GFAP Degradation in TBI: Linking Novel Modified Products to Astrocyte Pathology and Patient Outcomes

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Abstract

Monitoring patients with traumatic brain injury (TBI) is essential for mitigating secondary complications. TBI disrupts proteostasis, leading to increased protein degradation and proteinopathy, which heighten the risk of neurodegeneration and underscore the need for early detection via noninvasive biomarkers. Glial fibrillary acidic protein (GFAP), an astroglial intermediate filament, is a well-established TBI biomarker, yet pathomechanisms and proteoforms of GFAP release are incompletely understood. This translational study defined the GFAP degradome in TBI through mass spectrometry (MS) sequencing of immunopurified GFAP breakdown products (BDPs) from patient biofluids. The TBI-GFAP fragments differed from those of Alzheimer's and Alexander Diseases, revealing novel coil1/coil2-harboring cleavage products in TBI CSF, while TBI serum samples had only coil1-products. Trauma-specific citrullinations and acetylations were validated via MS spectra. Epitope mapping identified a novel trauma-specific cleavage site and revealed coil1-BDPs as selectively released into fluid, while coil2 fragments remained intracellular in a human trauma culture model. Citrullinated GFAP-specific antibodies exhibited distinct binding patterns from those of unmodified, native GFAP in TBI patients' CSF and detected unique granular aggregates in dystrophic astrocytes without staining filamentous GFAP. TBI-GFAP fragments were produced by stepwise trauma-activated calpain and caspase activities shown by in vitro inhibitor studies. Live cell imaging with fluorescent protease reporters demonstrates coexisting calpain and caspase activities, along with membrane disruption jointly identifying distinct astrocyte injury states. The trajectory of GFAP proteolysis was analyzed in CSF samples from 24 TBI patients over 10PIDs, via calibrated, band-specific scaled immunoblot densitometry. These profiles covaried with patient age and body temperature. Full-length GFAP and 45-49kDa fragments peaked on injury day and declined over time, whereas calpain-generated 37-39kDa BDPs remained elevated. Small BDPs exhibited either delayed rises or remained undetected. Compared to the 20-26kDa BDPs, 15-19kDa products were notably underrepresented, consistent with coil1-BDPs' preferred release and coil2-BDPs' selective cellular retainment. Fluid levels of calpain-generated GFAP fragment were time and severity-dependent in the trauma culture model. TBI CSF trajectories of GFAP-BDPs differentiated TBI patients with poor versus those with good outcomes on the extended Glasgow Outcome scale (GOSE), whereas uncleaved GFAP trajectories failed in early prediction of patient outcome at six months. These findings define trauma-specific GFAP degradome dynamics and highlight novel longitudinal proteolysis profiles for patient monitoring and stratification. This work is significant as it supports GFAP fragment profiling for potential context of use (COU) in neurocritical care TBI monitoring, and offers preliminary insights into diagnosing astroglial proteinopathy-linked neurodegeneration after TBI.

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Running title: GFAP degradome and astrocyte pathology in TBI

Data availability

The data masterlist comprised of de-identified TBI patient clinical variables and outomes, CSF collection and all GFAP band-specific measurements of the DANA study cohort are uploaded to the Open Data Commons for Traumatic Brain Injury, Wanner lab and is accessible on ODC-TBI with Data dictionary here:
https://odc-tbi.org/lab/dataset?labid=387&datasetid=1432

Introduction

Traumatic brain injury (TBI) is the most common neurological disorder surpassing Alzheimer's disease and stroke with a global prevalence of 56 million cases annually[1]. A rigorous meta-analysis showed that 18 % of the adult US population suffered a TBI with loss of consciousness during their lifetime[2]. Among survivors, 53% of mild TBI and 85% of moderate-to-severe TBI patients fail to achieve long-term recovery[3,4]. TBI recovery is profoundly shaped by secondary injury mechanisms, as an initial trauma disrupts cerebral homeostasis, which amplifies neural and biochemical dysfunction[5,6]. Effective monitoring of patients with TBI is therefore critical to minimize exacerbation and attenuate secondary injury processes. Although intracerebral catheters provide valuable information, their placement is invasive and reporting remains focal and is reserved for patients undergoing surgical intervention[7,8]. Thus, developing biomarkers for wide use to noninvasively monitor, evaluate, and improve outcome prediction in a broad spectrum of TBI patients is of high interest[9].

The FDA has approved a blood test that includes glial fibrillary acidic protein (GFAP) for use within 12 hours (hrs) post-injury to detect brain abnormalities typically identified by CT scan among patients with mild TBI Glasgow Coma Scale score of 13-15[10,11]. This narrow context of use (COU) excludes a wider population of TBI patients and does not address longitudinal patient monitoring[12,13]. To improve TBI patient evaluation, the National Institute of Neurological Disorders and Stroke (NINDS) has recently introduced a new TBI Classification framework that integrates biomarkers such as GFAP, and other assessment tools[14,15]. Effective broader use of GFAP to inform and guide future clinical applications for patient monitoring and stratification requires an understanding of GFAP's fate during TBI pathophysiology[16]. Two key interrelated questions remain: (1) What are the pathobiological underpinnings of GFAP signals in TBI? (2) What is the temporal dynamic of GFAP release and degradation following TBI?

The astroglial intermediate filament protein GFAP is highly enriched in the brain and is well studied, both as a biofluid marker and as a neuropathology indicator of reactive astrogliosis. GFAP levels are elevated in CSF and blood in patients with intracranial injuries such as hemorrhage, as observed in brain CT scans[10,17,18]. Elevated serum GFAP levels have been shown to predict poor outcome and mortality after TBI[9,19-21]. Therefore, GFAP is extensively investigated as a diagnostic and prognostic biomarker for TBI evaluation in clinical trials and translational studies. GFAP levels in biofluids are transient, rise within hrs after injury, reach their peak 6-18hrs post-injury, and then decline[21-24].

It is not well understood how a filament-tethered protein appears rapidly in circulation and how proteases shape GFAP fluid profiles. This requires integrating longitudinal biochemical analysis of GFAP degradation and post translational modifications (PTMs) with insights into cellular injury states[16].

In this translational research study we determined novel proteolytic breakdown products (BDPs) of GFAP and trauma-induced PTMs in TBI patients. Using a unique human cerebral trauma culture model we link these trauma-proteoforms to astrocyte injury states and trauma-activated proteases. Mass spectrometry (MS) of immunopurified GFAP fragments from TBI patient CSF identified,

for the first time, a set of TBI-generated GFAP BDPs with citrullination and acetylation modifications. Some groups of GFAP BDPs were preferentially released while others were selectively retained. We tracked longitudinal GFAP degradation patterns in Neuro-ICU patients and demonstrate fragment-specific correlations with six-month outcomes. This work conceptually connects GFAP biofluid patterns to an underlying non-filamentous proteinopathy, to astrocyte injury states, and neurodegeneration. These new insights into GFAP degradation, modification, and fate of specific GFAP proteofoms support a future for their broader clinical use.

Materials and methods

TBI patients, demographic and clinical data and brain tissue specimen.

The study comprised 112 CSF samples and 3 serum samples from 26 TBI patient and one control (Precision Med), average age 42±19, 22 male and 4 female. Their Glasgow Coma Scale (GCS) averaged a score of 6.6 ±3.4 along with anonymous demographic and clinical parameters (Suppl.Tables1,2). Human subjects protocols for the DANA foundation funded cohort included local IRB approved informed consent procedures from three medical centers (1) UCLA Ronald Reagan Hospital Neuro-Intensive Care Unit (Principal Investigator, PI Dr. Paul Vespa) and (2) University of Florida Dept of Anesthesiology (PI Dr. Steven Robicsek). Deidentified human fetal brain tissue from voluntarily aborted pregnancies was donated adhering to stringent ethical regulations. They were pre-shelved (exempt from IRB-review) at the UCLA Translational Pathology Core Laboratory (TPCL; tissue procurement by Dr. Sara Dry). Consent was established through UCLA Family Planning Services, Dept of OB-GYN (Dr. Angela Chen). Signed informed consent occurred after decision on elective termination, complying with US Public Law 103-43 and NIH policy, ensuring no incentivizing (The Public Health Service Act, 42 U.S.C. § 289g-2). Cerebral astrocyte cultures were prepared from 17 donated human fetal cranial specimen aged 16-19.5 gestational weeks.

Immunoprecipitation and mass spectrometry (MS) sequencing of GFAP from TBI patients.

