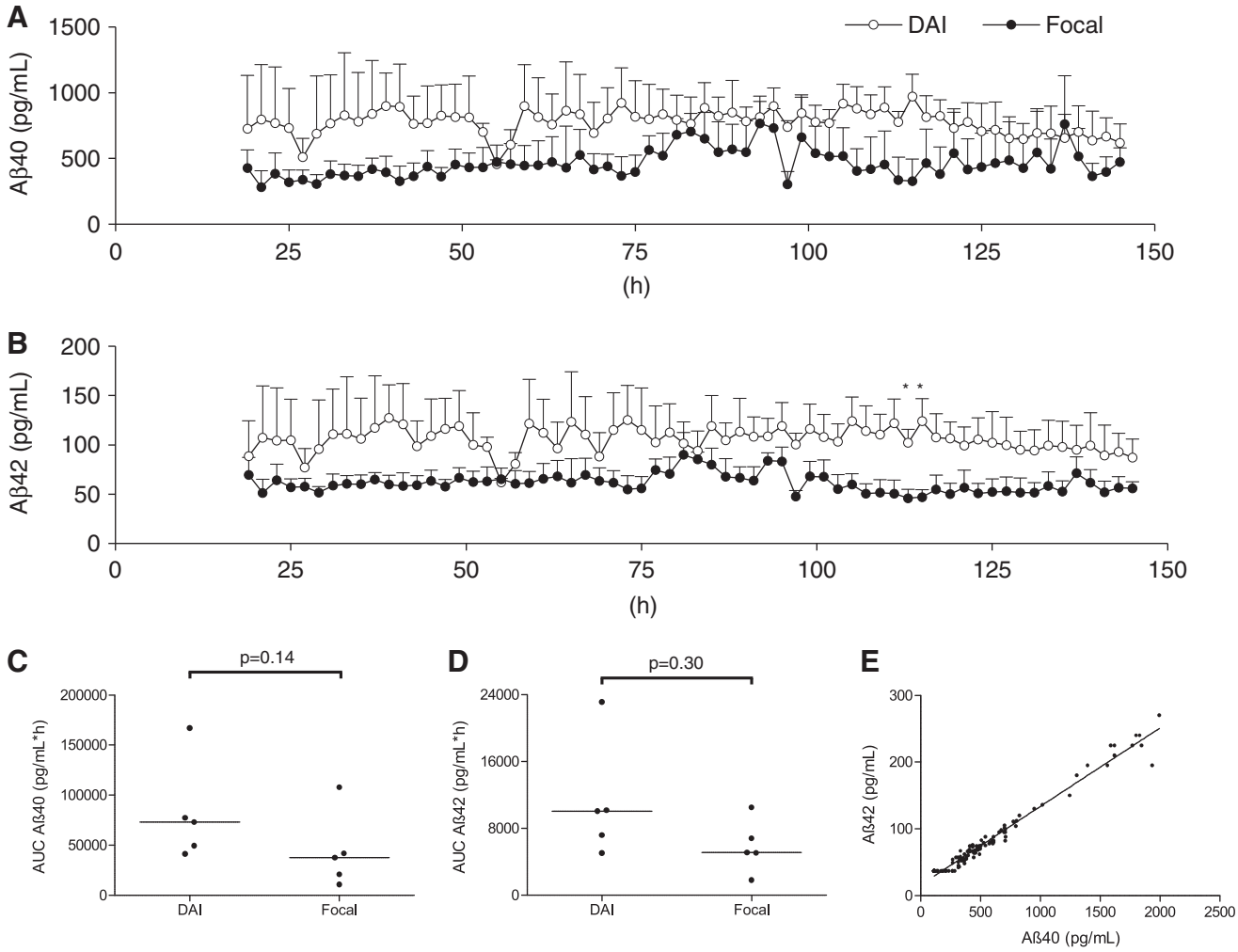

FIG. 1. Interstitial amyloid-β (Aβ)40 **(A)** and Aβ42 **(B)** concentrations were determined in 2 h sample fractions. For clarity, the data are presented as means ± SEM. Patients with diffuse axonal injury (DAI) (open circles) had consistently higher values than patients with mixed/focal traumatic brain injury (filled squares). A significant difference (< 0.05) is indicated with an *. **(C–D)** Area under the curve (AUC) values for each patient in the DAI (five patients) and mixed/focal (five patients) TBI groups. The AUC value for each patient was calculated for Aβ40 **(C)** and Aβ42 **(D)** between 19 and 146 h post-injury, presented with the median value (black line) and the individual values. **(E)** There was a very strong correlation between the Aβ40 and Aβ42 levels in the microdialysis (MD) samples.

8/12 samples from 111 to 123 h post-injury; Fig. 6A). Age (Fig. 6B) or the level of consciousness (data not shown) did not significantly influence the MD-glyc levels.

