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Tau elevations in the brain extracellular space correlate with reduced amyloid-β levels and predict adverse clinical outcomes after severe traumatic brain injury

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Axonal injury is believed to be a major determinant of adverse outcomes following traumatic brain injury. However, it has been difficult to assess acutely the severity of axonal injury in human traumatic brain injury patients. We hypothesized that microdialysis-based measurements of the brain extracellular fluid levels of tau and neurofilament light chain, two low molecular weight axonal proteins, could be helpful in this regard. To test this hypothesis, 100 kDa cut-off microdialysis catheters were placed in 16 patients with severe traumatic brain injury at two neurological/neurosurgical intensive care units. Tau levels in the microdialysis samples were highest early and fell over time in all patients. Initial tau levels were >3-fold higher in patients with microdialysis catheters placed in pericontusional regions than in patients in whom catheters were placed in normal-appearing right frontal lobe tissue ($P = 0.005$). Tau levels and neurofilament light-chain levels were positively correlated ($r = 0.6$, $P = 0.013$). Neurofilament light-chain levels were also higher in patients with pericontusional catheters ($P = 0.04$). Interestingly, initial tau levels were inversely correlated with initial amyloid-β levels measured in the same samples ($r = -0.87$, $P = 0.000023$). This could be due to reduced synaptic activity in areas with substantial axonal injury, as amyloid-β release is closely coupled with synaptic activity. Importantly, high initial tau levels correlated with worse clinical outcomes, as assessed using the Glasgow Outcome Scale 6 months after injury ($r = -0.6$, $P = 0.018$). Taken together, our data add support for the hypothesis that axonal injury may be related to long-term impairments following traumatic brain injury. Microdialysis-based measurement of tau levels in the brain extracellular space may be a useful way to assess the severity of axonal injury acutely in the intensive care unit. Further studies with larger numbers of patients will be required to assess the reproducibility of these findings and to determine whether this approach provides added value when combined with clinical and radiological information.

Introduction

Axonal injury has been hypothesized to be a primary determinant of adverse outcomes following traumatic brain injury (Smith *et al.*, 2003*b*). In autopsy studies, this pathology has been found to be highly prevalent in both severe and milder cases (Strich, 1956, 1961; Adams *et al.*, 1982, 1989; Blumbergs *et al.*, 1989, 1994, 1995; Christman *et al.*, 1994; Bennett *et al.*, 1995; Gorrie *et al.*, 2002; Adams *et al.*, 2011). In experimental studies, the extent of axonal injury after rotational acceleration injury in non-human primates was directly proportional to the depth of coma in the absence of other significant pathology (Gennarelli *et al.*, 1982). Similar findings were observed in a pig model (Smith *et al.*, 2000). Advanced MRI indicators of axonal injury have been found to be correlated with a variety of adverse clinical outcomes (Huisman *et al.*, 2004; Wilde *et al.*, 2006; Kraus *et al.*, 2007; Levin *et al.*, 2008; Niogi *et al.*, 2008*a, b*; Wang *et al.*, 2008; Kumar *et al.*, 2009; Lipton *et al.*, 2009; Lo *et al.*, 2009; Perlbarg *et al.*, 2009; Chang *et al.*, 2010; Kondo *et al.*, 2010; Marquez de la Plata *et al.*, 2010; Warner *et al.*, 2010; Kinnunen *et al.*, 2011). However, there has been comparatively little study of axonal injury in patients with severe traumatic brain injury during the acute phase (Petzold *et al.*, 2011), and new approaches are needed to assess the severity of axonal injury in the living human brain.

Tau is an intracellular, microtubule-associated protein that is highly enriched in axons. Its release into the CSF has been interpreted as indicative of axonal injury (Zemlan *et al.*, 1999; Emmerling *et al.*, 2000; Franz *et al.*, 2003; Ost *et al.*, 2006; Zetterberg *et al.*, 2006). Likewise, neurofilament-light chain (NF-L) is an integral part of the axonal cytoskeleton and NF-L elevation in the CSF may serve as a complementary marker of axonal injury (Norgren *et al.*, 2003; Van Geel *et al.*, 2005; Zetterberg *et al.*, 2006).

While CSF-based studies have provided significant insights, tau and NF-L release into the brain extracellular space as detected by microdialysis could be an even more direct measure of axonal injury. Microdialysis sampling can be used to assess local intracranial processes (Hillered *et al.*, 2005), whereas CSF sampling likely reflects more global events (Fishman, 1992). Importantly, brain extracellular interstitial fluid is not in full equilibrium with the CSF (Fishman, 1992; Brody *et al.*, 2008). Likewise, many patients with severe traumatic brain injury do not have external ventricular drains placed so serial CSF samples are not always available. While invasive, microdialysis catheters are relatively safe and readily placed at the bedside in conjunction with other cerebral monitoring devices.

Previously, we demonstrated that microdialysis could be used to serially measure amyloid-beta peptide (Aβ) levels in the brain interstitial fluid of severely injured patients undergoing invasive intracranial neurosurgical procedures for other clinical indications (Brody *et al.*, 2008). We found that brain interstitial fluid Aβ dynamics correlated with neurological status, a result that fits nicely with a body of experimental evidence indicating that synaptic activity is directly linked to Aβ production and release into the extracellular space (Kamenetz *et al.*, 2003; Cirrito *et al.*, 2005, 2008;

Kang *et al.*, 2009; Bero *et al.*, 2011).These interesting Aβ dynamics were not apparent in concomitantly sampled ventricular CSF (Brody *et al.*, 2008).

However, we are aware of only one previous study of tau levels in the human brain interstitial fluid. Marklund and colleagues (2009) found that patients with microdialysis catheters placed near areas of focal injury apparent on CT scans had higher brain interstitial fluid tau levels than patients with presumed diffuse axonal injury. There was a negative correlation at later time points between tau levels and $A\beta_{1-42}$ levels, but the sample size ($n = 8$ patients) and low sensitivity of the Aβ detection methods used limited the conclusions that could be drawn. NF-L has not previously been measured by microdialysis to our knowledge. Recently, proteolytic cleavage products of neurofilament heavy chain have been measured using high-molecular weight cut-off microdialysis catheters and found to be predictive of mortality following severe traumatic brain injury (Petzold *et al.*, 2011).

We hypothesized that microdialysis measurements of tau and NF-L in the extracellular space of the injured human brain would represent a useful approach to assess the severity of traumatic axonal injury. Furthermore, we were interested in the relationship between axonal injury markers and Aβ based on our previous studies of Aβ dynamics. With these aims in mind, we developed sensitive methods to allow assessment of tau and NF-L levels with high time resolution in the same microdialysis samples used for measurements of Aβ. We used these methods in a total of 16 patients with severe traumatic brain injury, and found that tau levels were highest in pericontusional regions, inversely correlated with Aβ levels, concordant with NF-L levels and most importantly predictive of 6 month adverse clinical outcomes.