MS experiments were performed on six CSF and serum samples from five TBI patients. GFAP was immunoprecipitated using a rabbit polyclonal anti-GFAP antibody that was cross-linked to protein G conjugated magnetic beads (Dynabeads) using dimethylpimelimidate-2 (freshly prepared 20mM DMP in 200mN triethanolamine, ph8.6) followed by heat/ice denaturing and reduction in 1% ß-mercaptoethanol to maximize yield[25-27]. Eluted material was separated by preparative SDS-PAGE using 10% NuPAGE gradient gels in morpholinoethansulfonic acid (MES) buffer in reducing conditions along with a molecular weight standard (Benchmark). Gels were Sypro-Ruby stained and fixed in 10% methanol, 7% acetic acid. GFAP bands were cut out under UV light and placed into tubes for in-gel trypsin digestion. A fraction (~1/9th) of the eluate was

blotted and GFAP was detected using a mouse monoclonal GFAP antibody (mab1A64-244, Abbott Diagnostics or MCA3E10, EnCor Biotechnology Inc.) to provide reference signals for gel band excision. Bands at 50 and 25kDa were omitted to avoid immunoglobulins (Suppl.Fig.1). Gel bands were fully destained, dried, and then reduced with 10 mM tris(2-carboxyethyl) phosphine in 100 mM ammonium bicarbonate buffer at 56°C for 30min followed by alkylation with 100 mM iodoacetamide in the same buffer at 37°C for 15min. The bands were equilibrated with acetonitrile (ACN), dried, and digested using 0.01mg/ml sequencing grade trypsin in 100mM ammonium bicarbonate buffer at 37°C for 12hrs. Peptides were extracted from the gel bands by three rinses of 50% ACN/0.1% formic acid solution at 37°C for 15 min and the combined extracts were dried in a vacuum concentrator. The peptides were measured by liquid chromatography-tandem MS (LC-MS/MS) using an Ultimate 3000 nano LC instrument (1hr gradient) coupled to a Q-Exactive Plus Orbitrap mass spectrometer. Full spectra (MS1) were collected at a resolution of 70,000 and the 15 most abundant ions of each scan were subsequently fragmented by higher-energy collisional dissociation (HCD; MS2) with a resolution of 17,500 and a dynamic exclusion of 45 seconds. Varied amounts of recombinant human GFAP were employed as technical controls. Raw MS data were searched against the human GFAP protein sequence (Uniprot (https://www.uniprot.org/uniprotkb/P14136/entry) by Proteome Discoverer (PD) version 1.4. SEQUEST-engine searches of the peptides confirmed that the sequences are unique to GFAP, excluding other proteins (e.g., vimentin, neurofilaments). GFAP sequence map regions marked green show "high" confidence filtering with at least two peptides per region and ≤1% false discovery rate (FDS) correction for Xcorr Score versus charge; while only few regions marked yellow were detected by at least two "medium" confident peptides with ≤5% FDR (Figure 1). Identified PTMs including acetylation and citrullination were further validated by: (a) unique signatures of citrullination (immonium ion at m/z 130.0975 and/or neutral loss of isocyanic acid at 43.0058 Da) or lysine acetylation (cyclized immonium ion at m/z 126); (b) MS2 product ions (b/y) revealing mass increments consistent with modification (Suppl.Fig.9); (c) comparison of identified citrullination sites after treatment with peptidylarginine deiminase (PAD) enzyme[28].

Partial chemical cleavage of GFAP for monoclonal antibody epitope mapping.

The binding region of monoclonal anti-GFAP clone 5C10 was determined initially by binding to the peptide covering amino acids 233-261 of the human coil 2A sequence (AALKEIRTQYEAMASSNMHEAEEWYRSKF) using dot blotting (not shown). The specific epitope was subsequently identified using partial chemical cleavage at the single tryptophan in GFAP, amino acid W256. Recombinant GFAP (33µg, EnCor Biotechnology Inc.) was treated in excess with 1% BNPS-Skatole (100µg; 1mg/ml glacial acetic acid; 3-bromo-3-methyl-2-(o-nitrophenylsulfenyl)indolenine, Sigma), an oxidant and brominating agent that cleaves proteins at tryptophan residues[29,30]. An equal volume of distilled water precipitated BNPS-Skatole and was removed by centrifugation (5min at 12,000rpm). The supernatant was lyophilized and the GFAP and tryptophan-cleaved BDPs were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) blotted followed by antibody incubation of MCA-5C10, MCA-3E10, a coil1 antibody, and

a pan-intermediate filament antibody, IFA (hybridoma supernatant from ATCC, epitope coil2B aa 358-377: ALDIEIATYRKLLEGEENRI; see Suppl.Fig.5)[31,32].

Western blotting using scaled densitometry and biomarker calibration.

SDS-PAGE was conducted as previously described using 7.5% acrylamide for αII-spectrin, 12% gels for GFAP[23]. Protease inhibitors (cocktail CompleteMini, Roche 1:50) and EDTA to a final concentration of 10mM were added to TBI patient CSF and the samples were added to Laemmli sample buffer, boiled for 5min followed by ice incubation and addition of ß-mercaptoethanol to 1%, prior to loading 34 µl/lane. The same procedure was followed for astrocyte lysates and concentrated media was prepared as described below. Samples and molecular weight standards (Kaleidoscope, Biorad) were collected upon initial run for 30min at 100V and separated for 1h at 120V until the bromophenol blue front reached the gel bottom. Proteins were cold transferred at 120V for 3hrs onto nitrocellulose and stained with Ponceau S. Membranes were blocked in 10% non-fat milk in Tris-buffered saline with Tween (TBST) at room temperature (RT) prior to incubation with primary antibody in 5% bovine serum albumin (BSA) in TBST overnight at 4°C. Secondary antibodies conjugated with peroxidase (Pierce) were incubated in 5% BSA in TBST for 1hour at RT. Enhanced Chemiluminescence (ECL) substrate was applied to washed membranes for 5min at RT (Pico and Femto ECL)and multiple exposures of Kodak films as well as an iBright imager were used. Calibrants were recombinant human full-length GFAP and a 20kDa His-tagged BDP (72-217, EnCor Biotechnology Inc.) that were diluted with 0.5% BSA as carrier in Tris-buffered saline (TBS) at various concentrations ranging from 0.08ng-2.6ng that were measured at different exposures (Suppl.Fig.11). Densitometry was done on eight exposures of each blot (2s-20min) after background correction. Sub-saturated band measurements were standardized to 1min by multiplying shorter exposures and by dividing longer exposures using scaling factors between exposures that were empirically determined from same unsaturated bands found on two exposures (Suppl.Table 5). This stepwise scaling corrected for limitations of enzyme-substrate reaction and film saturation and expanded the detection range continuously over 5 orders of magnitude. Band sizes were computed using distances of each molecular weight marker from the gel-front and curve-fit equations.

Human neocortical astrocyte cultures, trauma, microscopic imaging.

This human culture trauma model uses in vitro differentiated fetal neocortical astrocytes as previously described[33]. Briefly, neocortical tissue was mechanically dissociated, filtered and astrocytes were enriched by Percoll gradient centrifugation. Cells were expanded in DMEM/F12 with 10% fetal bovine serum (FBS). Residual oligodendrocytes and progenitor cells were removed by shaking. Astrocytes were seeded on collagen I coated deformable silastic membranes at 138,000-180,000 cells/9.62cm^2 and differentiated in DMEM/F12: Brainphysiology Medium supplemented with SM1 and N2A (Stem Cell Technologies) by stepwise serum removal. Astrocytes were traumatized by 50ms pressure pulses (4.9-5.3psi) that abruptly stretched the

culture bottom, shearing the cells. This inflicts membrane integrity compromise and delayed cell death[23].

Live microscopy imaging.

Cerebral fetal human brain cells were differentiated for three weeks by serum withdrawal in a 1:1 mixture of DMEM/F12: brainphysiology medium (StemCell Technology). Live imaging was done 5hrs after stretch injury on a Leica DMI8 microscope in a gas and temperature-controlled chamber (Okolab). Cells were bathed for 5min in 60µM propidium iodide (PI, Sigma) to label leaky membranes. Washed cultures were live-imaged in the presence of two permeable fluorescent protease substrate reporters for calpains (30µM CMAC tBoc Leucyl-methionine, ThermoFisher), and for caspases (5µM DEVD-NucView488, Biotium).

Astrocyte protein sample preparation for gel electrophoresis.

