David L. Brody*, Sandra Magnoni*, Kate E. Schwetye, Michael L. Spinner, Thomas J. Esparza, Nino Stocchetti, Gregory J. Zipfel, and David M. Holtzman

Amyloid-β Dynamics Correlate with Neurological Status in the Injured Human Brain

SUPPORTING ONLINE MATERIAL

METHODS

Summary

Intracerebral microdialysis was performed by inserting standard CMA70 or CMA71 microdialysis catheters into the brain parenchyma of severely brain injured patients. All procedures were combined with clinically indicated invasive intracranial procedures performed by experienced neurosurgeons. Most catheters were placed in the right prefrontal subcortical white matter (Fig. 1A, C, E, Fig 2A). Because of the small size (0.6 mm diameter) and flexibility of the catheters, microdialysis catheter insertion presents little additional risk (*1*). Catheters were removed after 3-7 days of monitoring. All protocols

were approved by appropriate regulatory bodies, and written informed consent was obtained from next of kin for all procedures.

Sterile isotonic perfusion fluid was continuously pumped through the catheters at 0.3 µl / minute for most studies. Sterile human albumin was added to the perfusion fluid to allow recovery of Aβ. Microdialysis occurs as the fluid and solutes exchange with the brain extracellular fluid across the catheters' semipermeable membrane. The returning fluid was collected in sealed vials every 1-2 hours. Aβ was measured in this microdialysate using previously described enzyme-linked immunosorbent assay (ELISA) techniques (*2, 3*). All clinical assessments such as Glasgow Coma Score data were collected prospectively without knowledge of results of Aβ measurements. Ventricular CSF was obtained through external ventricular drains placed for clinical indications.

Statistical tests were performed using Statistica 6.0 or Prism 5.0. Spearman correlations and Wilcoxon signed rank tests (nonparametric) were performed as raw and normalized Aβ concentrations were not normally distributed (Shapiro Wilk Test, $P<0.0001$). A total of ~100 Spearman correlations were tested in both a hypothesis driven and exploratory fashion; therefore based on the Bonferroni method, Spearman correlations were reported as statistically significant when P values were <0.0005. Wilcoxon signed rank tests were reported as significant when P values were <0.05. Comparisons between two correlation coefficients were performed using a difference test (Statistica 6.0). Exponential fits to zero-flow extrapolation data were performed using the Microsoft Excel Solver.

Human Subjects. All protocols were approved by the Human Research Protection Office at Washington University, St. Louis and by the Hospital Ethical Committee at Ospedale Maggiore Policlinico, Milan. Written informed consent was requested in 27 patients and received in 21 patients. 1 patient was discontinued from the study due to a change in clinical management resulting in a contraindication to participation (need for acute anticoagulation), and 1 was discontinued for technical reasons (microdialysis catheter failure). A total of 19 patients aged 17 to 60 with acute traumatic brain injury (n=12), aneurysmal subarachnoid hemorrhage (n=6), or unruptured cerebral aneurysm (n=1) participated in this study. Potential participants were referred by treating physicians. Written informed consent was provided by next of kin of all injured patients, and by the unruptured aneurysm clipping patient himself prior to surgery. The principal investigators (DLB and SM) at each site were responsible for obtaining all informed consents. No participant or family member was paid or compensated in any way for participation. Patients with any clinical or laboratory evidence of coagulopathy were excluded. Members of potentially vulnerable populations such as prisoners, pregnant women, known HIV positive patients, military personnel, employees or family of employees at the treating institutions were excluded. Patients not expected to survive more than 3 days were excluded. All data was recorded in a coded fashion to mask identifying information and the linked identifying information was stored in a HIPAA compliant fashion. The complete protocol and supporting documents are available on request.

At Washington University, safety was reviewed by an independent monitor (Dr. M. Diringer) after each participant, and a subsequent participant was not enrolled until the interim safety report from the previous patient was received. At Ospedale Maggiore

Policlinico, Milan microdialysis was used as part of a multimodal brain monitoring system along with brain oxygen tension and cerebral temperature monitoring; clinical data and safety information were reviewed by the treating physicians every 6-12 hours. There was one complication possibly resulting from microdialysis catheter placement: a small, asymptomatic subdural hemorrhage near the site of catheter placement. This was detected after the participant was treated with anticoagulant medications following aneurysm coiling. At the time of enrollment, the plan by the treating physicians had been aneurysm clipping, without anticoagulation. As a result, the protocol was changed to include placing microdialysis catheters in subarachnoid hemorrhage patients only at the time of surgical aneurysm clipping, rather than at earlier times when clinical management could still potentially include coiling with anticoagulation. No other adverse events were considered likely or possibly related to the experimental procedures. Specifically, there were no infections, CSF leaks, other hemorrhages, cerebral infarctions, skin reactions, or retained foreign bodies.