Materials and methods

Patients

All research protocols were approved by the human research protection offices at the Ospedale Maggiore Policlinico in Milan and Washington University in St Louis. Next of kin or legally authorized representatives provided written informed consent for every patient. A total of 19 patients admitted to intensive care for severe closed-head traumatic brain injury between 2006 and 2010 met inclusion criteria and were approached for this study. These inclusion criteria were: (i) clinical indication for placement of invasive intracranial pressure monitoring; (ii) age ⩾16 years at Ospedale Maggiore Policlinico, age ⩾18 years at Washington University; (iii) no severe coagulopathy defined as clinical evidence of excessive bleeding, platelet counts <100 000, International Normalized Ratio >1.4, or partial thromboplastin time >50; (iv) no clinical indication for future anticoagulation, e.g. life-threatening deep vein thrombi/pulmonary embolisms; (v) not a member of a vulnerable population, e.g. prisoners; (vi) not pregnant; and (vii) family or next-of-kin available to provide written informed consent. Of these, family members of two patients did not consent, and useful microdialysis samples were obtained in all but one patient. In this patient, the microdialysis catheter was placed in the subarachnoid space, rather than in the brain parenchyma. This did not result in any adverse clinical events but the samples were uninterpretable and the catheter was promptly removed. This yielded a total sample size of

16 patients. Aβ levels from 10 of these patients have previously been reported (Brody *et al.*, 2008).

Microdialysis

CMA71 catheters (CMA Microdialysis AB) with a nominal molecular weight cut-off of 100 kDa were inserted by trained neurosurgeons at either the Ospedale Maggiore Policlinico or Washington University. All catheters were inserted in conjunction with another clinically indicated intracranial procedure. In 10 patients, this was the insertion of an intracranial pressure monitoring device. In six patients, this was evacuation of an intracranial or extracranial haematoma with or without decompressive craniotomy. Microdialysis catheters were placed a mean of 21.5 h (25–75th percentile: 15–28 h) after injury (Supplementary Table 1). Sample acquisition was started 1–2 h after catheter insertion. CT scans were used to confirm the placement of the microdialysis catheters. CNS perfusion fluid (CMA Microdialysis AB) + 1.5% sterile human albumin was perfused at a rate of 0.3 μl/min, as described previously (Brody *et al.*, 2008). Samples were collected every 1 to 2 h for at least 72 h. Samples were immediately placed on ice and frozen at −80°C within 12 h of collection.

In vitro recovery of tau, NF-L and Aβ were measured for microdialysis catheters that were functioning normally at the time of removal and did not appear damaged. Catheters were inserted into vials of human CSF from patients with either traumatic brain injury or another neurological condition expected to cause elevated CSF tau and NF-L levels. Perfusion at the same flow rate with the same perfusion fluid was performed for 2–6 h. Aliquots of the CSF were placed into CMA collection tubes before and after the *in vitro* microdialysis. *In vitro* recovery was defined as the average concentration of each analyte in the microdialysis samples divided by the average concentration of each analyte in the CSF aliquots taken before and after *in vitro* microdialysis.

Analytical methods

$Aβ_{1−x}$ levels were measured by enzyme-linked immunosorbent assay (ELISA), as previously reported (Brody *et al.*, 2008). The capture antibody was m266 recognizing $Aβ_{13−26}$ and the detection antibody was biotinylated 3D6 recognizing $Aβ_{1−5}$ with a free N-terminus. Standard curves were made using synthetic $Aβ_{1−40}$ and ranged from 1.6 to 1200 pg/ml. The sensitivity of this ELISA was typically 4.9 pg/ml (Supplementary Table 2).

Total tau levels were measured by ELISA using a commercial kit (#KHB0042, Invitrogen). Recombinant human tau standards provided by the manufacturer were run with each set of assays to generate standard curves. The sensitivity of this ELISA was 32 pg/ml (Supplementary Table 2).

NF-L levels were measured by ELISA, as previously reported (Zanier *et al.*, 2010). The capture antibody was the rabbit polyclonal AHP 286 (Serotec) and the detection antibodies were DA2 (Zymed) and 8A1 (Leinco). AHP 286 was raised using purified primate neurofilaments and recognizes NF-L with low levels of phosphorylation. DA2 is a mouse monoclonal antibody raised against dephosphorylated porcine NF-L that recognizes a phosphorylation-independent epitope (Shaw and Chau, 1988). 8A1 is a mouse monoclonal antibody raised against bovine NF-L (Guillemot and Cepko, 1992). All three cross react with human NF-L. Standard curves were made using purified bovine spinal cord NF-L (MP Biomedical). Two protocol modifications were made: biotinylated rabbit anti-mouse immunoglobulin-g (DAKO E0354) was used at 0.1 ng/ml and incubated for 1 h at room temperature. Poly-HRP 40-strep was used at 1:16 000 and incubated for 30 min at room temperature. The protocol was otherwise the same as

previously published. The sensitivity of this ELISA was 12 pg/ml (Supplementary Table 2).

Lactate, pyruvate, glucose and glutamate measurements were made with a commercial bedside kinetic enzymatic analyser (CMA600) immediately after collection with standardization per the manufacturer's instructions before freezing. Urea was measured using the QuantiChrom (BioAssay Systems) colorimetric urea assay kit. A standard curve was run with each set of assays using urea standards provided by the manufacturer.

Sequential enzyme-linked immunosorbent assay

Due to the limited volume of samples available from human microdialysates (18 μl/h), the sequential ELISA method was used to measure multiple analytes in each sample (Osuchowski and Remick, 2006). The principle of the sequential ELISA strategy is that, in general, only one or a few specific analytes bind to the antibody coated wells of the ELISA plate; the rest remain in the solution phase. Instead of discarding the unbound analytes when the plate is washed after incubation, a portion of the solution phase can be transferred to another ELISA plate coated with a different capture antibody. This transfer step can be repeated several times.

Most samples were analysed as follows: original thawed samples were diluted between 1:10 and 1:30 and loaded onto a plate coated with m266 for measurements of $Aβ_{1−x}$; after overnight incubation, 90 μl from each well was transferred to a plate coated with AHP286 for measurement of NF-L; after incubation, 50 μl from each well was mixed 1:1 with sample buffer from the Invitrogen human total tau kit and loaded into wells pre-coated with anti-tau antibody from the Invitrogen kit and; an additional 10–20 μl was diluted to 100 μl and assayed for urea. We used low protein binding polypropylene tubes (Axygen), and low protein binding plates and tips throughout. This process required a total of 4 days. Quantitative values have been reported after correction for all dilutions. All primary results reported are based on samples frozen and thawed once.

We verified that the sequential ELISA methods used to assess multiple analytes in the same samples resulted in accurate quantitation of the later analytes in the series. We performed sequential ELISAs on mock samples containing known quantities of Aβ, NF-L, tau and urea, and compared the results with the measurements obtained using standard (non-sequential) ELISA measurements on the same samples. For measurements of NF-L after $Aβ_{1−x}$, tau after NF-L and tau after $Aβ_{1−x}$, the results agreed very well [Fig. 1A–C, $r^2 = 0.99$, 95% confidence interval (95% CI) of the slope of the best fit line close to 1 and 95% CI of the intercept always containing the origin]. Bland-Altman plots (Bland and Altman, 1999) demonstrated that there did not appear to be systematic errors due to the sequential ELISA procedure (Fig. 1D–F). We also tested the accuracy of the sequential ELISA measurements in five sets of pooled human microdialysis samples. This revealed that the sequential ELISA approach overestimated the true tau concentration by 3–9% compared with tau measurements made on naïve samples. The sequential ELISA approach underestimated the true NF-L concentration by 1–17%. We have not corrected for these errors, as none of the fundamental conclusions of the study would be altered as a result.