Whole cell lysate (WCL) of adherent astrocytes was prepared by adding 150µl lysis buffer/9.62 cm^2 wells (150mM NaCl, 20mM Tris, 5mM EDTA, 10mM NaPyrophosphate, 30mM phosphatase substrate, 5mM dithiotreitol, DTT (Calbiochem)), 1% NP40 and 1 tablet/10ml protease inhibitors (Mini Protease Inhibitor Cocktail, Roche). Lysis buffer was incubated for 5min at 4°C, collected by scraping into low binding tubes and homogenized by 3x5sec sonification bursts without foaming (Branson Digital Sonifier, 90% or constant amplitude duty cycle, output level 5). Culture medium without serum or protein supplements was collected and 5mM DTT and 1tablet protease inhibitors /10ml added. Particles from lifted cells and debris were pelleted by initial centrifugation (5000g, 5min, 4°C) then lysed for 5-10 min in 0.5% lysis buffer and sonicated 3x5sec. Remaining insoluble fractions were removed by centrifugation (10min at 16,000g 4°C). Media were concentrated (Vivaspin, 3kDa cutoff) by centrifugation (4-8h at 5000g, 4°C) to ~1/10th-20th of original volume to obtain 1-1.5 µg/ml protein concentration. All samples were immediately frozen at -80°C in aliquots.

Antibodies used on TBI CSF and astrocyte samples.

All antibodies used with their known epitopes, sources and dilutions are tabulated with approved RRIDs (Suppl.Table3). A rabbit polyclonal GFAP antibody (DAKO) was used for immunoprecipitation and immunoblotting. For densitometry the antibody was applied in two steps, high dilution (1:20,000-150,000) for detecting full-size GFAP and large GFAP BDPs, followed by a higher concentration (1:5,000-7,000) for detecting small GFAP products followed by scaling for adjustment (see below). A mouse anti-GFAP monoclonal antibody, MCA-3E10 (EnCor Biotechnology Inc.), was generated to a recombinant construct containing amino acids 72-217 of human GFAP, the entire coil1A and 1B sequence. The epitope for MCA-3E10 was localized to amino acids 196-210 (EIRFLRKIHEEEVRE), using peptide competition with a set of nested 20 amino acid peptides, each overlapping by 5 amino acids. Additional antibodies clones were MCA-5C10 (EnCor Biotechnology Inc.), mab1A64-244 (Abbott Diagnostics), a mixture of three clones (4A11, 1B4, 2E1, BD Pharmingen) and mabGA5 (Sigma[34,35]). Citrullination studies used clone CTGF-1221 that was kindly provided by Dr. Akihito Ishigami (Tokyo Metropolitan Institute for

Geriatrics and Gerontology) to detect citrullinated GFAP at R270 and R416[36]. Anti-peptidyl-citrulline antibody clone F95 (EMD Millipore) validated citrullinations. Several additional rabbit polyclonal citrullination site-specific GFAP antibodies, including cit-GFAP-270, were generated and characterized (BRAINBox Solutions, Inc.). A mouse antibody to αII-spectrin was used at 1:3,000-1:5,000 dilution (Enzo Life Sciences).

Protease inhibition of traumatized human astrocytes.

Pan-calpain inhibitor calpeptin (Z-Leu-nLeu-H, Millipore/Sigma), a cell permeable peptide aldehyde inhibitor[37,38] was specific to calpain 1 without introducing toxicity, and was used at 13μM. Pan caspase inhibitor ZVAD-FMK (Benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (Enzo/ThermoFisher) is a cell-permeable, irreversible pan-caspase inhibitor effective against a spectrum of caspases (1-10, except 2; Invitrogen)[39]. ZVAD-FMK is reported showing also calpain inhibition after controlled cortical impact to the mouse brain[40]. Here ZVAD-FMK was used at 11μM. Inhibitors were applied 10min to cultures after stretching in the absence of gentamicin in the medium for 5, 24 or 48hrs and were replenished every 8-10hrs. Other tested calpain inhibitors such as leupeptin (20μM) and SJA6017 (10μM Calpain Inhibitor VI, Calbiochem) had limited effect, or were harmful by either increasing cell permeability as did PD1500606 (100μM, Calbiochem) or elevating cell death as did MDL28170 (Z-Val-Phe-al, 4μM, Calbiochem; data not shown) and were not further pursued.

Statistical analysis

All GFAP band measurement residuals were assessed for normality by the Shapiro Wilk criterion for Gaussian distribution fit. Due to significant lower W-values, all GFAP measurements were log-transformed replacing zero with half of the lower limit of detection, which was 0.7pg in 22μl CSF or a concentration of 27pg/ml using a polyclonal GFAP antibody.

Reproducibility of immunoblot densitometry was determined on technical replicates using the coefficients of variation (CV=SD/mean x100). The average between-experiment CV was 27.8% (n=6 pairs of multiple scaled measurements, specifically 37% for full-length GFAP, 21.5% for fragment bands and 25% for 37kDa band).

Covariates: Patient age, race, sex, and concurrent body temperature and their interactions as well as temperature interaction with postinjury elapsed time were evaluated as potential covariates for GFAP levels by using restricted maximum likelihood (REML) linear mixed model (LMM) with patient identifiers (IDs) as random effect and knotted spline modeling of injury elapsed days (Suppl.Fig.12 for data distribution). Partial omega squared (ω^2) was used to report the proportion of population variance fixed effects as it adjusts for variable sample size (age effect shown for GFAP bandsets in Suppl.Table6)[41]. It was computed using fixed effect F-statistic (F) results of the REML LMM output in SAS/Jmp along with degrees of freedom (df) and its demoninator term (DFDen) for the respective covariate effects following: (1)

$$\omega^2 = \mathrm{F} * \mathrm{df}_{\mathrm{effect}} - (\mathrm{df}_{\mathrm{effect}}/\mathrm{F} * \mathrm{df}_{\mathrm{effect}}) + \mathrm{DFDen} + \mathrm{df}_{\mathrm{effect}}. \quad (1)$$

For Glasgow Outcome Scale Extended (GOSE), demographic covariates were assessed and included in the linear mixed model was patient age as it was covariate for GFAP levels and GOSE outcome.

Temporal profiles: Longitudinal means were graphed by spline fit lines from resampled daily CSF draws, weighting observations, along with 95% confidence intervals (Figs.7C,8). Temporal GFAP and fragment trajectories were evaluated using REML-LMM with age and concurrent body temperature as indicated (Suppl.Table6 and Suppl.Fig.13). Cohen's D effect sizes on each postinjury day (PID) were computed using adjusted means (Adj.Mean) on each PID compared with overall means weighted for sample availability on each day[42,43] (2).

$$\text{Cohen's } D_{\text{forGRAP by PID}} = (\text{Adj.Mean}_{\text{PID}} - \text{Adj.Mean}_{\text{Overall}})/\sqrt{pooled}\ SD \quad (2)$$

In (2) the pooled standard deviation (SD) was the random variance component[44].

Multivariate correlation matrix. To assess GFAP substrate-product and product-product relationships, log-transformed GFAP bands were pairwise correlated reporting uncorrected Pearson correlation coefficients (Figure 7, Suppl.Fig.14). Biplots use orthogonal fit for equal variance between GFAP bands were reported with R^2 and false discovery rate (FDR)-corrected Logworth, $-\log_{10}$(p-value) to differentiate highly correlated GFAP bands (Fig. 9). Outlier portion is documented with robust correction defined as percentage of datapoints with a distance exceeding 3X the residual SD. Outliers that might affect the correlations across all variables were reported using the Mahalanobis distance with threshold (upper control limit, UCL) calculated using sample size and the number of variables used, by applying robust weighting to better discern outliers[45-47].

Outcome prediction: The GOSE score was used to assess six months recovery prediction by longitudinal GFAP profiles. GFAP trajectories are shown for good (GOSE 5-8), and poor (GOSE 1-4) outcomes using bootstrapped cubic splines with same curvature fit and 95% confidence bounds $\lambda = 0.28$ (Figure 10). GFAP profiles were assessed for distinguishing good versus poor outcome using REML-LMM model with ID as random and elapsed PIDs with knotted splines as fixed effects, adding age as joint covariate. Differences in GFAP Adjusted Means between good and poor recovering patients were computed using (3).

$$\text{Cohen's } D_{\text{forGFAP by outcome}} = (\text{Adj.Mean}_{\text{GOSE poor}} - \text{Adj.Mean}_{\text{GOSE good}})/\sqrt{pooled}\ SD \quad (3).$$

Cohen's D in standard deviational (SD) units here uses the residual variant component capturing within-subject variability (Suppl.Table7). Inserts in Figure 7 show least square means model adjusted means with standard errors and post-hoc t-statistics and patient ID as random effect and time as fixed effect.