Microdialysis Catheter Placement and Removal: All microdialysis catheters were placed by experienced neurosurgeons in conjunction with another interventional procedure, typically placement of an intracranial pressure monitoring device. In 6 patients, the intracranial pressure monitoring device was an external ventricular drain. In the remaining patients it was an intraparenchymal pressure monitoring system. Microdialysis catheter placement occurred in either the intensive care unit or in the operating room within 48 hours of initial injury. Catheter placement was performed using rigorously sterile technique either by the percutaneous method (*4*) or at the time of craniotomy. In most patients, the

right frontal lobe white matter was targeted. In 13 patients, a single CMA71 high molecular weight microdialysis catheter was used. In 6 patients a single CMA70 catheter was used. The cutoff of the microdialysis membrane was either 20 kDa (CMA70) or 100 kDa (CMA71), whereas Aβ is approximately 4 kDa in size. The active membrane length was 1 cm. No patient had more than 1 microdialysis catheter placed. All catheter placements were confirmed radiologically as these catheters have gold tips that render them highly visible on CT.

All catheters were removed by an experienced neurosurgeon using rigorous sterile technique at the bedside. Careful inspection for CSF leaks was performed. All participants were monitored clinically for at least 24 hours in the Intensive Care Unit after catheter removal. No adverse events related to catheter removal were noted. The CMA71 catheters are not approved in the US (though they are approved in Europe) therefore, a waiver from the FDA was obtained for their use in this investigational context at Washington University.

Microdialysis Sample Acquisition: Sterile human albumin was added to sterile CMA perfusion fluid to a final concentration of 1.5%. Without albumin, no Aβ recovery was obtained (unpublished data) likely due to nonspecific binding of Aβ to the membrane and/or plastic tubing. Several concentrations of albumin were tested; lower concentrations resulted in a net fluid loss across the CMA71 membrane, and higher concentrations resulted in a net fluid gain at 0.3 μl/min. This indicated that at 1.5% albumin, the oncotic pressures were approximately balanced on both sides of the microdialysis membrane. Human albumin is not approved for this use in the US. Therefore, a specific waiver was

obtained from the US FDA to allow its use in this study. Perfusion of this fluid through the microdialysis catheters was initiated immediately after catheter placement and was continued for 3 to 7 days. For all brain injured patients, the flow rate was 0.3 μl/min, and samples were acquired every 1-2 hours in CMA microdialysis tubes. All samples were immediately cooled on ice, and frozen at -80°C within 12 hours of acquisition. In most patients, microdialysis vials were weighted before and after collection in order to ensure appropriate recovery of perfusion fluid. Less than 5% of samples had low volume recovery (<80% of expected) and were not analyzed.

Clinical Assessments, Monitoring and Data Acquisition. Clinical data including Glasgow Coma Scores(*5*), sedation, intracranial pressure measurements, mean arterial pressure measurements, brain tissue oxygen tensions, and cerebral temperatures were recorded by treating nurses in the medical record hourly or bihourly without knowledge of any microdialysis-based information. This clinical data was extracted from the charts and tabulated in a masked fashion. A Licox triple lumen Brain Oxygen Monitoring System was used to measure brain tissue oxygen tension ($P_{ti}O2$) and cerebral temperature in 11 patients. Mean arterial pressure (MAP) transducers were calibrated to zero at the tragus. Intracranial pressure (ICP) was measured via an external ventricular drain calibrated to zero at the tragus in 6 patients, and an intraparenchymal Codman microsensor catheter in 12 patients. ICP, MAP, $P_{ti}O2$, and brain temperature were continuously recorded. The MAP, ICP, $P_{ti}O2$ and temperature data were transmitted to a computer through an analog-digital converter for storage and analysis. Cerebral perfusion pressure (CPP) was calculated on-line as the

difference between MAP and ICP. The mean hourly values of these parameters were calculated and matched with the corresponding microdialysis samples.

Ventricular Cerebrospinal Fluid Sample Acquisition: In 5 of 6 patients with external ventricular drains (2 with traumatic brain injury and 3 with aneurysmal subarachnoid hemorrhage) cerebrospinal fluid was withdrawn every 2 hours for 3 days. Using rigorous sterile technique, an initial aliquot of 1 ml of CSF was withdrawn directly from the drain and discarded. Then a second aliquot of 1 ml was withdrawn to obtain CSF that was in the lateral ventricle, rather than in the drain tubing. The CSF was spun at 5000 rpm for 5 minutes at room temperature and the supernatant was transferred to a new tube. Low protein binding tubes (Axygen) were used throughout. Samples were immediately refrigerated on ice and frozen at -80°C within 12 hours of acquisition.