Discussion

The aim of this study was to evaluate the dynamics of the Aβ peptides Aβ40 and Aβ42 after TBI using intracerebral MD. The refined analysis method allowed for monitoring of both Aβ40 and Aβ42 in 2 h fractions in a small but thoroughly characterized TBI cohort. These results provide a unique data set for evaluation of Aβ peptide dynamics in the acute phase following injury, with higher temporal resolution and analytical specificity than in previously published studies.[25,26,45] The Aβ results were also compared with injury type, biomarkers for cerebral energy metabolism (glucose, lactate, pyruvate), cellular distress (glycerol, and glutamate), and clinical factors including patient age and level of consciousness, to test our main hypotheses. Higher Aβ levels in patients with diffuse brain injury compared with focal TBI were observed, supporting our previous data suggesting that Aβ peptides may be valuable biomarkers for axonal injury.[25] In contrast, the energy metabolic situation evaluated using MD biomarkers of ischemia and cellular distress in the same brain region only weakly correlated with Aβ peptide levels. Additionally, patient age or level of consciousness did not clearly influence Aβ levels in this patient cohort.

We observed that the levels of the Aβ peptides Aβ40 and Aβ42 were higher in patients with diffuse brain injury than in those with focal TBI, and there are several lines of evidence suggesting an increased Aβ formation following diffuse TBI. First, in approximately one third of TBI patients, Aβ plaques have been detected in the postmortem brain,[56] in surgically resected tissue,[11,12] and in injured axons of DAI patients dying < 9 days post-injury.[14] This TBI-induced Aβ pathology can remain for many years in the brains of TBI survivors.[5,57] Second, following diffuse TBI in the pig, Aβ plaques were observed in the one third of animals displaying the most severe axonal injury.[58] Importantly, such plaques occur not only in cortical tissue but also in white matter tracts.[14] Finally, axonal accumulation of Aβ, APP, and BACE has been observed in

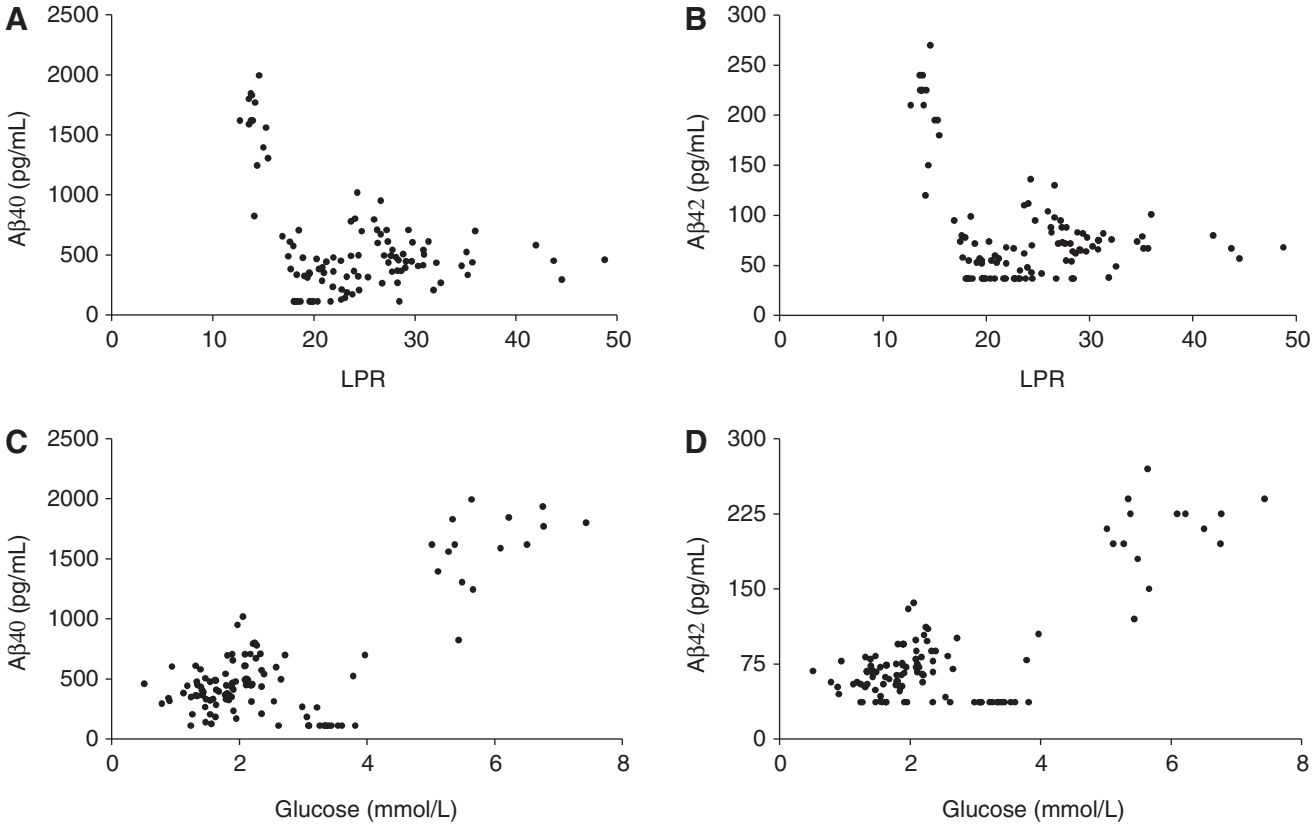

FIG. 2. We correlated amyloid-β (Aβ)40 **(A,C)** and Aβ42 **(B,D)** to the lactate-pyruvate ratio (LPR; **A,B**), where an LPR >30 was considered critical and to microdialysis (MD)-glucose levels **(C,D)**, where a level <1.0 mmol/L was considered critical. We used the initial 48 h post-injury values and no correlation was found between Aβ40 or Aβ42 and LPR or any other marker of energy metabolic crisis.