Label-free MS analysis used A) Peptide presence/absence frequency was compared between coil1 and coil2 peptides using a mixed model with unequal variances, patient ID as random and GFAP-BDP sets as fixed effects. B) The peak ion abundance of coil1 versus coil2 ions was compared using a mixed model analysis with ID as random and fragments as fixed effects while undetected peptides were excluded for peak heights (Suppl.Fig.3).

Results

Novel set of trauma-specific GFAP breakdown products (BDPs) in TBI patients

GFAP fragments in TBI patients differ from Alzheimers and Alexander Disease BDPs. As GFAP shows many degradation products and TBI facilitates proteolysis, we interrogated TBI-specific GFAP degradation by MS sequencing fragments from gel-slices of immunopurified GFAP of six CSF samples/five TBI patients covering key molecular weight ranges aligned with matching immunoblot GFAP signals (Suppl.Fig.1, Bandsets I-IV). Molecular masses were estimated after pooling slice-specific sequences from all patient samples based on high confidence identified tryptic peptides covering the majority of the fragment sequences in the identified GFAP-BDPs (Fig.1B). Band I contained a 41.7kDa fragment. Band II gel-slice ran between 36-39kDa yet sequenced peptides covered a calculated mass of 40.6kDa. This slightly higher mass than suggested by the gel migration resulted from pooling the peptides of all patient samples. Hence, the larger peptide range suggest patient-specific variability in GFAP proteolysis. Remaining charged residues in the head and tail regions of acidic GFAP may have also contributed to slightly faster gel migration[48-51]. Band III comprises two GFAP-BDPs, a 21.8kDa product from coil1 and a 17.5kDa product from coil2 of the GFAP rod. Band IV also includes coil1 with a slightly shorter, 21.3kDa product and also coil2 with an estimated 15.2kDa product. Coil1-covering fragments display the same N-terminus and slightly differing C-termini producing a four-residue larger Band III and a shorter Band IV coil1 product (Fig. 1C). These coil1 products both extend beyond the previously reported caspase 6 cleavage product associated with Alexander disease at VELD225[34]. Two coil2 product sequences with the same N-terminus (F261) and distinct C-termini, K410 (Band III) and R390 (Band IV) comprise coil2 products differing by 22 amino acids or ~2.4kDa (Fig. 1C). Notably, TBI patient associated15-17.5kDa coil2 products are smaller than the 30kDa fragment associated with Alzheimer's disease, yet consistent with the reported caspase 3 cleavage site (residues 258-266, RSKFA↓DLTD)[52]. Interestingly, no peptides from the linker region between coil1B and 2A and the coil2A region were detected by MS in these small BDPs. Yet these aa were readily detected in larger fragments (not shown) and in control analysis of full-length recombinant GFAP (Suppl.Fig.2). Immunoblots using a polyclonal antibody detected small GFAP-BDPs with these observed molecular masses, although monoclonal MCA-3E10, specific for the C-terminus of coil1b, missed the 17kDa band (Fig.1A, Suppl.Fig.1, Fig.2). This data indicates that stepwise and TBI-specific proteolysis produces the previously observed trauma induced GFAP-BDPs of 42, 37 and 18-25kDa[23]. Lastly, coil1-BDPs display greater coverage than coil2 BDPs (Fig.1B), an imbalance further supported by the significantly higher frequency of coil1 peptide presence compared to coil2 peptides which were consistently underrepresented across MS runs irrespective of sample or gel-slice (Suppl.Fig.3A, Methods). Further, among detected peptides ion peaks indicated significantly greater coil1 than coil2 peptide abundance, providing added evidence

on a biofluid imbalance between these two sets of BDPs controlling for patient and irrespective of gel-slice.

GFAP BDPs in Serum. We then immunoprecipitated GFAP from TBI patient sera and identified a coil1-product spanning S51 to R217 (97 residues, 18.2kDa, Suppl.Fig.4). With 59% sequence coverage, the termini could not be confidently identified. Noteworthy, coil2-containing peptides were missing in 4 different TBI blood samples, from 2 patients and one control (PrecisionMed).
In summary, exhaustive MS sequence analysis of GFAP in TBI patient biofluids revealed proteolytic processing that would be expected to generate equimolar co-products; however, coil1 products were more abundant and robustly detected than coil2 products. This disparity was further confirmed by greater abundance of 20-26kDa bands versus 15-19kDa GFAP-BDPs in immunoblot densitometry of 24 TBI patients (Fig.6, see below). Notably, the detailed MS data indicate that GFAP degradation in TBI differs from that of other neurodegenerative diseases and cannot solely be predicted from recombinant GFAP digested with purified enzymes in cell-free systems or toxin treatments[34,52,53]. Additionally, we did not recover peptides spanning the region between coil1B and coil2B, suggesting the involvement of previously uncharacterized trauma-specific cleavage site(s). To address these unresolved issues, we next employed a trauma culture model using human primary cerebral astrocytes.

Human trauma model provides evidence of GFAP cleavage and reveals distinct distribution fates of coil1 and coil2 products.

Trauma-generated GFAP co-products have different fates. The MS-based conclusions regarding coil1 and coil2 GFAP products were further investigated using monoclonal antibodies in a human trauma culture model[23,33]. Mechanical pressure-pulse stretching led to GFAP filament disassembly, release and degradation in traumatized astrocytes[23]. Immunoblots of cell lysates and concentrated culture medium of unstretched controls and stretched cultures from the same donors were probed using mAbs with defined epitopes (Fig.2; Suppl.Table 3). All clones detected full-length GFAP and large fragments, but differed in their detection of small BDPs (Figure 2)[54]. MCA-3E10, a coil1 marker, detected a 20kDa GFAP product in astrocyte medium that increased after trauma, but failed to detect it in cell lysates (Fig.2C, red). The more C-terminally targeted clone GA5 detected an epitope at the end of coil2 and the beginning of the “tail” sequence[35]. It detected a set of 17-28kDa GFAP-BDPs in cells but not in fluids (Figure 2C, purple). A cocktail of three monoclonal antibodies served as positive controls, revealing GFAP-BDPs in cells and fluids from the same donors (Fig.2C, blue). These experiments demonstrate that following mechanical trauma of human astrocytes, coil1 products were preferentially released, while coil2 products were selectively retained within cells. This provides new insight to explain the unequal presence of coil1/2 BDPs in trauma-released fluids.
Novel GFAP trauma cleavage is site defined by partial chemical breakdown of GFAP for mab5C10 epitope mapping. Clone MCA-5C10 detected full size and large GFAP fragments in

cells and fluids after trauma. MCA-5C10 failed to bind any fragment smaller than ~40kDa, suggesting that it recognized an epitope that was destroyed in further trauma-associated GFAP proteolysis in both compartments when probed on the same donor samples as the other clones (Fig.2C, dark yellow). MCA-5C10 showed weak but reproducible binding on dot blots to amino acids 233-261 of the human coil2A sequence (not shown). To pinpoint this clone's epitope further, a chemical-mediated partial digest of GFAP was used. The single tryptophan in GFAP, W256, lies within the region recognized by MCA-5C10. BNPS-skatole cleaves after tryptophan residue[29,30] to produce N-terminal 34.6kDa and C-terminal 20.5kDa fragments from recombinant human GFAP (Suppl.Fig.5). Clone MCA-5C10 bound avidly to this C-terminal fragment on western blots (Suppl.Fig.5). The identity of this 20.5kDa fragment was confirmed using the pan-intermediate filament antibody anti-IFA antibody to the C-terminal of coil2B (amino acids 358-377, ALDIEIATYRKLLEGEENRI)[31,32]. The MCA-3E10 antibody bound the N-terminal 34.6kDa band. We conclude that the sequence following the tryptophan: from tyrosine 257 to tyrosine 260, YRSKF, is an important component of the MCA-5C10 epitope, which likely also includes elements of the neighboring caspase 3 cleavage site, YRSKFA↓DLTD. Consistent with this, MS defined TBI-generated coil1 peptides extended no further than R239 with the N-terminus of coil2 products observed consistently at F261. This unique mapping data suggests novel trauma-cleavage(s) occur upstream of F261 combined with potential caspase 3 activity downstream of it.

Calpains and caspases cleave GFAP and vary in activity among astrocyte types and injury states.