***In vivo* zero flow extrapolation to calculate Aβ recovery by microdialysis:** Using a CMA61 variable speed pump, flow rates (μl/min) were changed as follows: 0.3, 0.1, 1.0, 0.2, 0.5, 0.3, 0.1, 1.0, 0.2, 0.5. A single 2 hour sample was taken at each step. Each sample was started after the 5 μl of fluid in the tubing (dead volume) from the previous sample had cleared. This required between 5 minutes (at 1 μl/min) and 50 minutes (at 0.1 μl/min). The complete collection protocol required 24 hours. This protocol was performed on one unruptured aneurysm patient who remained in the ICU for observation during the entire protocol. His mental status was entirely normal (GCS 15) throughout.

***In vitro* calibration of Aβ recovery by microdialysis:** After removing catheters from patients, *in vitro* recovery of Aβ was tested by placing the catheter in a vial of cerebrospinal fluid at room temperature. 3 to 5 samples were acquired over 1 to 2 hours each at 0.3 µl/min and immediately frozen at -80 °C. An aliquot of the CSF was taken before and after *in vitro* microdialysis and similarly frozen. *In vitro* recovery was calculated as the mean of the values from each microdialysis sample divided by the mean of the values of the two CSF samples. Additional CMA70 and CMA71 catheters never used in a patient were tested similarly; results were indistinguishable and data have been pooled.

ELISA measurements of Aβ: 96-well plate format ELISAs were used as previously described (*6*). Briefly, samples were thawed on ice, diluted to 100 µl and loaded onto 96-well plates coated with one of the following monoclonal antibodies: m266 ($A\beta_{1-x}$), 2G3 ($A\beta_{1-40}$) or 21F12 ($A\beta_{1-42}$). M266 recognizes amino acids 13-28, 2G3 recognizes amino acids 33-40, and 21F12 recognizes amino acids 33-42(*3*). After incubation, the detection antibody 3D6-biotin was applied followed by streptavidin conjugated horseradish peroxidase (HRP). 3D6 recognizes amino acids 1-5. Colorimetric detection of HRP reaction with TMB at 650 nm was performed using either a Biotek 600FL or Biotek Synergy 2 plate reader. Standard curves were made by performing serial dilutions of synthetic $A\beta_{1-40}$ or $A\beta_{1-42}$ (American Peptide) into CMA perfusion fluid + 1.5% albumin to match the composition of the microdialysis samples as closely as possible. The limit of reliable Aβ assessment by interpolation between valid points on the standard curve was 4.9 pg/ml for $A\beta_{1-40}$ and $A\beta_{1-42}$ assays, and between 1.6 and 4.9 pg/ml for the $A\beta_{1-x}$ assay. The

upper limit of each standard curve was 1600 pg/ml. All samples were diluted between 1:2 and 1:50 in duplicate or triplicate.

Urea colorimetric measurements: The QuantiChrom (BioAssay Systems) colorimetric urea assay kit was used according to the manufacturer's instructions http://www.bioassaysys.com/DIUR.pdf and read at 520 nm. A standard curve of 7 serial dilutions of urea ranging from 0.3125 to 20 mg/dl was run in duplicate with each set of microdialysis samples. Coefficients of variation were <5% for these assays.

Lactate, Pyruvate, Glucose and Glutamate measurements: A commercial bedside kinetic enzymatic analyzer (CMA600) was used with standardization per the manufacturer's instructions http://www.microdialysis.se/public/file.php?REF=da4fb5c6e93e74d3df8527599fa62642&art=110&FILE_ID=20070627100441_1_1.pdf. These measurements were performed for samples from 13 patients.

SUPPORTING FIGURES

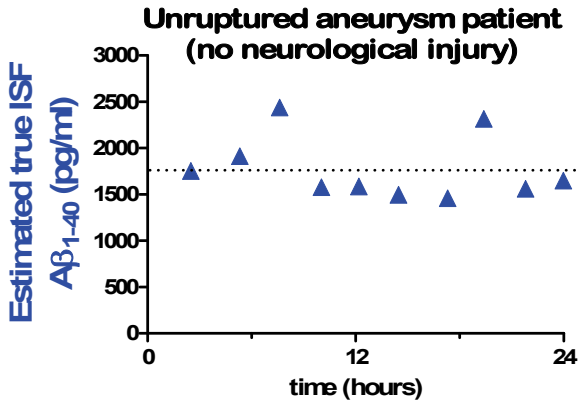

Figure S1: Estimated true ISF Aβ levels in a neurologically normal patient who had undergone clipping of an unruptured cerebral aneurysm. Measured ISF $A\beta_{1\text{-}40}$ levels were corrected for estimated fractional recovery to yield estimates of true ISF $A\beta_{1\text{-}40}$ concentrations. Estimated fractional recoveries were calculated using the zero-flow extrapolation method (see Fig 1C). Overall, estimated $A\beta_{1\text{-}40}$ levels appeared to remain stable throughout the 24 hour monitoring period, as is required to satisfy the assumptions of the zero flow extrapolation method (*7*).