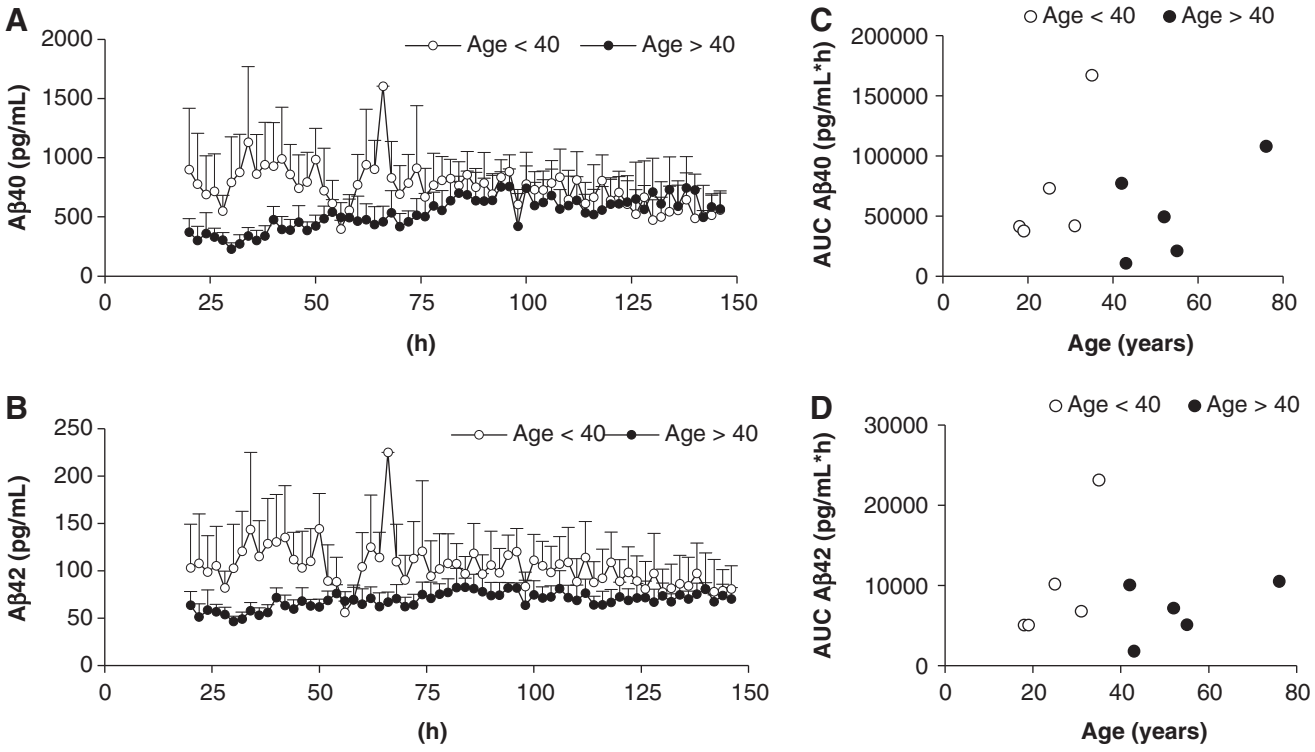

FIG. 3. We analyzed the influence of age on amyloid-β (Aβ)40 **(A,C)** and Aβ42 **(B,D)** levels. Open circles represent the five patients <40 years of age and filled squares represent the five patients >40 years of age. Data are presented using means $\pm$ SD. **(C,D)** Area under the curve (AUC) Aβ40 and Aβ42 values, calculated for each traumatic brain injury (TBI) patient between 19 and 146 h post-injury and presented for patients <40 years (five patients, open circles) and >40 years old (five patients, filled squares). **(A–D)** Age defined as $<$ or $>$ 40 years old did not significantly influence Aβ levels.