Calpain and caspase inhibitors block trauma-activated GFAP proteolysis steps. The contributions of TBI-activated calpains and caspases to generating these TBI fragments was investigated with inhibitors of calpains and caspases[55-58]. αII-spectrin breakdown products (SBDPs) served as positive controls with their common caspase and calpain indicator fragments, yet the protein's ubiquitous expression, (e.g., muscle, endothelial, epithelial and nucleated blood cells) limits its utility as a TBI biomarker[59-69]. Human traumatized astrocytes were treated with calpain inhibitor calpeptin, or with caspase inhibitor ZVAD-FMK, and GFAP-BDPs were analyzed in whole cell lysates, fluids and pelleted lifted cells and debris. αII-spectrin and SBDPs were abundantly expressed in differentiated human fetal astrocytes (Suppl.Fig.6), as reported in human gray and white matter astrocytes[70-72]. Both calpain (145/150kDa) and caspase (120kDa) SBDPs increased two days after stretch injury in cells, debris, and fluids and were decreased by their respective inhibitors (Suppl.Fig.6). Both, 145kDa and 150kDa calpain bands were observed in whole cell lysates, while the 150kDa showed in lifted cell pellets and 145kDa in culture fluids, respectively. The SBDP120kDa caspase indicator was enriched in the fraction of detached material and demised cells. This indicates that αII-spectrin calpain fragments, alike GFAP-BDPs, originated intracellularly, and also had distinct compartment preference after trauma, suggesting that selective distribution of specific cytoskeletal fragments was not unique to GFAP.

GFAP trauma proteolysis was analyzed from the same sample sets using MCA-3E10, a coil1 marker. Some GFAP appeared fragmented in unstretched viable cells due to baseline cytoskeleton turnover (Figure 3A)[73]. Two days post-stretching, all BDPs were elevated in cells, fluid and lifted material. Calpeptin reduced the 35-38kDa BDP and small BDPs while undigested GFAP initially accumulated in adherent cells and later in lifted material (Fig. 3A; Suppl.Fig.7). ZVAD-FMK inhibited the 19-26kDa small BDPs effectively. Combined inhibitors (CV) had an additive effect, indicating that both calpains and caspases were involved in TBI-initiated GFAP degradation yet ZVAD-FMK is reported to also inhibit calpains[40]. Inhibitors reduced GFAP fragmentation in all three fractions. This data provides the first evidence of *endogenous* trauma-inflicted GFAP BDPs calpain and caspase activities going beyond non trauma experiments[53,74]. These findings connect traumatic GFAP proteolysis in human astrocytes with the well-known roles of calpains and caspases in TBI and cell death processes. The work supports the goal of using GFAP-BDPs as CNS-specific protease indicators after TBI[75,76].

Identifying acute astrocyte injury states using live-cell protease reporters and membrane permeability. To begin identifying the cellular pathophysiology we imaged traumatized astrocytes using fluorescent calpain and caspase indicators together with the membrane wounding dye propidium iodide (PI). Cerebral in vitro differentiated astrocytes displayed two morphotypes: fibrous astrocytes with elongated processes and bushy-to-star-shaped astrocytes that resemble white and gray matter astrocyte morphologies[77] (Fig. 3). Under control conditions, both morphotypes displayed calpain activity (blue), but rare and low caspase activity (green). Fetal neocortical cells express calpains 1(μ) and 2(m) along with low endogenous calpain inhibitor calpastatin during the gestation times used here[78]. Five hrs post-stretching, injured astrocytes displayed blebbing with phase-dark vesicles and vacuoles and beaded processes, known as clasmatodendrosis (Figure 3B,C)[79,80]. PI-uptake revealed a mixed population of membrane-wounded astrocytes with varied PI-uptake that suggest dynamic membrane damage and resealing (Suppl.Fig.10). Phase-dense shrunken astrocytes with bright nuclear PI-stain lacked calpain or caspase activity indicating cell integrity loss that suggests acute necrosis. Membrane-damaged low-intensity PI-positive cells had variable caspase activity and either lacked calpain activity (elongated cells) or displayed active calpains and caspases (bushy cells, Fig.3B, Suppl.Fig.10). PI-positive caspase activity was associated with swollen cell bodies, vesicles and vacuoles. Caspase activity varied in cytosol and was strongest in nuclei, suggesting ongoing apoptosis.
Overall, preliminary imaging documents a range of parallel human astrocyte trauma phenotypes that are relevant to GFAP degradation after trauma. The findings suggest that distinct astrocyte subpopulations with different vulnerability and protease activities coexist soon after mechanical trauma. Endurance of traumatized astrocyte subpopulations, their integrity and enzymatic activity states warrant further studies.

Trauma uncouples citrullinated from regular GFAP, defining novel CSF and astrocyte proteinopathy features.

Validated PTM mapping of TBI GFAP fragments. We analyzed mass spectra for TBI-associated PTMs such as acetylation and citrullination to identify TBI signatures crucial for a) biomarker assay development, and b) detecting cytoskeleton destabilizing proteinopathies that may impair astrocyte functions[81-86]. To our present knowledge, PTMs have not been characterized in human TBI patient samples, although GFAP citrullination was observed in chemically stressed cell lines, in cell-free reconstituted systems, and in the context of other neurodegenerative diseases[28,83,87]. Therefore, we validated the MS-detected acetylated and citrullinated peptide fragments by inspecting MS2 spectra for the presence of signature ions and neutral mass losses (Methods). Validated were four citrullines in coil1 and three in coil2, including a potential novel citrullination site in coil1 (R79), which were consistent with observed mass deviations (neutral losses) in MS2 spectra (Fig.5A, Suppl.Fig.9; Suppl.Table4). An additional five MS-detected citrullinations were previously reported to be citrullinated by PAD treatment in a cell-free system[28]. The robustness of these MS-detected and validated citrullinations is supported by their repeated detection (Suppl.Table4).

TBI patients had different citrullinated GFAP signatures. Further evidence of citrullination was provided using citrullination-site-specific GFAP antibodies characterized in human traumatized astrocyte lysates (Suppl.Fig.10). Two antibodies to neoepitopes in citrullinated GFAP were selected and tested in nine CSF samples from five TBI patients in addition to the samples analyzed by MS (See Suppl.Table 3 and Methods)[36]. Citrullinated GFAP and fragments had patient-specific bands had distinct signal patterns that differed from that of unmodified, native GFAP signals detected in parallel (Fig.5B). Both citrullinated-GFAP antibodies robustly detected 13-16kDa and to a lesser extent, 26-30kDa BDPs that belonged, based on antibody epitopes, to coil2 products. Additional citrullination was detected among the large GFAP BDPs by CTGF-1221, with its epitope at R416. Additional citrullinated bands larger than 50kDa likely reflect GFAP bearing other PTMs (such as ubiquitination) or aggregated GFAP multimers[88,89]. Protease and deiminase activities occurred with patient-to-patient variability, documenting differences in GFAP modification and destabilization during TBI progression.

Citrullinated GFAP in human traumatized astrocytes labels non-filamentous aggregates. Citrullinated GFAP signals were investigated in traumatized and control human astrocytes combining cit270-GFAP, coil2-tail antibody GA5 and a polyclonal GFAP antibody (Figure 5). Traumatized cultures had more citrullinated GFAP than controls, both at 5 and 24hrs after stretch injury. Cit270 marked clusters of small speckles and large granules in a subgroup of astrocytes, but never appeared fibrous, as shown by GA5 and the polyclonal antibody (Fig. 5, Suppl.Fig.10). Aggregated and filamentous signals were generally not colocalized pending ultrastructural confirmation. Citrullinated GFAP either clustered in locally depleted GA5 regions or associated with exceptionally bright GFAP reported from disassembled GFAP in acutely wounded, calcium ionophore-treated, or calpain activated astrocytes[23,90]. Astrocyte morphotypes with cit-GFAP

granules included reactive, atrophic (shrunken) and dystrophic astrocytes, named for swollen cell bodies and damaged or missing processes[91]. Cit270 signals were also detected as speckles peppered around astrocyte processes confirmed by phase-contrast images delineating astrocyte shapes (Suppl.Fig.10). Thus, traumatic injury-induced GFAP citrullination and filament breakdown was associated with aggregation and fluid release showing heterogeneity in astrocyte microstructures and patient CSF signals. Jointly, these findings provide new tools to study astrocyte degeneration using such pathological GFAP proteinopathies in connection with TBI biomarker signatures.

TBI patients' CSF profiles share acute GFAP peaks but have variable fragmentation patterns.