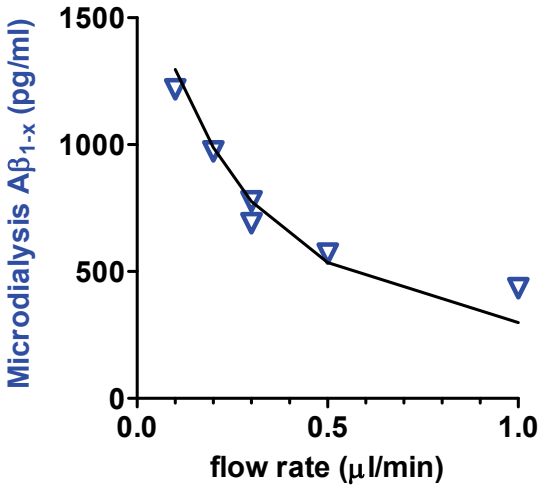

Figure S2: *In vitro* zero flow extrapolation. An unused CMA71 microdialysis catheter was immersed in a vial of previously frozen cerebrospinal fluid stirred continuously at 37°C. Flow rates (µl/min) were varied as follows: 0.3, 0.1, 1.0, 0.2 0.5, 0.3. Single 2 hour samples were acquired at each flow rate. Samples of the original CSF were removed at the start and end of the protocol. Aβ levels in the CSF were stable: 1447 pg/ml at the start of the experiment, and 1362 pg/ml 24 hours later. Zero flow extrapolated value of Aβ from the exponential fit to the microdialysis data was 1435 pg/ml, which agrees remarkably well with the measured values. This confirms the validity of the zero flow extrapolation method for Aβ using this microdialysis system.