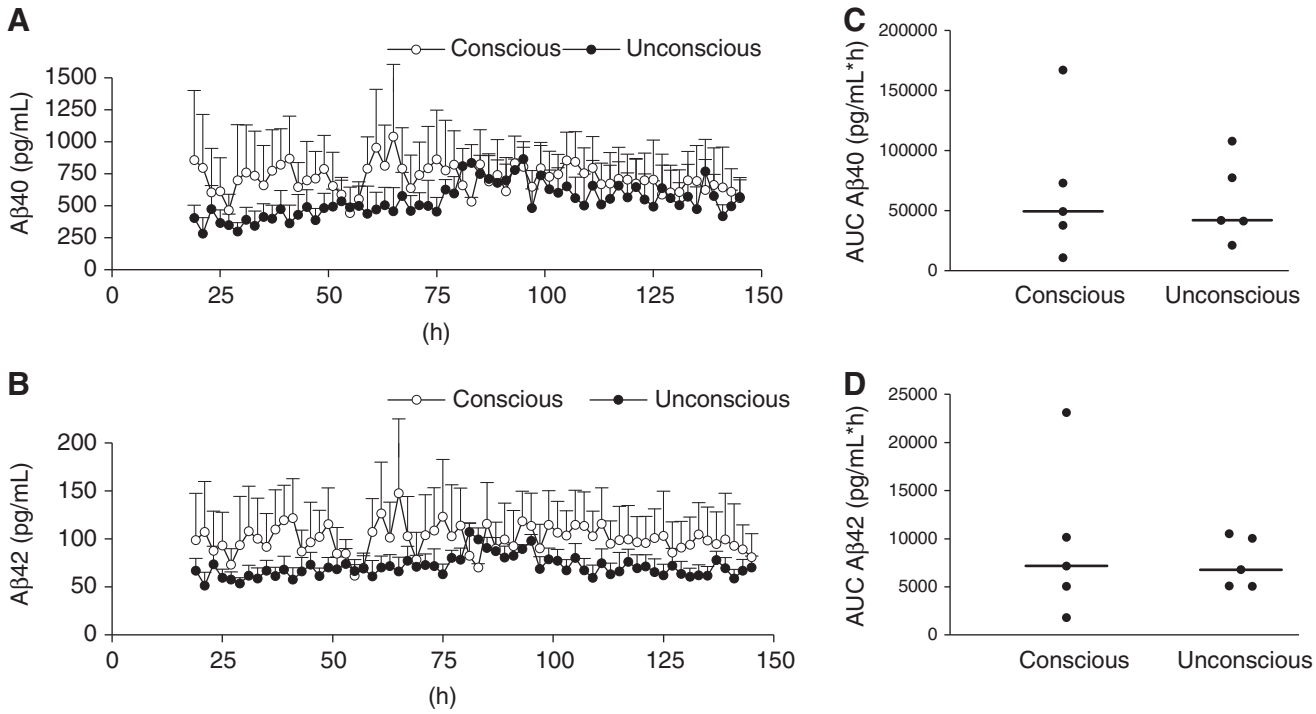

FIG. 4. **(A,B)** We evaluated the relationship between the level of consciousness and the interstitial amyloid-β (Aβ)40 **(A)** and Aβ42 **(B)** values, data presented as means ± SD. Aβ levels were similar in conscious patients (defined as patients obeying command when assessed using the neurological wake-up test; open circles) or unconscious patients (filled squares). **(C,D)** Area under the curve (AUC) values for each patient in the conscious and unconscious groups. The AUC value for each patient was calculated for Aβ40 **(C)** and Aβ42 **(D)** between 19 and 146 h post-injury, presented with the median value (black line) and the individual values. There were no significant differences among the groups.

both human and pig DAI up to 3 years post-injury.[21,22,59] Therefore, it appears likely that a prolonged APP accumulation in swollen axons results in an increased formation of Aβ peptides[5,56,60] released into the interstitial compartment following axonal rupture.

It should be noted that the classification of focal TBI versus DAI was solely based on the Marshall classification of CT scans, which is based on a 1991 analysis of the Traumatic Coma Data Bank. Although this classification was found to be predictive of a poor outcome, its ability to predict detailed neuropsychological deficits has not been established.[61] MRI is clearly more sensitive than CT for small lesions such as those observed in DAI patients.[62] In our material, no patient in the DAI group had a focal lesion, and the largest hematoma in this group was < 4mL. However, with ever increasing sensitivity of MRI, it is becoming clear that a high proportion of patients with predominately focal TBI display components of axonal injury.[63] As standard CT and MRI may underestimate the extent of white matter damage after TBI,[64,65] additional neuroradiological tools such as diffusion tensor imaging (DTI) could further increase the detection of white matter damage post-injury.[66] Ideally, MR scans following the implantation of the MD catheters could in more detail have evaluated the details of axonal injury in the vicinity of the probes in our present study.

The Aβ40 and Aβ42 levels did not markedly fluctuate during the initial post-injury days following human TBI, in contrast to previous MD studies in which they gradually rose over time.[26,45] Our observations are also in contrast to experimental data analyzing Aβ levels using MD early following focal TBI in mice. In awake, moving mice, the hippocampal Aβ levels declined by up to 50% post-injury, depending upon injury severity. In animals with severe TBI, the Aβ decline was more marked and prolonged than in animals with a milder, focal TBI.[67] In the present series, the pre-injury levels were unknown, and initial Aβ levels may have been different. It is likely that human TBI represents a less marked focal TBI than what is used in the rodent model,[68] making direct comparisons difficult.

Although the evidence cited in the previous paragraphs suggest that Aβ levels may be increased post-injury, we must also consider that the Aβ levels presented in our study may actually be reduced in some TBI patients.[45] In a previous study, patients with contusions had lower Aβ peptide levels than did DAI patients,[26,45] findings similar to our present and previous report.[25] In our focal TBI group, three patients had their MD probes close to an evacuated mass lesion, and the lower values observed in the focal TBI group may also represent reduced levels in the vicinity of contusion caused by Aβ consumption in the formation of plaques. Although the lack of histology makes any explanation hypothetical, early plaque formation is observed in only one third of TBI patients (see earlier description). Therefore, it is unlikely that the reduced Aβ levels in focal TBI patients are the result of plaque formation. Although CSF Aβ concentration may differ from the interstitial concentration,[5] levels of Aβ42 have been reported to increase following TBI.[60,69–72]