TBI trajectory of temporal GFAP degradation. Examples of the longitudinal GFAP heterogeneity is illustrated in two patients' sets of immunoblots with variable GFAP bands and signal intensities over consecutive CSF samples within two time windows (Fig.6A). Here, we measured full-lengths and GFAP fragments in CSF of 24 TBI patients collected from Neuro-ICU care (Suppl.Tables 1, 2) over 10 PIDs using recombinant 50kDa and 25kDa GFAP proteins for range-fitted calibration (Methods; Suppl.Fig.11). Each band was quantified by scaling various exposures expanding the dynamic range from the lower limit of detection (0.027ng/ml CSF) to the upper limit of quantification (767ng/ml CSF; Suppl.Table5). GFAP proteoforms with similar profile and sizes were grouped, pooling migration variability within and across patients also seen in the MS data (Fig.1). GFAP trajectories had patient-specific peaks and troughs (Fig.6C-H) with acceptable variance spread (Suppl.Fig.12). Age had an effect on GFAP release and proteolysis with older patients showing higher CSF levels (Suppl.Table.6, Suppl.Fig.13A). While this reflects known increases in GFAP biofluids in healthy elderly, age is also linked to altered clearance and worse TBI outcomes (see Discussion)[92,93]. Body temperature, concurrent with CSF draw, had a slight and significant effect on GFAP 45-49kDa fragments with smaller fragments in patients with fever (Suppl.Table6, Suppl.Fig.13B). Both covariate effects were included in the analysis model (Methods). Total GFAP levels had an injury day peak that was 1.8 SD units above the weighted mean[42] and displayed overall fewest extremes ($p=0.035$, Fig.6C). Full-length GFAP had the highest relative injury day peak with 2 SDs above its weighted mean followed by decrease with lowest variability ($p=0.012$, Fig.6D). Mean levels of large GFAP fragments decreased sharply over first two PIDs, then remained flat around their overall mean ($p=0.028$; kurtosis: -1.3, Fig.6E). Mean levels of calpain-generated 37-39kDa fragments peaked with 1.5 SDs above their overall mean, stayed elevated on PIDs 2-5 followed by a decrease during the second week (Fig.6F). Secondary rises were common among small BDPs 20-26kDa and their data had highest kurtosis (-1.8) and marked dispersion driven by both, sporadic peaks and undetectable values (interquartile range:3.18; variance: 2.8 log units). BDPs 15-19kDa peaked on injury day with 1.8 SD above their mean followed by secondary peaks and a more frequent non-detections than other BDPs ($p=0.028$; variance: 1.8 $\log(ng/ml)^2$, Fig.6H). Most bands' daily means exceeded the overall mean on until

PID4 but fell below it from PID7 onward, highlighting a general decline over time. This result suggests that continued proteolysis of GFAP and its large fragments are associated with the temporary accumulation of smaller BDPs and an overall 10-day progressive clearance of GFAP in TBI CSF.

Fragment covariation and selective underrepresentation. We observed a strong correlation between the 50kDa GFAP and calpain-generated 37-39kDa fragments that suggests their co-release (Pearson coefficient, r=0.82, LogWorth=24.3; Fig.7A, B). The 40-42kDa fragments correlated also strongly with the 37-39kDa fragments indicating consecutive cleavage (r=0.73, LogWorth=16.3). The 15-19kDa BDPs related noticeably weaker with precursors 40-42kDa and 37-39kDa bands than 20-26kDa small BDPs (Fig.7; Suppl.Fig.14). Regarding the co-product sets, we noticed that their co-variance was weaker than expected considering they originated from same cleavage, based on MS and epitope mapping data done on a subset of this cohort. The 15–19 kDa BDPs had 28% greater absence than the 20–26 kDa BDPs across all 24 patients (Fig.7C). This co-product imbalance is consistent with less robust MS sequence coverage and lower aa detection frequency and signal peaks (Suppl.Fig.3). Thus, jointly these TBI patient findings support a selective astrocyte retaining of coil2 fragments and preferential coil1 product fluid release found in trauma cultures. Future work is warranted to investigate GFAP's in vivo fragment fates. Taken together, our findings suggest astrocyte-associated pathological coil2 aggregation co-occurs with fluid release of coil1 GFAP. These findings are consistent with the concept that both TBI pathologies initiate from the same cleavage event, yet coil-specific preferred fates could lead to differential compartment accumulation. This is translationally relevant, because linking any pathological cerebral protein aggregation, here GFAP proteinopathy with biomarker signals carries important implications for biomarker interpretation as discussed later. Disrupted proteostasis - particularly cytoskeleton derangement- is an important part in TBI pathophysiology and neurodegeneration as we discuss below[94,95]. To begin addressing how GFAP, as a widely used biomarker, might be a proxy for overall injury burden, we have: (1) evaluated GFAP fragment levels under two severities in the trauma cultures and (2) evaluated GFAP BDPs in predicting TBI patients' outcome.

GFAP fragments inform on trauma severity and their early profiles predict TBI patient recovery.

Time-sensitive calpain-specific GFAP-BDP release relates to trauma severity. The release of a 37kDa GFAP fragment was measured after 3psi versus 5psi pressure pulse stretching in 4-9 donor's astrocyte preparations from 0.5-48hrs postinjury and controls (Suppl.Fig.15). After severe stretch-injury, GFAP 37kDa fragments were elevated already at early timepoints (0.5-5hrs) over controls ($p<0.03$). Later postinjury (24-48hrs), these calpain-fragments were elevated in fluids of both severities ($p<0.001$). Their levels rose over time in both injury severities with differing kinetics (Suppl.Fig.15). This resulted in a severity of calpain-GFAP cleavage into 37kDa at 5hrs

postinjury (p=0.036). Although further studies are warranted, these findings suggest that time-course of GFAP degradation besides their levels could become useful to evaluate trauma severity.

TBI outcome is predicted by levels of GFAP fragment trajectories not by those of full-length GFAP.

Proteolysis could relate to the underlying TBI patient burden following injury. To investigate how GFAP degradation related to patient recovery after TBI, we compared longitudinal CSF levels of full-length GFAP and its fragments in patients with favorable versus unfavorable six-month outcomes on the extended Glasgow Outcome Scale (GOSE). Age was included as joint covariate in the mixed model analysis (Methods). Adjusted CSF means of calpain-generated 37-39kDa GFAP BDPs differed with an effect size of 1.1 between patients with poor recovery (GOSE 1-4) and those with favorable outcomes (GOSE 5-8, p=0.02; Fig.8, Suppl.Table7). Small 20-26kDa BDPs separated good-versus-poor GOSE early postinjury and during the second week, while adjusted means overlapped 3-7PIDs due to frequent undetected values (Fig.8B). Full-length GFAP levels and their adjusted means substantially overlapped during 10PIDs. Similar outcome predictions for same GFAP proteoforms were observed using stricter favorable outcome (GOSE7+8), indicating the findings did not depend on dichotomization (not shown). In summary, longitudinal profiling of GFAP degradation products offers greater prognostic resolution than full-length GFAP alone, and hence may support early risk stratification and outcome prediction in TBI patients.

In conclusion, TBI triggers coordinated and specific GFAP destabilization via citrullination, other PTMs and stepwise proteolysis that is associated with astroglial integrity lapses and astrodegeneration. Pathological GFAP aggregation and fragment-specific biofluid profiles are interrelated as co-products generated by same TBI-GFAP cleavages and thus offer monitoring of evolving TBI pathophysiology and recovery.

Limitations: The clinical study had limited patient sample collection, as it was conducted during the Covid pandemic under substantially short-staffed Neuro-Intensive Care unit personnel. Large cohort studies are needed for further covariate inclusion, yet within the cohort, same GFAP band-signals were measured in all 102 samples using subsaturated, calibrated, and scaled raw data. The longitudinal profiling was truncated at 10 PIDs to avoid violating unequal variances. The remaining sample size used omega squared to compute age and temperature effects. Cohen's D effect size was used to predict good versus poor outcomes, as area-under-the-curve accuracy testing was inadequate for simultaneously including elapsed time postinjury. MS sequencing was done using TBI CSF and only limited serum. Ventriculostomy for CSF drainage is employed in 45–80% of severe TBI offering unique biomarker insights for TBI monitoring while carrying a small risk of infections[96,97]. UCLA surgical pathology procurement of donated fetal brain specimen allowed setup of cerebral astrocyte cultures[23,33], yet primary tissue amounts are limited to cover all relevant conditions simultaneously to address coil1 & 2 fragments, key timepoints, controls and severity settings for various protease inhibitors. Shown data was pooled from six cultures and was

representative for other donors. Live-cell staining limited the use of high-intensity or repeated fluorescence imaging but enabled simultaneous monitoring of both enzymes and membrane permeability at a single timepoint. This approach provided insight into coexisting astrocyte injury states, as well as GFAP degradation, modification, and distribution, offering relevant information on astrodegeneration and TBI monitoring.