Clinical outcome assessments

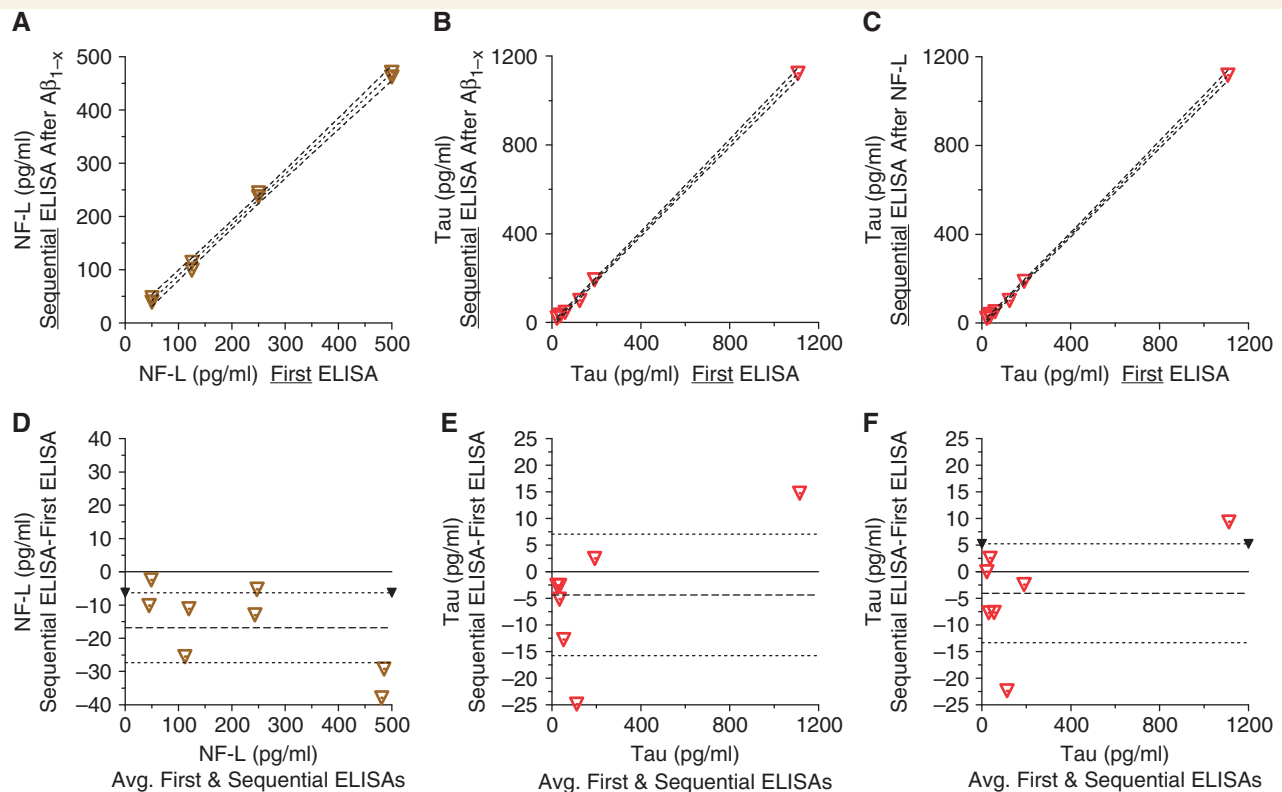Telephone or in-person assessments of overall clinical outcome were performed using the Glasgow Outcome Scale-Extended

Figure 1 Verification of the accuracy of sequential ELISA methods using mock microdialysis samples. Symbols indicate measured sample points. Dashed lines indicate linear best fits and 95% CIs. (**A**) NF-L sequential ELISA after $A\beta_{1-x}$ ELISA versus NF-L as the first ELISA: $r^2 = 0.99$, slope 95% CI 0.90–0.99, y-intercept 95% CI -17.6 to 8.1. (**B**) Tau sequential ELISA after $A\beta_{1-x}$ ELISA versus tau as the first ELISA. $r^2 = 0.99$, slope 95% CI 0.99–1.05, y-intercept 95% CI -20.5 to 2.3. (**C**) Tau sequential ELISA after NF-L ELISA versus tau as the first ELISA. $r^2 = 0.99$, slope 95% CI 0.99–1.03, y-intercept 95% CI -17.7 to 3.4. (**D–F**) Bland-Altman plots for the data presented in panels (**A–C**).

(Teasdale *et al.*, 1998; Wilson *et al.*, 1998; Pettigrew *et al.*, 2003). These assessments were performed at least 6 months after injury, and typically based on information obtained from both the patient and a reliable collateral source.

Statistical methods

All data were analysed using GraphPad Prism version 5 (GraphPad Software). Data were typically not normally distributed, so Mann–Whitney U-tests were used for unpaired group comparisons, Wilcoxon paired rank tests were used for comparisons across time and Spearman's rank order correlations were used for analyses of the relationships between variables. Two sided tests were used and were reported as significant for $P < 0.05$. P-values have not been corrected for multiple comparisons. Error bars and ranges represent 25–75th percentile confidence intervals.

To assess tau and $A\beta$ dynamics in a homogenous fashion across all patients, time bins of 1–12 h and 61–72 h after the initiation of microdialysis were used. This made maximal use of the available samples, since microdialysis duration was variable (Supplementary Table 1). Parametric methods such as exponential curve fitting were not used, as there was a great deal of variability in the quality of such fits, and no *a priori* reason to presume a specific form (e.g. monoexponential) for the changes in analytes over time. Per cent changes in tau and $A\beta$

were used as well as absolute changes because of the wide variation in initial levels of both markers.

Results

Brain extracellular tau and amyloid-β: diffuse injury versus contusion

We studied a total of 16 patients with severe traumatic brain injury from the Neurosurgical Intensive Care Unit at the Ospedale Maggiore Policlinico in Milan and the Neurological/Neurosurgical Intensive Care Unit at Barnes Jewish Hospital in St Louis. Clinical characteristics of the patients and position of microdialysis catheters are summarized in Table 1. No catheter placement-related intracranial haemorrhages were detected. There were no other adverse events related to microdialysis procedures.

In nine patients, there were no apparent focal lesions on CT scans in the frontal lobes that could be targeted for microdialysis. In eight of these patients, the microdialysis catheters and intracranial pressure monitors were placed in the right frontal subcortical white matter (e.g. Fig. 2A and C). In one patient (Case 1), the catheter was placed in the left frontal subcortical white matter.

Table 1 Clinical characteristics of patients

Case number	Age (years) M/F	Initial GCS	GOS-E at 6 months	Marshall CT classification	Type of surgery	Microdialysis catheter position
1	32 F	5	6	Evacuated mass lesion	EDH, ICH, Dec Crani	L Frontal
2	49 F	5	7	Evacuated mass lesion	SDH, ICH, Dec Crani	R Frontal
3	50 F	7	3	Evacuated mass lesion	EDH, ICH, Dec Crani	R Frontal
4	21 F	7	5	Evacuated mass lesion	EDH, SDH, ICH, Dec Crani	Pericontusional
5	24 M	7	3	Diffuse injury II	None	Pericontusional
6	31 F	4	3	Diffuse injury II	None	Pericontusional
7	16 F	4	4	Diffuse injury III	None	R Frontal
8	40 M	6	3	Diffuse injury II	None	R Frontal
9	41 M	5	4	Diffuse injury II	None	R Frontal
10	32 F	13	3	Evacuated mass lesion	SDH, Dec Crani	Pericontusional
11	35 M	11	6	Evacuated mass lesion	ICH, Dec Crani	Pericontusional
12	41 F	6	3	Diffuse injury II	None	R Frontal
13	29 M	5	3	Diffuse injury II	None	R Frontal
14	30 M	5	7	Diffuse injury II	None	R Frontal
15	63 M	5	1[a]	Non-evacuated Mass lesion	None	Pericontusional
16	39 M	3	3	Evacuated mass lesion	SDH	Pericontusional

Dec Crani = decompressive craniectomy; Diffuse injury II = cisterns present, midline shift <5 mm, and/or small <25 cc high or mixed density lesions; Diffuse injury III = compressed or absent cisterns but midline shift <5 mm and/or small <25 cc high or mixed density lesions (Marshall *et al.*, 1992); EDH = evacuation of epidural haematoma; GCS = initial post-stabilization total Glasgow Coma Score (Teasdale and Jennett, 1974); GOS-E = Glasgow Outcome Scale-Extended at 6 months (Wilson *et al.*, 1998); ICH = evacuation of intracerebral haematoma; SDH = evacuation of subdural haematoma.
a This patient died of causes unrelated to traumatic brain injury ~2 months after injury.