Any biomarker may also have an effect on the injury process itself. Based on experimental data, interstitial Aβ may have deleterious effects following TBI.[24] Neurotoxic and synaptotoxic effects of Aβ oligomers *per se* have also been observed.[42–44] Therefore, the interstitial Aβ could influence the secondary injury cascade after TBI. In addition, alternate splicing of the APP molecule may occur, and generation of other Aβ species than Aβ42 evaluated in our present report is possible. The shorter Aβ1–40 (Aβ40) makes ~80–90% of all generated Aβ peptides, although Aβ42 is more hydrophobic and prone to aggregate into plaques.[26] In

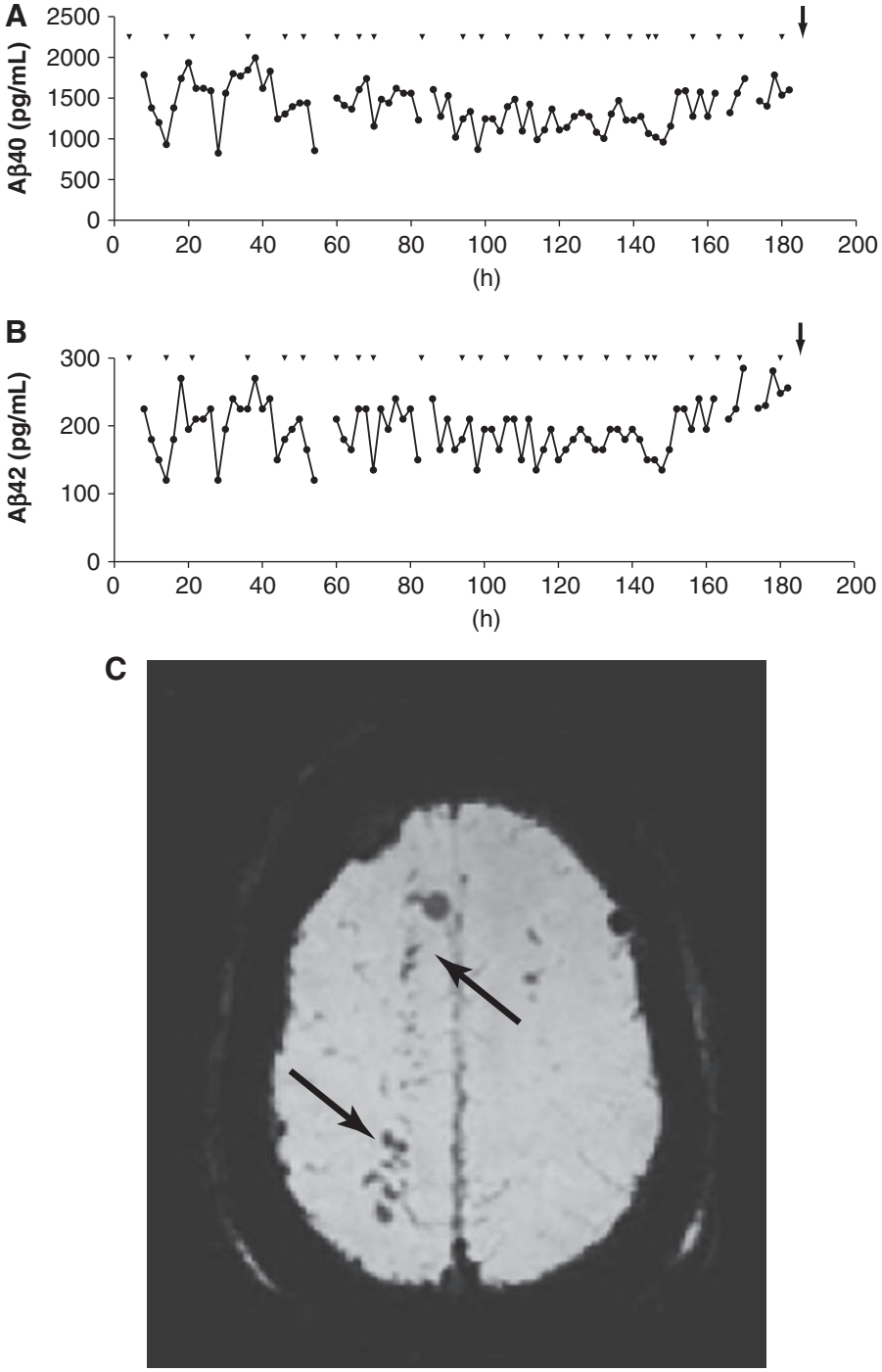

FIG. 5. Microdialysis data from a patient who sustained a Marshall diffuse brain injury II traumatic brain injury after a fall during horseback riding. Graph showing changes in amyloid-β (Aβ)40 **(A)** and Aβ42 **(B)** levels detected using microdialysis. **(C)** An MR scan obtained at 2 days post-injury showing multiple hemorrhages at the gray–white matter interface, indicated with arrows, suggestive of diffuse axonal injury (DAI). The highest interstitial Aβ40 and Aβ42 levels of this patient series were observed in this patient. Despite gradually improving neurological status during the monitoring time, the Aβ did not change and remained at a high level. The time for interruption of sedation in order to evaluate the level of consciousness using the neurological wake-up test, shown with arrowheads, did not clearly influence Aβ levels. The time for extubation is shown with an arrow.

the pathophysiology of TBI, other Aβ subspecies than Aβ42 may be of interest, which could be evaluated using MD.[73] The cutoff for the MD catheter used in the present study was 100 kDa, and although the molecular weight of Aβ42 is ~4.5 kD, longer peptides resulting from APP splicing or Aβ oligomerization, presumably a key event in the pathogenesis of AD, may not be detected using MD.[74]