Discussion

This study describes novel TBI-induced GFAP degradation products and proteoforms and demonstrates trends over time with unique patient-specific profiles. GFAP proteolysis and release are progressive processes while distinct cerebral astrocyte injury states may coexist. We highlight a novel TBI cleavage that produces two sets of products with distinct distribution fates from a common fragment precursor. Specific GFAP BDPs have clinical significance for pathophysiology, longitudinal monitoring, and predicting outcome. These findings are novel and inform on the complexity of astrodegeneration that could advance our understanding of pathomechanisms during the subacute setting after TBI.

With this use-context in mind, interpreting biofluid GFAP signals is desired. Assaying of biofluids after TBI is now frequently used for acute brain injury using commercial antibody-based assays. Beyond a general specificity for GFAP it is not clear what proteoforms are being measured, as this information is either proprietary or unknown[10,14,98]. Yet it is presumed that total trauma-released GFAP is being captured. We used MS and domain-specific and modification-selective antibodies to identify TBI-induced GFAP proteoforms, their profiles and distribution after trauma. This study informs that coil1 and coil2D products exhibit distinct release patterns, crucial knowledge for designing sensitive and specific assays. Underlying perturbed proteostasis with pathological citrullinated coil2-fragment aggregation after injury is documented. Together, our findings provide an important first step toward understanding how GFAP proteoforms that reflect pathological processes following TBI.

Cellular and biochemical pathophysiology of mechanical force induced traumatic injury. Mechanical trauma or whiplash shear brain tissue, causing diffuse axonal injury and astroglial clasmatodendrosis -membrane wounding that can lead to protracted cell loss[23,79,99-101]. Trauma ruptures plasma membranes via mechanoporation, causing rapid initial protein release[102,103] -including full-length GFAP and large fragments- followed by later generation and release of small GFAP-BDPs linked to astroglial death[23]. Mechanical forces also stretch and can break intermediate filaments that may initiate protein aggregation[104,105]. Under biomechanical high-speed strain, filaments get stretched, disassemble exposing the rod center making it vulnerable to proteases[73,90,106] or may reach a molecular breaking point[105,107]. Indeed, non-filamentous GFAP has been documented minutes after stretch-injury in mechanoporated human astrocytes[23]. Thus, filament-disassembly is closely linked to membrane permeability perturbing ion balance that

threatens cell viability[23]. Ion dysregulation is followed by water leading to membrane blebbing and astrocyte swelling (oncosis). If unresolved, and with energy resources being depleted cell integrity maybe lost resulting in necrosis. Burden from reactive oxygen species, acidosis and hypoxia further impair mitochondria eventually shifting from apoptosis to necrosis[108-113]. This progressive pathophysiology may occur in human astrocytes with considerable delay (unpublished comparison of human and rodent astrocytes) and could contribute to secondary GFAP release in TBI patients reflecting cytotoxic edema and subacute astrocyte loss[114-119].

Linking proteases and destabilizing enzymes to GFAP proteinopathy and astrocyte pathology. Treating rat astrocytes with a calcium ionophore causes rapid and transient increase in cellular GFAP immunocytochemical signals due to filament unwinding that exposes additional epitopes[90]. As aforementioned, trauma-induced unwinding can also reveal a concealed proteolytic cleavage site in the rod center of GFAP. Calcium augments GFAP proteolysis, rapidly activating *calpain* through a conformation change leading to initial partial GFAP cleavage, consistent with calpains' limited proteolytic activity[73,90,103,120-128]. Calpains and caspases both participate in GFAP proteolysis in stretch-injured astrocytes as calpain cleavage activates caspases[125,129]. Our observation of unstretched calpain activity is consistent with calpains being part of the ubiquitin-proteasome, autophagy-lysosomes, and cytoskeleton remodeling systems, active at baseline and regulated by endogenous inhibitors such as calpastatin[78,130-132]. Our live imaging documents coexisting astrocyte injury states with active calpain and caspases, indicating that various progressed states of astroglial wounding coexist due to astrocyte subtype differences, bushy versus fibrous morphotypes, or variations in focal mechanical loading and cellular energy exhaustion. Active *caspase* activity in process-damaged and blebbing human astrocytes after trauma parallels early post-lesion caspase activity seen in clasmatodendrotic rat astrocytes following excitotoxicity, linking cytoskeleton remodeling, its caspase-mediated breakdown, and apoptosis[123,133,134]. Variability in membrane damage and energy depletion shape cell resilience and produce a spectrum of concurrent cell injuries[101,118,135]. These pathological GFAP changes impair astrocyte function, risk diminished glutamate buffering and neuronal support that could lead to neurodegeneration[136]. Calcium also activates peptidylarginine deiminases (PADs) among other enzymes that destabilize cytoskeleton proteins including GFAP by *citrullination* leading to proteinopathies in neurodegenerative diseases such as Alexander disease[81-86,137]. We show TBI patients with such GFAP destabilizing proteoforms. In our trauma model stretch-injury drives pathological GFAP aggregation in human astrocytes consistent with GFAP citrullination after mechanical stretching, hypoxia and chemical injury that upregulate and activate PADs in other culture models of rat and human astrocytes and Müller glia[88,138,139]. Assembled GFAP filaments are vital for maintaining astrocyte morphology, mechanical resilience, ATP release and other essential functions[73]. Thus, the *GFAP proteinopathy* shown in our study signifies *pathological astrocytes* with potential harmful consequences that, as seen in Alexander disease, critically impair astrocyte function, jeopardizing neural metabolism, network activity and the integrity of the neurovascular unit[140,141].

Trauma caused GFAP fragment sequences. TBI patient GFAP BDP sequences show novel cleavage patterns, distinct from established protease sites in other neurodegenerative diseases. A caspase 6 cleavage of GFAP ($VELD_{225}$-VAKP) was described in mouse culture models of Alexander disease, splitting GFAP at the linker2 sequence, with the C-terminal coil2 fragment detected by antibody GA5[34,35]. The present study identified TBI patient-defined coil1 products with ending sequences that repeatedly contained an additional 8 or 14 amino acids that are distinct from the cleavage seen in Alexander disease[34]. Hence, we conclude that a new proteolytic cleavage occurred between residues 240-260 after TBI that produced these trauma-related GFAP-BDPs.
The TBI patient coil2 products we identified begin at F261 and end at R390 or K410, making them shorter than the GFAP 20kDa and 30kDa fragment pair reported in Alzheimer's disease patients generated by caspase 3 cleavage at $_{258}$RSKFA↓DLTD in coil2A[52]. This cleavage does not explain the shorter coil1 or coil2B fragments we detected; rather, our findings point to coil2B being particularly sensitive to proteolysis. Our data implicate calpain and caspases in this process, though other proteases -including possible endogenous trypsin-like activity (YRSK↓F_{261})- may contribute. Such activity could generate the new termini found in both TBI-GFAP small products (LTAALKEIR↓T; R239) that would account for the observed sequence gap between them. Endogenous trypsin activity has been reported after brain injury inflicting astrocyte compromise and adverse inflammatory effects[142,143]. Our rigorous MS high confidence peptide maps and MCA5C10 epitope loss ($_{257}$YRSKF_{261}) flanks a neighboring caspase 3 cleavage site. The gap between the two co-products was unlikely an experimental artifact, as high confidence peptides were detected repeatedly covering that region during MS runs of longer GFAP precursor fragments and recombinant GFAP sequencing. Further studies are needed to delineate all proteases involved in GFAP cleavage after TBI. The variability in fragment presence and termini suggest different enzymes and fragments could reflect different TBI phenotypes and astrocyte injury populations.

Structural evidence for protease vulnerability of the novel TBI cleavage site. Intact GFAP molecules anneal by α-helical coiled-coil interactions forming parallel dimers, antiparallel tetramers and ultimately 10nmØ units that grow in length to mature filaments[54]. Computer modeling and structural data show that the entire rod segment of vimentin, closely related to GFAP, is a continuous α-helix[144]. This is despite several regions, historically referred to as linkers or stutters, lack the typical hydrophobic heptad repeat pattern of α-helical coiled-coils. These regions contain proline and other turn-promoting aa or feature 11aa (hendecad) sequences disrupting the heptad repeats. These regions were therefore thought to represent non-helical linkers. Notably, the Alexander disease-associated cleavage site discussed above is found within one such non-helical "linker12", consisting of several hendecad repeats. Likewise, the region containing the MCA5C10 epitope and caspase3 site were historically referred to as "stutter1", where a hydrophobic heptad repeat (7aa) is replaced by a single hendecad sequence, disrupting the packing and thereby can render both regions more susceptible to proteases[145].