These patients were all comatose (initial Glasgow coma scores 3–8) and such patients are often presumed to have diffuse axonal injury. These patients will be referred to as the 'non-contusional' group. In seven patients, there were frontal haemorrhagic contusions apparent on CT scans (e.g. Fig. 2E and G). In six of these patients, the microdialysis catheters were placed in pericontusional regions on either the right or left frontal lobe. In one patient (Case 6), the catheter was placed in the parietal lobe. These patients with apparent contusions near the microdialysis catheters will be referred to as the 'pericontusional' group.

In all patients, tau levels could be measured in single microdialysis samples collected every 1 to 2 h. In initial patients (e.g. Fig. 2B), all hourly samples were analysed and a high time resolution time course was obtained. In the majority of the patients (e.g. Fig. 2D, F and H), samples were analysed every 4–6 h. Absolute tau levels varied considerably from patient to patient, but they were typically highest at the start of microdialysis and generally fell monotonically over time (Fig. 2). Interestingly, tau levels remained quite elevated even 72–120 h after the start of microdialysis (e.g. Fig. 2B and F). Aβ levels measured in the same samples rose over time (Fig. 2), as has been previously reported for a subset of these patients (Brody *et al.*, 2008). Urea levels were measured as a control for microdialysis catheter function and were found to be stable, as previously shown (Brody *et al.*, 2008).

In vitro recovery of tau ranged between 1% and 2%. *In vitro* recovery of Aβ was 30%, as reported previously (Brody *et al.*, 2008). Despite several attempts, we have not been able to recover NF-L by microdialysis *in vitro*. These attempts have used human CSF with ~1300 pg/ml of native NF-L (Zanier *et al.*, 2010) and

purified bovine NF-L spiked into artificial CSF at concentrations up to 500 ng/ml. Data presented here has not been corrected for fractional recovery.

Patients with contusions near the microdialysis catheters had higher tau levels than patients with catheters placed into non-contusional regions. Median tau levels over the first 24 h of microdialysis sampling in the pericontusional group were 15 950 pg/ml (11 390–27 240 pg/ml) versus 3469 pg/ml (1684–8691 pg/ml) in the non-contusional group (Fig. 3A, $P = 0.005$, Mann–Whitney U-test).

In contrast, patients in the pericontusional group had lower Aβ levels than patients in the non-contusional group. Median Aβ levels over the first 24 h of microdialysis sampling in the pericontusional group were 270 pg/ml (83–417) versus 1023 pg/ml (778–1968) in the non-contusional group (Fig. 3B, $P = 0.0012$).

Tau dynamics were similar in the pericontusional and non-contusional groups. Tau levels fell from 1–12 h to 61–72 h in all patients in both groups (Fig. 3C, $P = 0.016$ for pericontusional group, $P = 0.0078$ for non-contusional group, Wilcoxon signed rank tests). The extent of the decrease between the pericontusional group (median decrease: 57%, range: 52–89%) and the non-contusional group (median decrease: 79%, range: 36–95%) was not significantly different ($P = 0.72$, Mann–Whitney U-test).