As cerebral ischemia may influence Aβ levels,[34–36] we evaluated biomarkers for energy metabolic perturbation obtained in the same location and brain compartment as Aβ. In contrast to a previous report,[26] which found a positive correlation between MD-Aβ and MD-glc and a negative correlation with LPR, we observed only weak correlations. In our series, pathological LPR and/or MD-glc

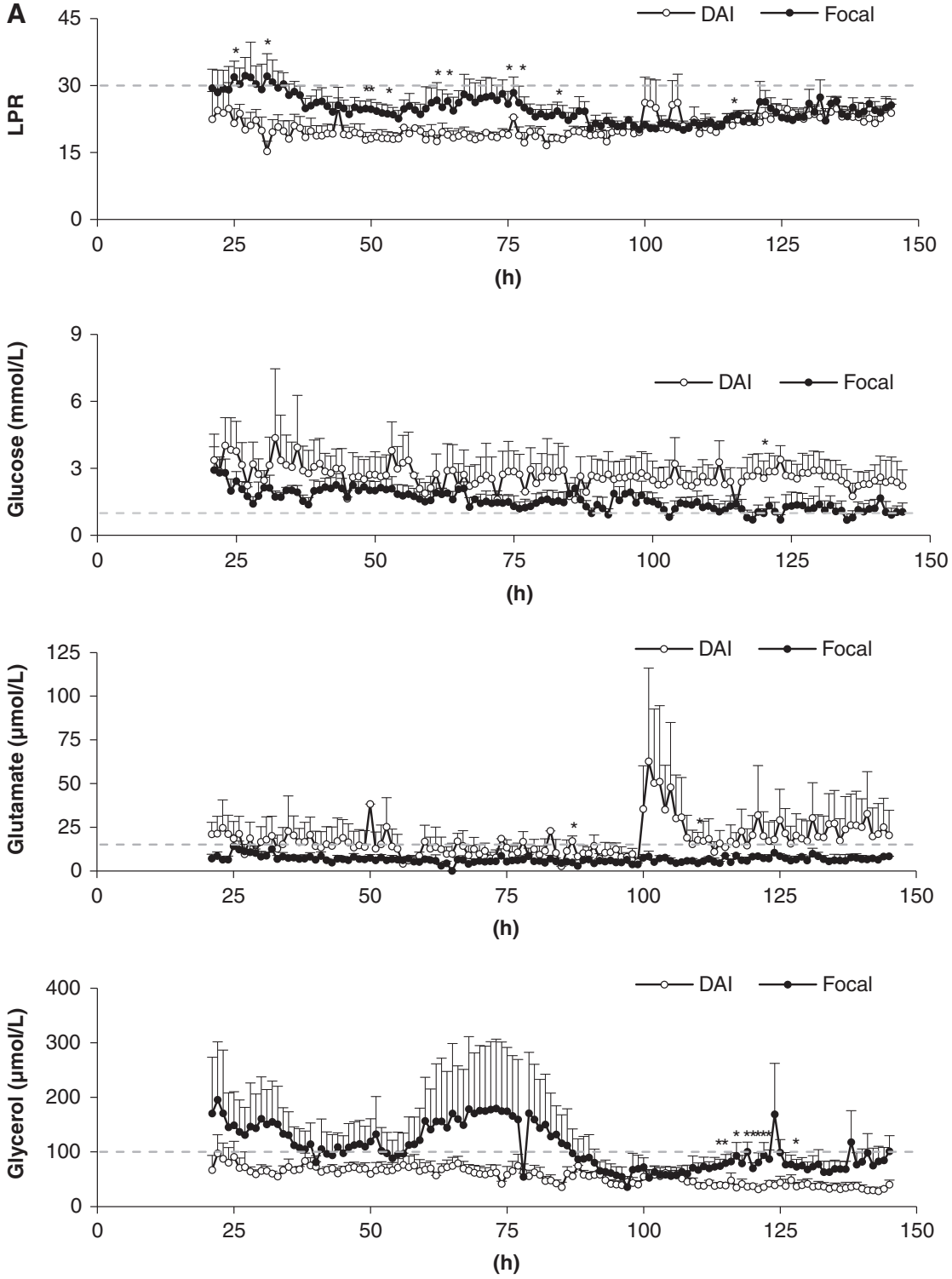

FIG. 6. A,B. Interstitial lactate-pyruvate ratio (LPR), glucose, glutamate and glycerol measured using microdialysis (MD), data presented as means ± SD. The energy metabolic markers showed similar values when the patients were divided into groups depending on injury type **(A)** and age **(B)**. **(A)** Graph showing the LPR, MD-glucose, MD-glutamate, and MD-glycerol levels in the diffuse axonal injury group (DAI; open circles) compared with the focal injury group (filled squares) in MD samples from 21 to 145 h post-injury. Significant differences between the two groups indicated with an asterisk (*). **(B)** The energy metabolic situation, analyzed showing the LPR and MD-glucose in five patients > 40 years old compared with the younger patients < 40 years old. No significant differences were seen between the two groups. The dashed lines in **A** and **B** indicate critical cut-off levels for the LPR (> 30), MD-glucose (< 1.0 mmol/L), MD-glutamate (18 μmol/L) and MD-glycerol (100 μmol/L).