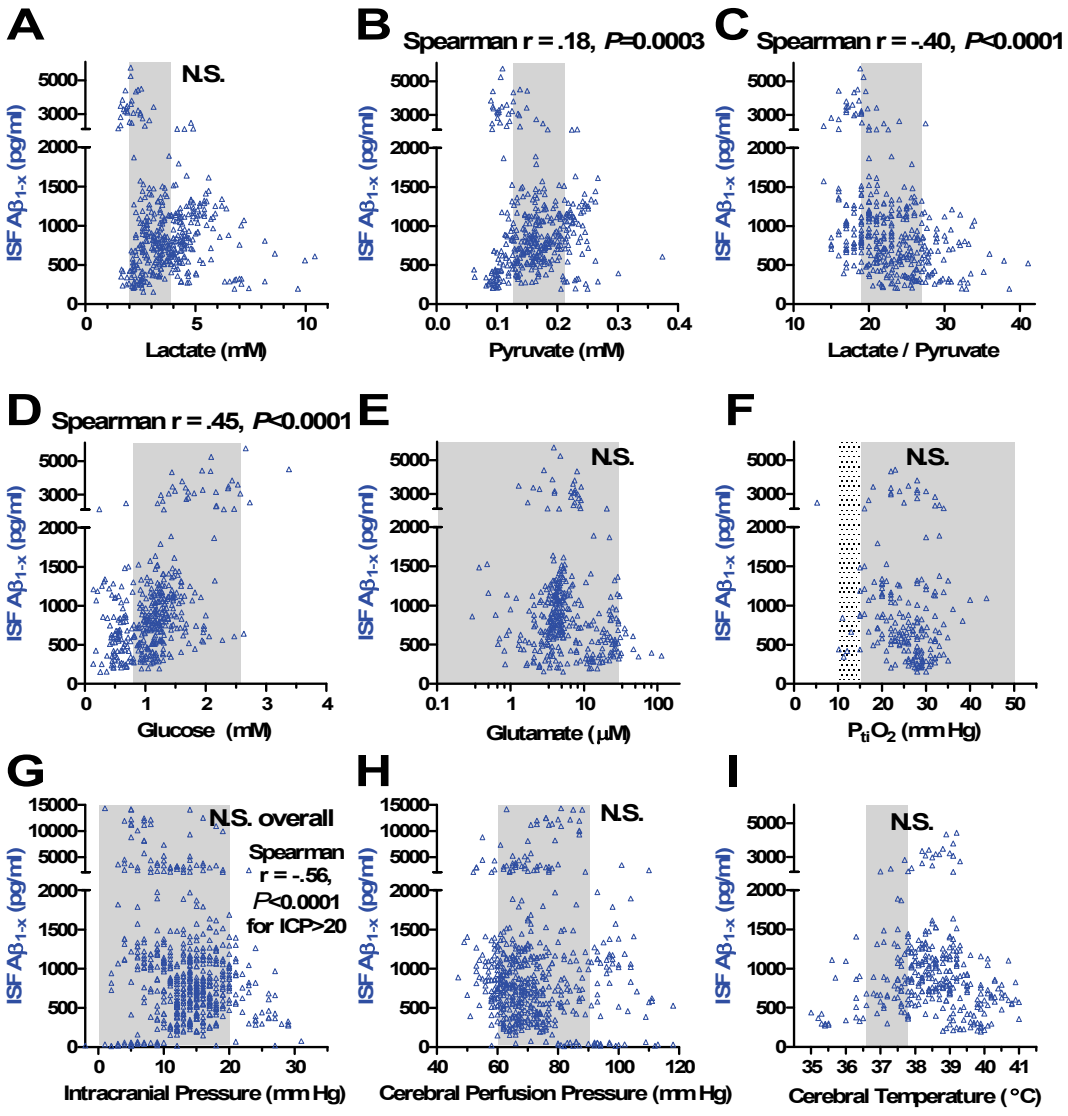

Figure S3: Correlations of brain ISF Aβ with other microdialysis parameters and physiological measures. **A.** No significant correlation of brain ISF Aβ with brain ISF lactate, measured in the same microdialysis samples. **B.** Weak positive correlation of brain ISF Aβ with brain ISF pyruvate. **C.** Moderate negative correlation of brain ISF Aβ with brain ISF lactate/pyruvate ratio. **D.** Moderate positive correlation of brain ISF Aβ with brain ISF glucose. **E.** No correlation with brain ISF glutamate. **F.** No correlation with brain oxygen tension ($P_{ti}O_2$). **G.** No correlation overall with intracranial pressure (ICP) but a

significant negative correlation between brain ISF Aβ and ICP for ICP>20 mmHg. **H.** No correlation with cerebral perfusion pressure (mean arterial pressure – intracranial pressure) **I.** No monotonic correlation overall with cerebral temperature (however, see Fig 3D). Shaded regions represent estimated mean ±1 standard deviation of estimated normal values (*1, 8, 9*).