Aβ levels rose in most patients (Fig. 3D). This rise was significant in the pericontusional group ($P = 0.031$), though not significant for the non-contusional group in isolation ($P = 0.29$). The extent of the rise was greater in the pericontusional group (median increase: 170%, range: 88–220%) compared with the non-contusional group (median increase: 47%, range: 16–62%, $P = 0.05$).
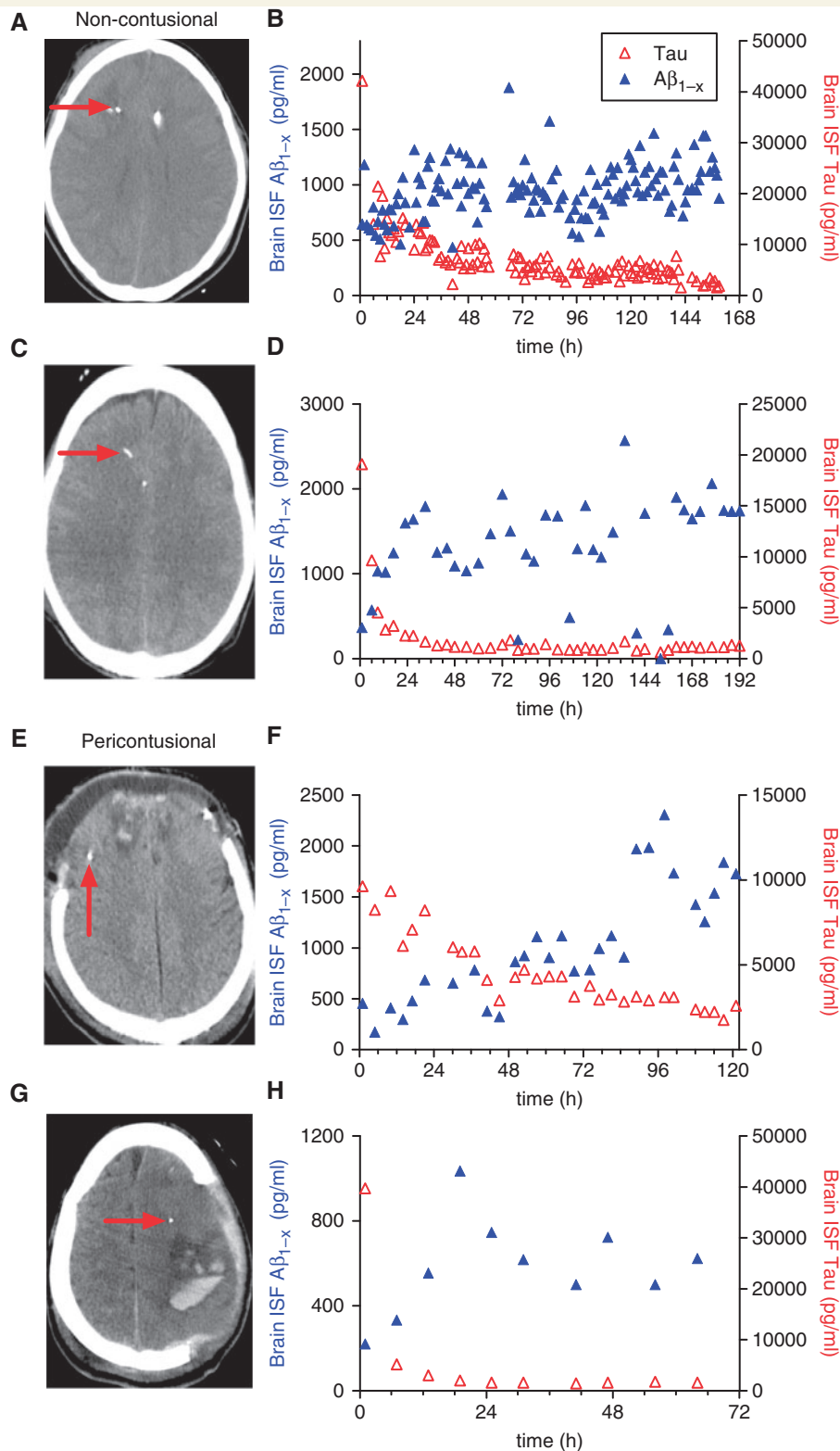
Figure 2 Examples of microdialysis-based measurements of tau and Aβ dynamics in the injured human brain. (**A**) CT scan of the brain from a patient in the non-contusional group. Microdialysis catheters tip (red arrow) apparent in normal-appearing right frontal subcortical white matter.(**B**) Brain interstitial fluid Aβ (*left y*-axis) and tau (*right y*-axis) levels as a function of time (h) since placement of the microdialysis catheter. (**C** and **D**) CT scan plus brain interstitial fluid Aβ and tau levels from another non-contusional patient. (**E–H**) CT scans plus brain interstitial fluid Aβ and tau levels from two patients with traumatic frontal contusions. Microdialysis catheter tips (red arrows) apparent near the frontal lobe contusions.
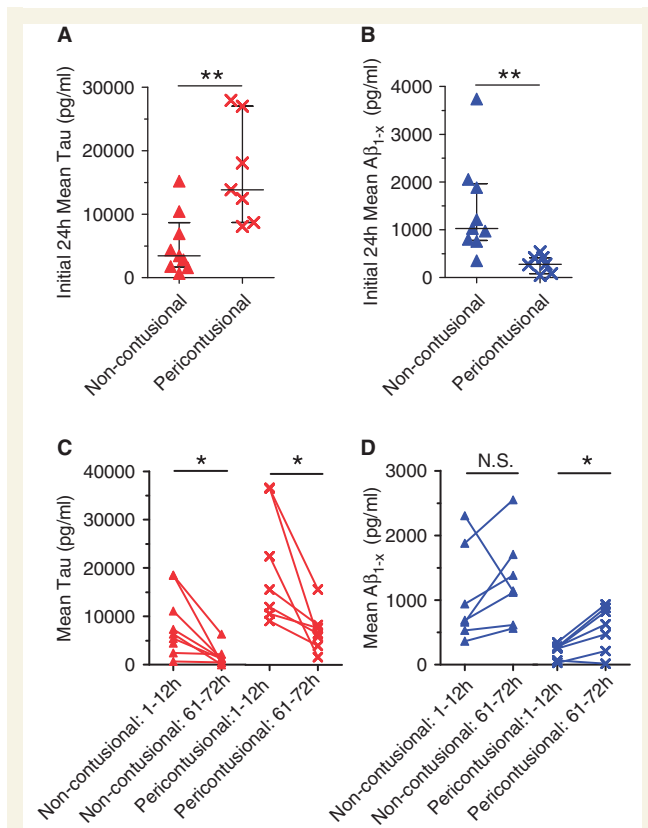
Figure 3 Tau and Aβ measurements in non-contusional versus pericontusional groups. (**A**) Mean tau levels during the initial 24 h of microdialysis sampling were higher in patients with catheters placed in pericontusional regions: Error bars represent median and 25–75th percentiles (**P = 0.005, Mann–Whitney U-test). (**B**) Mean Aβ levels during the initial 24 h of microdialysis sampling were lower in pericontusional group (**P = 0.0012). (**C**) Tau levels decreased with time in all patients (*P = 0.0078 for non-contusional group, *P* = 0.016 for pericontusional group, Wilcoxon paired rank tests). (**D**) Aβ levels rose over time in all but one non-contusional patient (*P* = 0.29) and all but one pericontusional patient (*P = 0.031). NS = not significant.

Correlations between tau and other microdialysis analytes

Patients with the highest tau levels typically had relatively low Aβ levels. This inverse correlation was highly statistically significant (Spearman $r = -0.87$, $P = 0.000023$) and present for both pericontusional and non-contusional patients (Fig. 4A).

There was not a strong correlation between the dynamics of tau and Aβ. The extent of the reduction in tau from 1–12 h to 61–72 h after the initiation of microdialysis did not significantly correlate with the extent of the rise in Aβ during the same time period, as measured using per cent changes (Fig. 4B, $r = -0.16$) or absolute changes (data not shown).

The same microdialysis samples were assayed for commonly measured small molecule analytes (Hillered *et al.*, 2005). There was a statistically significant negative correlation between tau

levels and glucose levels (Fig. 4C, $r = -0.61$, $P = 0.016$); most glucose levels were within the normal range, but the two patients with abnormally low glucose levels also had high tau levels. There was a non-significant trend towards higher lactate/pyruvate ratios in patients with the highest tau levels (Fig. 4G, $r = 0.41$, $P = 0.13$) and no correlation between tau levels and glutamate levels (Fig. 4F). Microdialysis data for each individual subject are shown in Supplementary Table 1.

Tau as a marker of axonal injury: concordance with neurofilament light-chain levels

The dynamics of tau and NF-L levels were often very similar; both typically fell monotonically over time (e.g. Fig. 5A). There was a positive correlation between initial tau levels and initial NF-L levels (Fig. 5B, Spearman $r = 0.60$, $P = 0.0013$). NF-L levels were significantly higher in patients with microdialysis catheters placed near contusions (median: 1555 pg/ml, range: 1152–2012) than in patients with catheters placed in non-contusional regions (median 104 pg/ml, range: 0–1201, Fig. 5B, $P = 0.04$, Mann–Whitney U-test). This result was qualitatively similar to the findings for tau. NF-L levels were more variable than tau levels, and undetectable in some patients despite moderately high tau levels (Fig. 5B). Some of this may be due to instability of NF-L (Pant, 1988; Koel-Simmelink *et al.*, 2011). We found that freezing and thawing microdialysis samples resulted in a $29 \pm 12\%$ (mean ± SD) loss of NF-L, but negligible loss of other analytes. This may account for the increased variability of NF-L measurements relative to other microdialysis markers.

Microdialysis-based predictors of clinical outcome

We assessed the univariate relationship between early interstitial fluid tau levels and clinical outcomes in these patients and found a significant relationship in the expected direction: higher initial tau levels were associated with lower (worse) Glasgow Outcome Scale-Extended scores at 6 months after injury (Fig. 6A). This inverse correlation was statistically significant ($r = -0.60$, $P = 0.018$). The same fundamental relationship appeared to extend to both pericontusional patients and non-contusional patients. One patient died ~2 months after injury of causes that were unrelated to traumatic brain injury. Therefore, this subject was not included in outcome analyses. No patients were in a persistent vegetative state (Glasgow Outcome Scale-Extended score of 2). Setting a cut-off value of 10 000 pg/ml (Fig. 6A, dashed horizontal line) allowed a 70% sensitivity and 80% specificity for predicting poor clinical outcome, defined as Glasgow Outcome Scale-Extended <5 (Fig. 6A, dashed vertical line). A cut-off value above 14 000 pg/ml allowed 60% sensitivity but 100% specificity for predicting poor clinical outcome. The area under the receiver operating characteristic curve was 0.76.