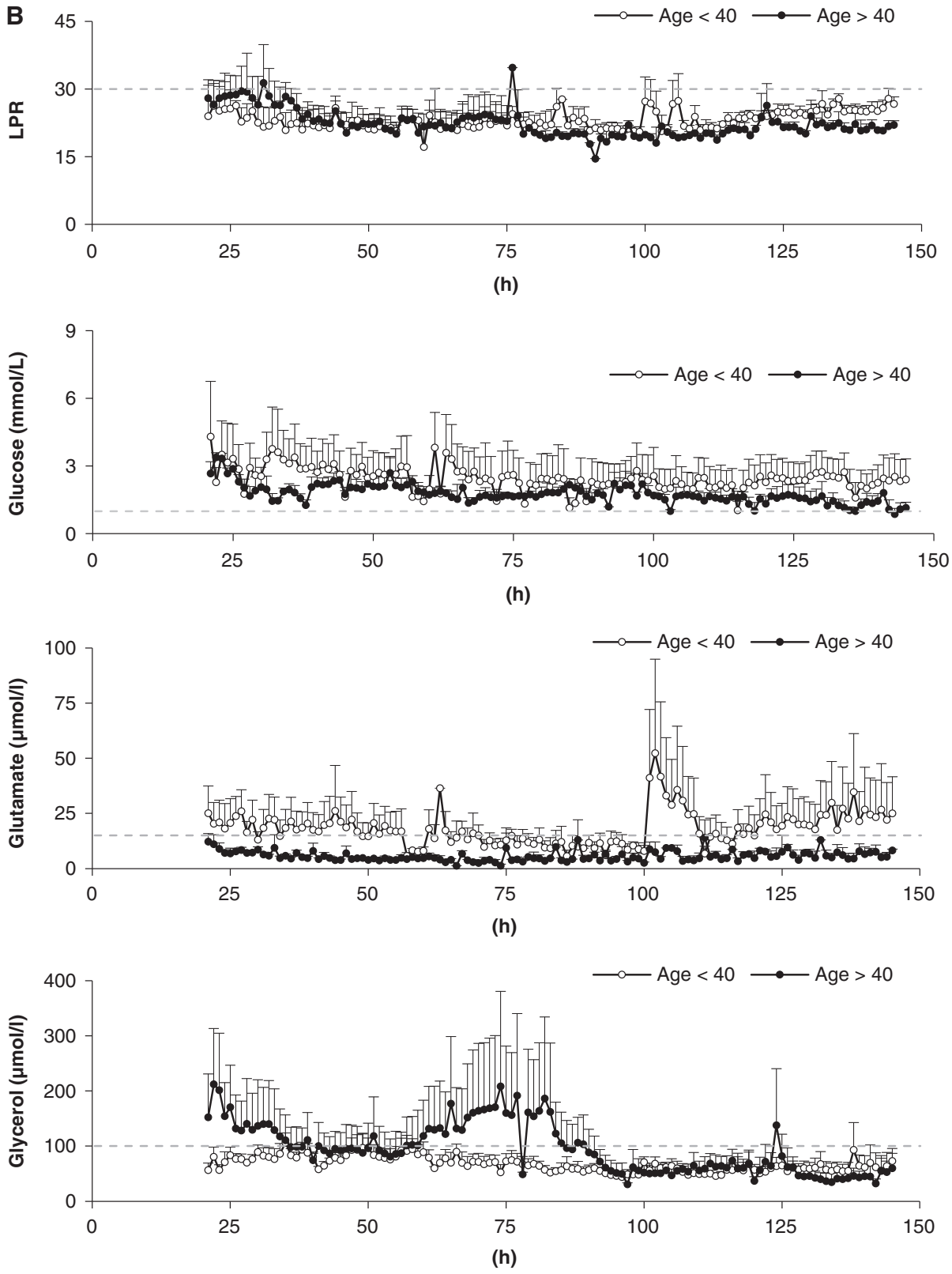

FIG. 6. (*Continued*)

values were rather infrequent and, at least for MD-glc, appeared less frequently than in the study by Brody and associates.[26] Additionally, in that report, six patients with subarachnoid hemorrhage (SAH) were included, and it cannot be excluded that those patients had a different neurochemical profile. The patients in our series had a severe TBI requiring sedation and controlled ventilation. Despite the injury severity, ICP problems were rare, which likely prevented marked neurometabolic perturbation of the brain. It cannot be excluded that another injury severity may have caused a different correlation between Aβ peptide levels and the focal neurochemistry.