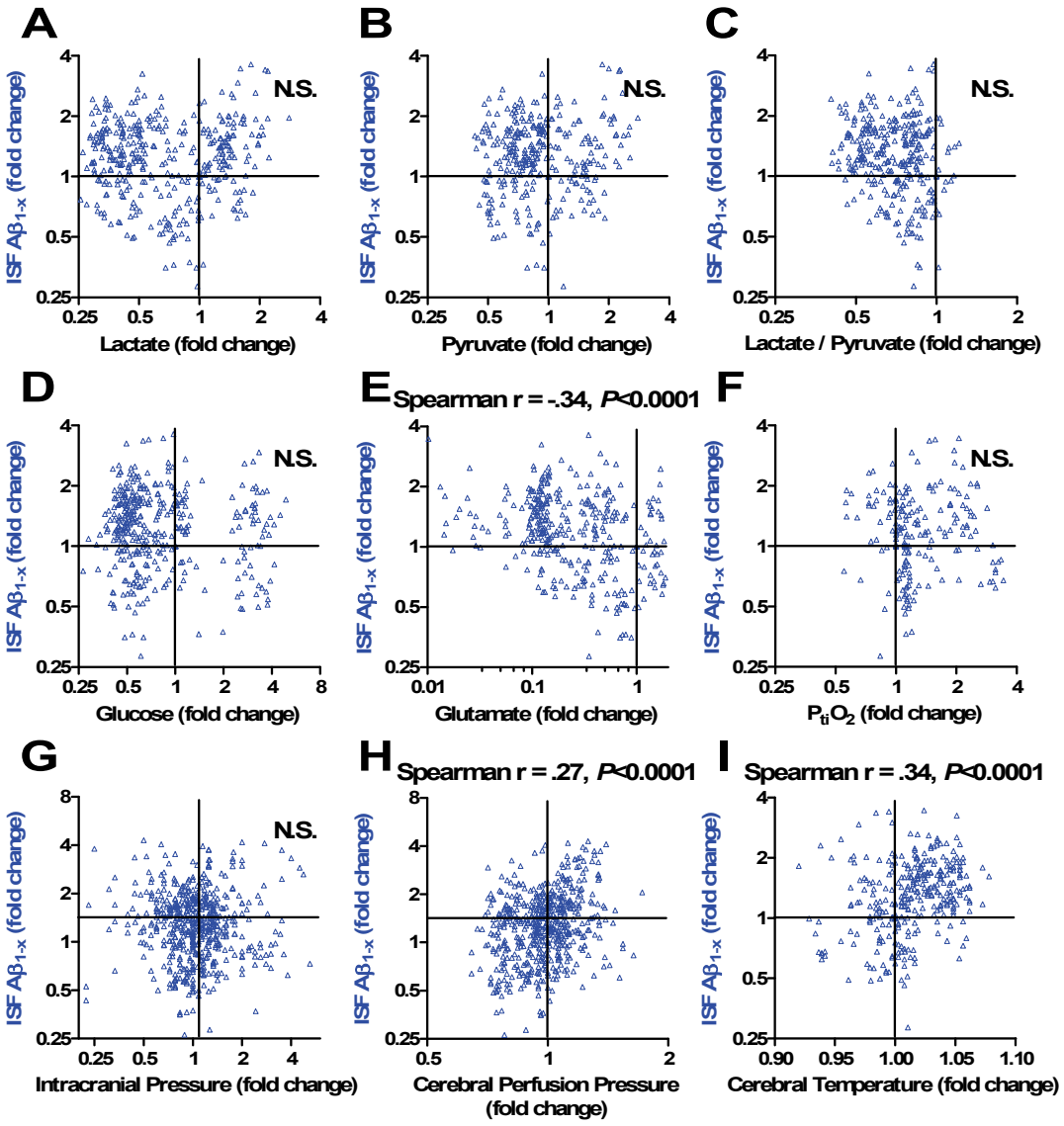

Figure S4: Correlations of relative changes in ISF Aβ with relative changes in other microdialysis parameters and physiological measures.

Fold change in brain ISF Aβ vs. **A.** change in brain ISF lactate, **B.** change in brain ISF pyruvate, **C.** change in brain ISF lactate / pyruvate ratio, **D.** change in brain ISF glucose, **E.** change in brain ISF glutamate, **F.** change in brain tissue oxygen tension ($P_{ti}O_2$), **G.** change in intracranial pressure, **H.** change in cerebral perfusion pressure, **I.** change in brain temperature. On both axes, the values for each parameter are normalized to the initial

values for each individual patient. Thus, values <1 represent decreases over time and values > 1 represent increases over time.

Negative correlations of change in brain ISF Aβ with change in lactate / pyruvate ratio (**C**) and with change in glutamate (**E**) were statistically significant ($P<0.0001$), as were the positive correlations with change in cerebral perfusion pressure (**h**) and change in cerebral temperature (**I**). These correlations were modest, yet overall reflect the trend of rising Aβ levels accompanying normalization of other physiological parameters.

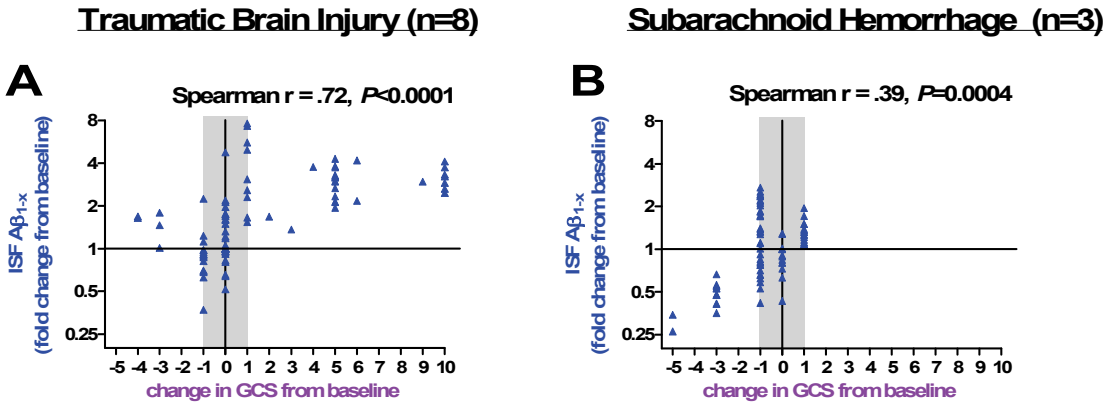

Figure S5: Correlations with brain ISF Aβ levels: analysis of subgroups of patients

A. Correlation of change in brain ISF Aβ from baseline with changes in neurological status across 8 traumatic brain injury patients with catheters placed in normal appearing right or left frontal lobe white matter. A strong correlation (Spearman r=.72, $P<0.0001$) was found.