There was not a strong relationship between Aβ levels and clinical outcomes in these patients. Lower initial Aβ levels were
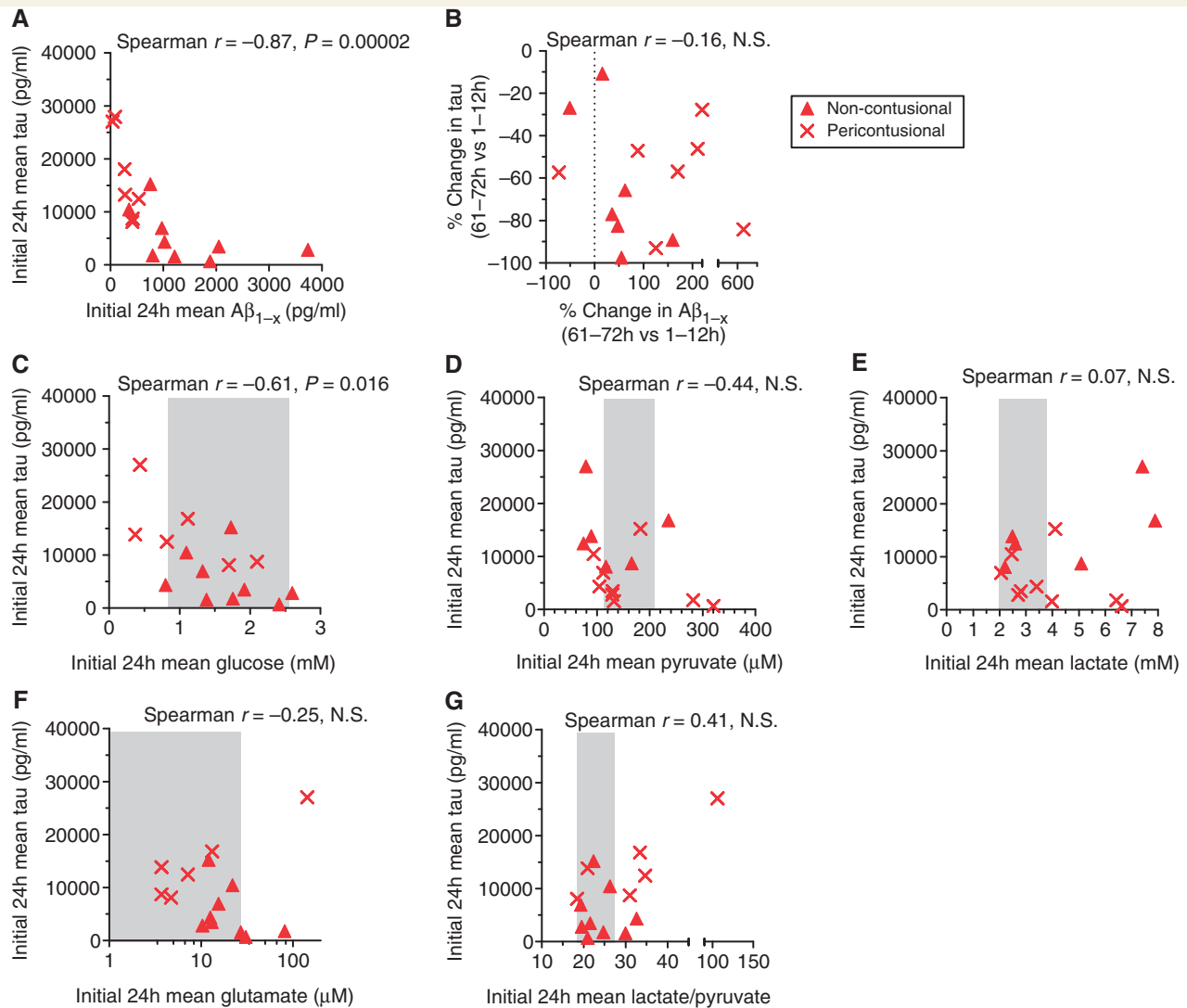
Figure 4 Correlations between tau and other microdialysis analytes. (**A**) Inverse correlation between initial Aβ levels and initial tau levels. (**B**) No correlation between the per cent change in Aβ levels and per cent change in tau levels. (**C**) Inverse correlation between initial brain interstitial fluid glucose levels and initial tau levels. (**D**) No significant correlation between initial brain interstitial fluid pyruvate levels and initial tau levels. (**E**) No correlation between initial brain interstitial fluid lactate levels and initial tau levels. (**F**) No correlation between initial brain interstitial fluid glutamate levels and initial tau levels. (**G**) No significant correlation between initial brain interstitial fluid lactate/pyruvate levels and initial tau levels. Shaded regions represent mean ± 1 standard deviation of estimated normal values (Reinstrup *et al.*, 2000; Hillered *et al.*, 2005). NS = not significant.

weakly associated with worse outcomes (Fig. 6B), but this was not statistically significant. Neither the dynamics of tau nor Aβ correlated with clinical outcomes (data not shown). Likewise, neither initial glucose levels (Fig. 6C), lactate/pyruvate ratios (Fig. 6D), nor glutamate levels (Fig. 6E) were significantly correlated with outcome in these patients.

Discussion

In summary, we have found that the axonal proteins tau and NF-L can be quantitatively assessed with 1–2 h time resolution by microdialysis in the injured human brain. The levels of these two putative markers of axonal injury were highest when microdialysis catheters were placed in pericontusional regions, but still elevated when catheters were placed in normal-appearing regions of the brain. Initial tau levels were inversely correlated with initial Aβ levels, and dynamics were opposite; Aβ rose and tau fell over time concordantly with the dynamics in CSF following severe traumatic brain injury reported by others (Kay *et al.*, 2003). Importantly, initial microdialysis tau levels directly correlated with poor clinical outcomes. Other microdialysis analytes including Aβ, glucose, lactate/pyruvate ratio and glutamate did not correlate with outcomes. However, glucose, lactate/pyruvate and glutamate were often within the normal range, making it difficult to directly compare our patients to those in other series (Zauner *et al.*, 1997;
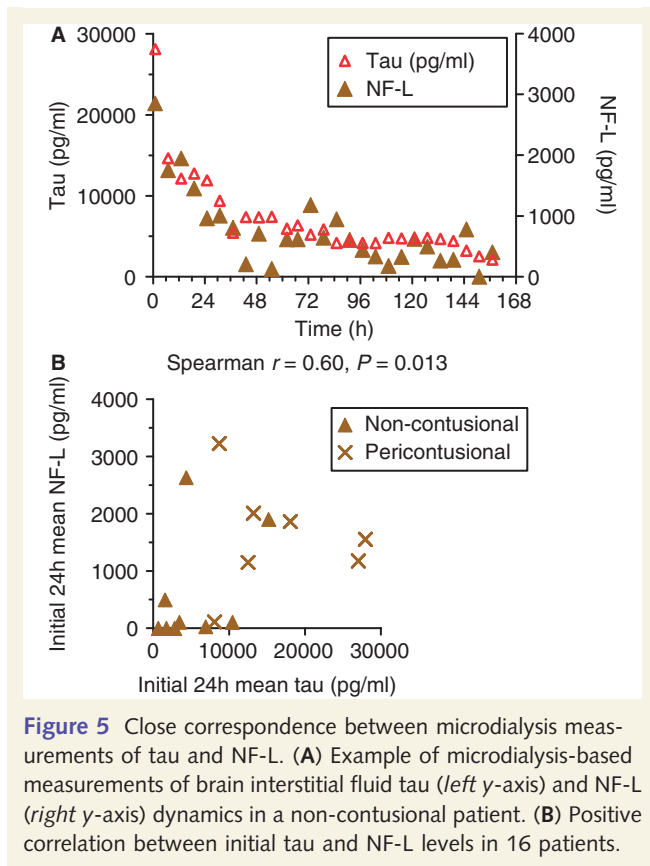
Figure 5 Close correspondence between microdialysis measurements of tau and NF-L. (**A**) Example of microdialysis-based measurements of brain interstitial fluid tau (*left y*-axis) and NF-L (*right y*-axis) dynamics in a non-contusional patient. (**B**) Positive correlation between initial tau and NF-L levels in 16 patients.