Previously, currently available ELISA methods were considered too insensitive to enable measurement of Aβ 1–42 levels, and in a previous report, an ELISA evaluating Aβ species from amino acid 1–28 or greater (A$\beta 1-x$) was used.[26] When using that analysis, the majority of the Aβ was neither Aβ40 nor Aβ42, and it was suggested that Aβ40 and Aβ42 concentrations were lower than the A$\beta 1-x$ concentrations by a factor of 35 and 2.5, respectively.[26] In our previous study, a high sensitivity variant of a commercially available ELISA kit was used.[25] In 12 h pooled samples, we were able to analyze Aβ40 in $\sim$50% of the patients, and were able to analyze Aβ42 in seven out of eight patients. These levels were comparable to those obtained in the present report. However, in the present report, the analysis of Aβ40 and Aβ42 in 2 h samples was possible in all patients, indicating a superior sensitivity using the microsphere-based Luminex xMAP technique. The high specificity of the antibodies used in the present report and their application in the Luminex technique has previously been established.[75–77] As long-term storage of CSF and plasma samples was found to influence the analysis of Aβ,[78,79] consideration of pre-analytical factors was crucial. Prior to analysis, the MD samples in our present study were stored at -70°C, and every precaution was taken to handle and store the samples properly. We believe that the minor fluctuations of Aβ during the course of the disease for the individual patient reflected this effort. In addition, the MD membrane may serve as a barrier separating Aβ from degrading enzymes such as insulysin and neprilysin,[80] contributing to sample stability. Finally, other groups have observed long-term stability of Aβ in CSF samples for >2 years of freezing,[81,82] arguing that pre-analytical factors did not markedly influence our results. However, further studies are clearly needed to define the effect of pre-analytical factors such as long-term storage on analyte levels.

Although astrocytes, microglia, and endothelial and smooth muscle cells may release Aβ, neurons express the highest levels of APP and may release Aβ into the extracellular space under normal cellular conditions.[83] In one previous report using MD, the Aβ levels were dependent on the level of consciousness.[26] In that report, studying a mixed TBI and SAH cohort, Aβ levels rose when the patient's neurological status improved and vice versa. Here, we did not detect a similar correlation among Aβ40, Aβ42, and the level of consciousness in a pure TBI cohort. As an important part of routine NCC monitoring, we frequently evaluate the neurological status (the motor component of the GCS), using the neurological wake-up test[46] requiring that the continuous sedation be interrupted. Despite the absence of sedation during the tests, interstitial Aβ levels were not markedly altered. There are several experimental reports suggesting a link between extracellular Aβ dynamics and neuronal activity.[83–86] It is plausible that TBI-induced factors are responsible for regulating neuronal activity and, conversely, Aβ levels. The wake-up test and reduction of sedation may not be sufficient to overcome TBI-induced depression of neuronal activity. In addition, the neurological evaluation performed in the NCC setting provides only a crude measurement of neuronal activity, and may be influenced by many additional factors other thanTBI severity. Therefore, the lack of correlation between Aβ levels measured using MD and the level of consciousness observed here may not be surprising. However, as there was a lack of pre-injury, baseline Aβ levels in the present data cannot resolve the possibility that our findings were the result of increased Aβ caused by axonal injury or a larger decrease in Aβ levels in focal TBI caused by a more marked regional decrease in neuronal activity. In addition, Aβ oligomers and protofibrils could be elevated after TBI, despite a reduction in MD Aβ levels. These issues should be addressed in future studies.

Limitations

There are obvious limitations to our study. The present cohort of 10 TBI patients is small, which may have prevented us from detecting significant correlations between Aβ levels and a number of clinical and radiological factors. Because of the heterogeneity of TBI, this number may be insufficient to enable firm conclusions, and even though each patient was thoroughly characterized, additional studies are needed. A routine use of MR imaging would more accurately have been able to determine the pathology and presence of axonal injury in the vicinity of the MD probe. Additionally, multimodal monitoring of each patient including electroencephalogram (EEG) and brain tissue oxygenation combined with, for example, positron emission tomography (PET) monitoring could have strengthened the interpretation of energy metabolic changes in relation to MD Aβ levels. The Aβ peptides evaluated in the present report may be relevant in the acute pathophysiology of TBI, and recent studies have indicated the importance of Aβ oligomers, not measured in the present study, in neurodegeneration.[87] Even though Aβ is produced by normal neuronal metabolism,[88] the normal extracellular Aβ levels using MD and our present analysis technique are unknown. Finally, the rates of enzymatic degradation, uptake into activated microglia, and receptor-mediated export of Aβ to other organs,[73] perhaps influenced by TBI, were not measured, and may have modulated our MD Aβ levels.

Conclusion

Using a microsphere-based Luminex xMAP technique, we were able to analyse and monitor Aβ40 and Aβ42 levels in bi-hourly samples in severe human TBI. Our data support the notion that MD is a useful tool for studying Aβ dynamics following TBI. We also confirm our previous hypothesis that these Aβ peptides occur at higher concentration in patients with diffuse injury than in those with focal TBI. However, we could not find a correlation between Aβ levels and the focal energy metabolic situation, biomarkers of cellular distress, age of the patient, or the patient's level of consciousness. Future studies using an increased number of TBI patients are needed to confirm these observations. Additional markers such as tau and neurofilament and/or Aβ subspecies including oligomers and protofibrils should also be used as previously suggested[73] for elucidating the potential role of Aβ in the pathobiology of TBI as well as the complex relationship between TBI and AD pathology.

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Author Disclosure Statement

Analysis of Aβ peptides were done at the time Dr. Vanmechelen was employed at Innogenetics, and results were transferred to the Institute of Neuroscience and Physiology and Division of Neurosurgery. All subsequent analyses and interpretations were done after September 2011. No other competing financial interest exists.

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Address correspondence to:
Niklas Marklund, MD, PhD
Department of Neurosurgery
Uppsala University Hospital
SE-751 85
Sweden

E-mail: Niklas.Marklund@neuro.uu.se