B. Correlation of change in ISF Aβ from baseline with changes in neurological status across 3 aneurysmal subarachnoid hemorrhage patients with catheters placed in normal appearing right or left frontal lobe white matter. A more modest (Spearman r=.39) but still statistically significant correlation ($P=0.0004$) was found.

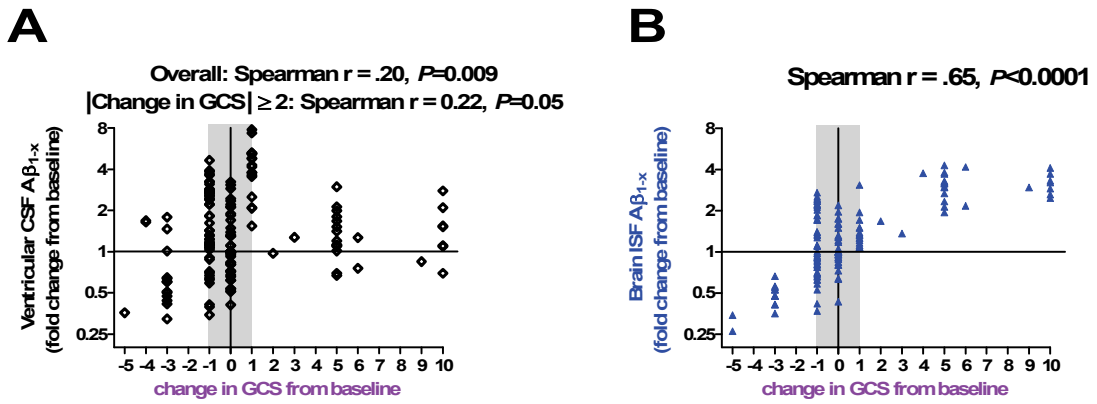

Figure S6: Ventricular CSF Aβ and clinical status. A. Correlation between changes in ventricular CSF Aβ levels and changes in neurological status, as reflected by GCS. Results were statistically significant, but weaker than for brain ISF Aβ (Fig. 4e). **B.** Because fewer data points were available for the CSF analysis than for the analysis of brain ISF Aβ, we reanalyzed a subset of brain ISF Aβ data from 5 patients where ventricular CSF was acquired concomitantly with ISF; in this subset the relationship with neurological status remained robust. The two correlation coefficients (r=.20 for ventricular CSF Aβ, r=.65 for brain ISF Aβ) were significantly different from each other (P <0.0001, difference test). All 5 patients had microdialysis catheters placed in normal appearing frontal white matter and external ventricular drains placed in the right frontal horn of the lateral ventricle.

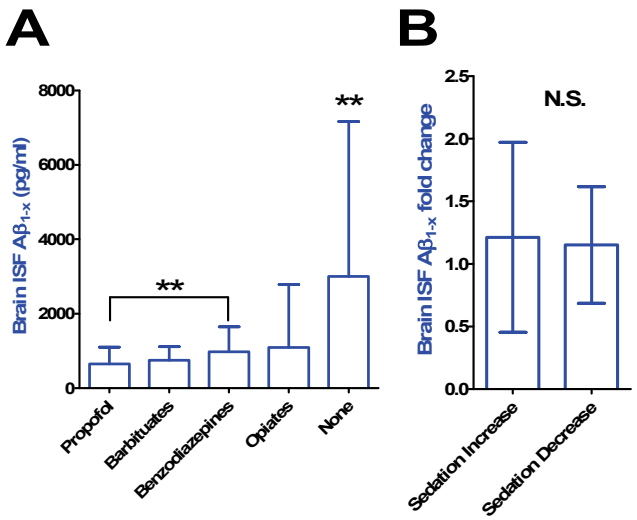

Figure S7: Sedating drugs and brain ISF Aβ levels. Charts from all patients were reviewed and the information was extracted regarding administration of all sedating drugs in time intervals corresponding to the microdialysis collection intervals. **A.** Comparison of several types of sedation. Overall, brain ISF Aβ levels were highest when patients were not sedated (**P <0.005, ANOVA followed Bonferroni pairwise post-hoc testing). Sedation with propofol was associated with lower Aβ levels than sedation with benzodiazepines (** P =.00025). However, sedation was not varied systematically; less severely injured patients typically received less intensive sedation, and the choice of agents was left to the discretion of the treating physicians. **B.** There was no difference in the fold changes in Aβ associated with times when sedation was increased vs. times when sedation was decreased (P=0.66). Ratios are slightly greater than 1 because brain ISF Aβ in general was rising over time. When this analysis was restricted to changes in short-acting agents (primarily propofol, midazolam, and fentanyl), the results were essentially unchanged (P=0.42).