Koura *et al.*, 1998; Goodman *et al.*, 1999; Marcoux *et al.*, 2008; Timofeev *et al.*, 2011). Overall, our findings complement and extend previous results on the topic of microdialysis measurements of tau (Marklund *et al.*, 2009).

We interpret these results as adding support for the hypothesis that axonal injury may be related to long-term impairments following traumatic brain injury. It is not surprising that the levels of tau and NF-L were higher in pericontusional regions; we expect that there is more neuronal loss and likely more extensive axonal injury in these regions than in regions that appear normal on CT. However, even the patients in whom catheters were placed into brain regions without CT lesions were in coma on admission to the hospital. Such patients are often presumed to have widespread or diffuse axonal injury. However, this diagnosis is rarely verified in the intensive care unit. The most likely interpretation of our findings is that there is substantial axonal injury also in normal-appearing regions of the brain following severe traumatic brain injury. Elevated brain interstitial fluid tau and NF-L levels detected by microdialysis support the diagnosis of diffuse axonal injury in these patients. This interpretation fits well with other lines of evidence that suggests that axonal injury is a primary determinant of many adverse outcomes following traumatic brain injury (Gennarelli *et al.*, 1982; Smith *et al.*, 2000, 2003*b*; Huisman *et al.*, 2004; Wilde *et al.*, 2006; Kraus *et al.*, 2007; Levin *et al.*, 2008; Niogi *et al.*, 2008*a*, *b*; Wang *et al.*, 2008; Kumar *et al.*, 2009; Lipton *et al.*, 2009; Lo *et al.*, 2009; Perlbarg *et al.*, 2009; Chang *et al.*, 2010; Kondo *et al.*, 2010; Marquez de la Plata *et al.*,

2010; Warner *et al.*, 2010; Kinnunen *et al.*, 2011; Petzold *et al.*, 2011).

An alternative explanation is that trauma to the brain due to insertion of the microdialysis catheter itself is responsible for the elevated tau levels in patients without apparent CT abnormalities. We cannot fully rule out a contribution of iatrogenic trauma, but argue that this is not likely to explain the majority of the tau and NF-L elevations. First, the levels and time courses of these proteins were quite variable, which is more consistent with variable initial injury severities and ongoing axonal degeneration, and less consistent with the relatively stereotyped catheter insertion procedure. Second, elevated levels of tau were associated with poor clinical outcomes. These poor outcomes are more likely to be related to injury severity and very unlikely to result from insertion trauma. Ultimately, experimental animal models including microdialysis data from uninjured control groups (Alessandri *et al.*, 2003; Timaru-Kast *et al.*, 2008; Schwetye *et al.*, 2010) will be required to definitively resolve this issue. Along these lines, recent tau microdialysis experiments in uninjured mice indicate that insertional trauma has little effect on tau levels (K. Yamada and D.M. Holtzman, unpublished results).

There are several limitations of this study that should be kept in mind. Most importantly, we have not directly verified the relationship between brain interstitial fluid tau levels and the severity of traumatic axonal injury. This would likely require an experimental animal model in which microdialysis measurements are correlated with histopathological assessments of axonal injury in each animal in the region surrounding the microdialysis catheter location. Pig models could be appropriate for this approach, as the same catheters used in human patients can be placed into pig brain subcortical white matter as is typically done in human patients (Alessandri *et al.*, 2003; Timaru-Kast *et al.*, 2008). Advanced imaging methods sensitive to traumatic axonal injury such as Diffusion Tensor Imaging (Mac Donald *et al.*, 2007*a*, *b*; Perlbarg *et al.*, 2009; Niogi and Mukherjee, 2010) in the same subjects studied by microdialysis would also be of great interest. This is a critical area for future investigation. While predominantly axonal tau has also been detected in other non-neuronal cell types after traumatic brain injury (Smith *et al.*, 2003), and a dendritic role for tau has been reported (Ittner *et al.*, 2010). Nonetheless, the concordance of tau and NF-L levels makes it likely that both of these markers fundamentally reflect the same axonal injury processes.

Second, we have not measured the levels of tau and NF-L normally present in the extracellular space of the human brain. One would expect the normal extracellular levels to be quite low as they are both intracellular cytoskeletal proteins. CSF studies, while not directly comparable to microdialysis, have demonstrated considerably lower levels of these proteins in normal controls than in patients with traumatic brain injury (Zemlan *et al.*, 1999; Franz *et al.*, 2003; Norgren *et al.*, 2003; Van Geel *et al.*, 2005; Ost *et al.*, 2006; Zetterberg *et al.*, 2006).

Third, the relatively modest sample size precluded meaningful multivariate analyses of the predictors of outcomes (Timofeev *et al.*, 2011). This will be an important avenue for future investigation. For example, it would be of great interest to assess whether the use of microdialysis-based measurements of initial tau levels improves upon the predictions of outcome made by
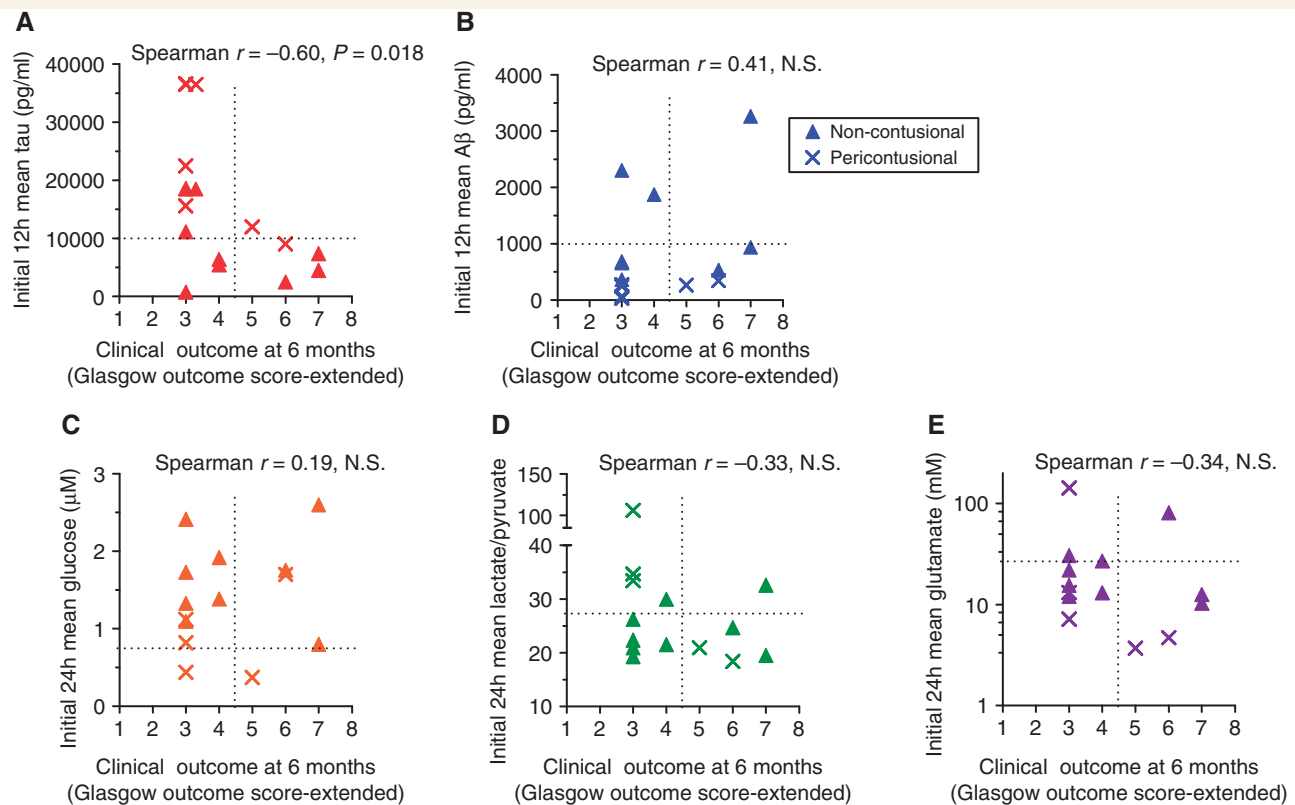
Figure 6 Tau levels, but not levels of other microdialysis analytes, predicted overall clinical outcomes 6 months after injury. (**A**) Significant correlation between tau levels in the first 12 h after catheter placement and overall clinical outcomes. Better outcomes indicated by higher Glasgow Outcome Score-Extended scores. (**B**) Non-significant correlation between initial Aβ levels and clinical outcomes. (**C**) No correlation between initial brain interstitial fluid glucose levels and clinical outcomes. (**D**) Non-significant correlation between initial brain interstitial fluid lactate/pyruvate levels and clinical outcomes. (**E**) Non-significant correlation between initial brain interstitial fluid glutamate levels and clinical outcomes. NS = not significant.

the IMPACT model, which incorporates early clinical factors and Marshall CT scan classification (Murray *et al.*, 2007).

Fourth, the relative recovery of tau was poor, and potentially variable between subjects. This potential variability between patients could have influenced our findings, but is not likely to have caused the correlation between tau levels and 6 month clinical outcomes. The use of even higher molecular weight cut-off catheters or additional carrier proteins in the perfusion fluid could be used to improve recovery in future investigations. We have not been able to determine the relative recovery of NF-L, despite several *in vitro* attempts, including catheter immersion in a large volume of human CSF (Afinowi *et al.*, 2009). This suggests one of the two interpretations: either that the recovery is <0.1%, or the characteristics of NF-L *in vitro* (i.e. oligomerization, binding to other proteins, degradation, phosphorylation, glycosylation etc.) are different from those *in vivo* (Petzold, 2005). We have not assessed the molecular weight, isoelectric point, phosphorylation status or hydrophobicity of the tau and NF-L recovered by microdialysis from the injured human brain. These are important areas for future investigation, but beyond the scope of the current manuscript.

An intriguing observation arising from this study is the wide variation in the rate at which tau and NF-L decreased over time.

It does not appear that changes due to dilution (e.g. from oedema) or systematic declines in microdialysis recovery are responsible; Aβ levels rose over time and urea levels remained stable. These variable decreases are likely to be due at least in part to heterogeneity in factors influencing diffusion and clearance, but could also in principle reflect variable ongoing release of these proteins into the interstitial fluid. In this study design, we cannot distinguish between these two possibilities. Local brain blood flow measurements could be helpful in future investigations. This is a critical question, as delayed axonal degeneration could be an important target for therapeutic interventions, whereas if all of the axonal injury occurs at the time of injury, efforts to improve prevention or restorative therapy would be more appropriate. Again, a valid experimental model will be required to fully address this issue.

As stated above, we have not assessed the phosphorylation state of tau in the interstitial fluid following traumatic brain injury. Hyperphosphorylated tau aggregations in the form of perisomatic and proximal dendritic tangles or neuropil threads are pathological features of sports-related chronic traumatic encephalopathy and a subset of severe cases of traumatic brain injury (Geddes *et al.*, 1999; Ikonomovic *et al.*, 2004; Jellinger, 2004; Uryu *et al.*, 2007; McKee *et al.*, 2009). These pathologies are

similar to those seen in several age-related neurodegenerative disorders (Lee *et al*., 2001). The relationship between elevated extracellular tau levels detected by microdialysis and these aggregations of intracellular tau detected by immunohistochemistry following traumatic brain injury is unknown. We speculate that injury to both axonal membranes and cytoskeletal structures could release tau protein into the extracellular space. Less severe axonal injury could release some tau into the extracellular space, but result in additional tau protein being dissociated from disrupted microtubules yet retained intracellularly. This tau could aggregate *in situ* or be transported back to the cell bodies. Alternatively, different pools of tau could be involved: axonal tau could be released into the interstitial fluid, and newly synthesized tau could aggregate proximally in part because it is not properly transported into axons.

Hyperphosphorylation of tau dramatically reduces its solubility. Therefore, the fractional recovery of these hyperphosphorylated tau species by microdialysis would be expected to be very low, at least for the 100 kDa molecular weight cut-off microdialysis catheters employed in this study. Catheters with a very high molecular weight cut-off (Winter *et al*., 2002, 2004) could be considered for future investigations along these lines.

The findings that interstitial fluid Aβ levels were lowest when tau levels were highest may seem surprising, but should be interpreted in the context of the established effects of synaptic activity on soluble extracellular Aβ release. Aggregation and elevated levels of Aβ have been reported in association with traumatic axonal injury in both human patients with traumatic brain injury and experimental animals (Smith *et al*., 1999, 2003*a*; Ikonomovic *et al*., 2004; Uryu *et al*., 2007). However, these aggregations were detected immunohistochemically and were likely to represent primarily insoluble Aβ. Microdialysis instead samples the soluble extracellular Aβ pool (Hillered *et al*., 2005; Schwetye *et al*., 2010). Aβ dynamics in this soluble extracellular pool have been shown to be tightly linked to synaptic activity in animals (Cirrito *et al*., 2005, 2008) and positively correlated with neurological status in humans (Brody *et al*., 2008). Thus, we interpret the low interstitial fluid Aβ levels in the patients with high tau levels as an indication that more severe axonal injury causes more profound synaptic and neurological impairment in these patients. It is unlikely that the arrow of causality is in the opposite direction, i.e. higher Aβ levels somehow prevent release of tau into the interstitial fluid or enhance its clearance.

From a clinical perspective, microdialysis-based monitoring of tau and NF-L to assess axonal injury could potentially be used together with other methods to add information about the status of brain white matter following severe brain injury. Further studies will be required to determine whether this or any other microdialysis-based monitoring improves outcomes or assists in prognosis. Rapid improvements in the speed and sensitivity of protein biomarker measurements may allow bedside assessments in the near future (Arruda *et al*., 2009). If fully validated, these microdialysis-based assessments of tau and NF-L could be useful for early phase clinical pharmacodynamic testing of candidate therapeutics targeting traumatic axonal injury.

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Supplementary material

Supplementary material is available at *Brain* online.

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