Chi square analysis of the direction of change in Aβ (increase vs. decrease) similarly revealed no differences: There were 22 increases in sedation associated with in an increase in brain ISF Aβ, 16 increases in sedation associated with a decrease in Aβ, 27 decreases in sedation associated with in an increase in Aβ, and 18 decreases in sedation associated with a decrease in Aβ (P=0.85). Thus, overall, there did not appear to be a strong relationship between the adminstration of sedative drugs and measured brain ISF Aβ.

SUPPLEMENTAL DISCUSSION

The results presented here raise several questions that may be addressed in ongoing and future studies. The $A\beta_{1-x}$ ELISA used for most of the measurements detects Aβ species from amino acid 1 to amino acid 28 or greater. Thus, many possible Aβ species are being measured in aggregate. Clearly, further work will be required to determine the full range of Aβ species present and their relative concentrations in human brain ISF. Oligomeric forms of Aβ have not yet been detected in microdialysis samples or ventricular CSF from these patients. Determination of whether these oligomeric Aβ species are present, and if so at what concentrations will be particularly important, given their reported potent neurotoxicity (*10-12*). Due to the small sample volumes obtained, the development of valid methods to assess the oligomerization state of Aβ in the human extracellular space by microdialysis will be a technical challenge.

It is possible that Aβ levels near the catheter are altered due to the tissue deformation induced by the catheter placement. The catheters used are 0.6 mm in diameter and quite flexible, so major tissue deformations are unlikely. Nonethelesss, a contribution

of tissue deformation-related Aβ alterations cannot be entirely ruled out based on the data presented. However, it is likely that this is not a major contributor, as the dynamic range of the changes in Aβ is quite large relative to the Aβ baseline: up to 4-fold increases and decreases (e.g. Fig 4E). Thus, if there are tissue deformation-related effects on Aβ levels, they are likely to be small compared to the Aβ dynamics associated with changes in neurological status. In addition, our microdialysis measurements of local brain metabolic parameters (glucose, lactate, pyruvate) stabilize within 2-3 hours after catheter implantation, as has been reported by others (*13*). This indicates that most effects of local tissue injury or tissue deformation are likely transient, and contribute little to our results.

Ongoing experimental microdialysis studies in a mouse model of traumatic brain injury (*14*) may complement the results presented here. Specifically, this will allow measurment of pre-injury and early post-injury ISF Aβ levels, and facilitate direct comparison of tissue homogenates with microdialysis samples. Furthermore, in future studies, intracerebral microdialysis measurements can be compared directly between experimental animals and human patients to address questions regarding the validity of experimental animal models.

Measurements of substances that change little in concentration over time can be used to control for changes in microdialysis catheter function. The measurement of urea in the same samples as Aβ provided an important control for such changes in microdialysis catheter function. Urea is not produced in the brain, diffuses readily through all tissues, and generally does not change rapidly in concentration. It has therefore been used as a control for the stability of the microdialysis catheter function (*15*). Additional controls involving substances of similar size and hydrophobicity to Aβ would be useful.

A potential clinical application of this work is that changes in brain ISF Aβ levels could provide advance notice of impending secondary insults, or provide reassurance that a course of therapy is having the desired effect. This approach could be used in a complementary fashion with other methods of monitoring (*16*).

Likewise, this microdialysis-based technique could be used for future pharmacodynamic measurements of the effects of various drugs or treatments on brain ISF Aβ levels. Pharmacodynamic studies could be performed in the intensive care unit in selected patients during periods when neurological status is relatively stable. During such periods, brain ISF Aβ levels overall are also likely to be relatively stable in the absence of intervention. Even studies involving a small number of human subjects could provide critical proof-of-concept data for Aβ-targeted therapeutics for Alzheimer's disease. Analogous microdialysis-based procedures could also potentially be used to measure many additional small proteins such as tau, neurofilament light chain, ubiquitin, prion protein, and alpha-synuclein that play key roles in other prominent human diseases.

AUTHOR CONTRIBUTIONS

DLB and DMH conceived the project. DLB and SM obtained regulatory approval for human subjects research and were responsible for informed consent. SM, GJZ, and NS recruited patients. GJZ placed all microdialysis catheters at Washington University. SM and KES supervised collection of samples. MS and TJE cataloged samples and performed Aβ ELISAs and urea assays. DLB, KES, and SM collected clinical data. DLB performed statistical analyses and made all figures. DLB wrote the paper with important contributions from SM, NS and DMH.

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