Novel GFAP co-product patterns-pathophysiological significance and biomarker implications. TBI proteolysis of GFAP generates co-products with unequal MS sequence coverage, peptide frequency and abundance in patient biofluids. MS disparities reflect coil1-BDPs to be more prevalent than coil2-BDPs in TBI CSF, while the later were undetected in serum. GFAP concentrations are generally lower in blood versus CSF due differences in dilution solute passage between the interstitial space and ventricles, versus capillaries and blood vessels[146-148]. Immunoblotting with a polycloncal antibody also shows an imbalance with of 20-26kDa BDPs over less abundant less frequent 15-19kDa BDPs in 24 TBI patients, paralleling MS data. The imbalance could reflect differences in release or susceptibility to further degradation. While we do not have data on GFAP fragment-specific clearance, stretch-injured astrocytes show preferred coil1-BDP trauma-release and selective coil2 BDP retention using coil-specific mabs. Traumatized astrocyte retention and modification of coil2-BDPs as non-filamentous GFAP aggregates mirrors Alexander disease, where mutant GFAP fragments disrupt proteostasis, form toxic Rosenthal fibers that impair astrocyte function driving neurodegeneration[34,35,136,149-151]. Thus, traumatized astrocytes with cit-GFAP aggregates are a) a signature for *astrocytopathy* and b) are linkable to GFAP in TBI biofluids. TBI CSF samples carry both co-products. However, coil2-BDP signals varied -were either missing or elevated- possibly indicating presence or absence of a pathological event leading to *integrity loss* with release of otherwise retained proteoforms. Astroglial membrane lysis from necrosis or oncosis during cytotoxic edema may account for such release (see below). Coil-specific and citrullinated GFAP antibodies may aid assay development for detecting astrocyte-driven TBI pathologies[80,152,153]. On one hand, coil1-GFAP fluid maybe matched by a latent coil2-GFAP burden in the injured brain. On the other hand, detecting coil2 in fluids may signal worsening conditions such as cytotoxic edema with unregulated astrocyte demise. These possibilities highlight two priorities for future research of present needs: (1) examine cit-GFAP-aggregates in postmortem TBI, BLAST, or CTE brains to establish traumatic astrocytopathy as new TBI neuropathological hallmark with modified GFAP proteoform antibodies; and (2) linking GFAP biofluid elevation to proteostasis disruption and neurodegeneration in animal TBI models[95,154]. Overall, our findings offer connecting GFAP as a TBI fluid biomarker with ongoing proteinopathy and may aid to explain recurring GFAP elevations due to secondary deterioration observed in TBI patients[21,23].

Potential clinical impact for new GFAP 'context of use' in neurocritical care TBI monitoring.

Possible new COUs for GFAP in TBI patient assessment warrant discussion: *(1) Longitudinal monitoring*; (*2) GFAP-BDP profiles associated with astrodegeneration may inform on secondary injury; and (3) Outcome prediction*.

(1) Longitudinal TBI monitoring: GFAP degradation kinetics offer a valuable longitudinal window for Neuro-ICU TBI patient monitoring during intensive care and early clinical trial endpoints[57,155-162]. In circulation GFAP-BDPs dominate, while CSF also contains both, uncleaved

GFAP alongside BDPs[23,163,164]. Temporal biomarker profiles show that all GFAP forms typically peak on injury day, a pattern shared with a subset of other cytosolic and cytoskeletal proteins (e.g. UCH-L1, S100β, aldolase C, neuron-specific enolase, αII-spectrin). Early peaks reflect acute mechanoporation, force-triggered filament breakage and calcium-driven proteolysis[23,24,63,102,165,166]. Hence, calpain-generated 37-39kDa GFAP BDPs correlated strongly with full-length GFAP supporting common origin in TBI CSF.
After initial trauma small BDPs occasionally display secondary rises (4-5; 9PIDs), reflecting caspase activation in some patients. This pattern aligns with secondary serum GFAP peaks (4PIDs) in a subset of older TBI patients with high CT burden, poor outcome, high mortality and pathophysiological features of more severe TBI patients[21]. We observed higher GFAP CSF levels among older patients as well. Age-related increases in baseline GFAP and neurofilament light (NfL) relate reduced clearance or ongoing parenchymal atrophy[92,93,167-169]. Elderly suffering a TBI clear CSF less efficiently through decreased CSF pulsation, ependymal cilia vulnerability, and disrupted cerebral blood flow, further elevating biomarker levels[170-172]. Finally, secondary GFAP-BDP spikes exhibit vary between patients, reflecting heterogeneity in secondary in secondary injury progression.

(2) GFAP-BDP profiles associated with astrodegeneration may inform on secondary injury. Neuroimaging finds expanding contusions after TBI affecting peri-lesional tissue with an underlying pathomechanism being cytotoxic edema[173-177]. Cumulative astrocyte swelling, secondary membrane integrity compromise, and loss underpin such microstructural changes driven at large by ion dysregulation and metabolic imbalance[114-116]. Ongoing injury events like these contribute to TBI patient heterogeneity that is also reflected in varying GFAP-BDP trajectories we report. Progressive astrodegeneration is quantified in autopsied brain tissues of TBI patients documented by reduced astrocyte numbers between acute (<6h) and subacute (>6hrs to 3PID) postmortem timepoints in cerebral white matter and hippocampus[119]. Accordingly, GFAP-BDP profiling in the Neuro-ICU may be considered as a tool to monitor secondary adverse postinjury events. An expanded window of biofluid testing in neurocritical care may aid individualized insights into an evolving pathophysiology, beyond single-timepoint GFAP testing in the emergency department.

(3) GFAP-BDP trajectories predict TBI patient outcome. Elevated GFAP in CSF and blood is well-documented as prognostic marker for severe TBI recovery[21,23,178-181]. Our longitudinal study further specified that among GFAP proteoforms, trauma-generated calpain GFAP-BDPs (37–39 kDa) differentiated poor versus good 6-months outcome, regardless of how recovery scores were dichotomized. This is important as evaluating "good" recovery is a very personal determination[182]. Profiles of small BPDs (20–26 kDa) followed this trend. In contrast, uncleaved GFAP levels failed to predict recovery. Jointly, our data emphasize that *ongoing pathological proteolysis* could inform on injury progression and recovery rather than merely the level of GFAP *release* as such. Current FDA-approved GFAP assays focus on a narrow injury-day window[9,178], missing this dynamic.

While GFAP assays may include detecting BDPs, they don't differentiate GFAP fragments and longitudinal detection is presently not part of the COU. Thus, collectively, while uncleaved GFAP release, conceivably from initial trauma, is less indicative of injury course and prognosis, monitoring GFAP-BDPs post-TBI have the potential to improve predicting mortality and functional outcomes[183], as magnitude and trajectory of GFAP proteolysis are shaped by the evolving burden of brain injury that may drive recovery.

In summary, this study delineates the stepwise proteolytic processing of GFAP following TBI, highlighting the distinct molecular signatures and PTMs that underpin astrocyte dysfunction and neurodegeneration. The identification of trauma-specific GFAP BDPs, their divergent fates, and the mechanistic link to both acute biomarker release and latent proteinopathy provide a novel foundational concept for improved TBI phenotyping and patient monitoring. These findings underscore the importance of assay design tailored to GFAP proteoform heterogeneity and support the use of longitudinal GFAP-BDP trajectories as robust neurocritical care predictors of progressing injury course and clinical outcome.

Authorship Contributions

IBW conceptualized and designed the study, wrote and revised the manuscript, curated data and conducted formal analysis, designed and prepared all visualizations, acquired funding, coordinated research activities, supervised laboratory assistants and students and conducted cell culture and live cell imaging studies. **JH** and **JL** conducted sample preparation and immunological experiments, assembled raw data and illustrations, curated and analyzed data. **JL, SS** and **YC** analyzed MS spectra. **YC** and **SS** conducted MS analyses. **HZ** provided the thumbnail infographic. **CS** analyzed data and provided visualizations. **ROL** and **JAL** provided MS data analysis guidance and **JAL** was involved in the study conceptualization. **TVM** and **GS** provided antibodies, peptides and recombinant proteins and offered biochemical guidance. **GS** also conducted antibody binding and chemical cleavage studies and wrote the structural part of the discussion. **BME** interpreted data and revised the manuscript. **JG** provided guidance on data curation and statistical analysis approaches. **SR** and **PMV** had oversight over clinical aspects of the study including TBI patient enrollment, IRB approval and sample provision, further, **PMV** provided guidance on clinical interpretation of the findings. Resources included **IDDRC** laboratory infrastructure including cell culture, centrifuges, gel imagers. The **MIC** provided mass spectrometry instruments and proteomics facilities for the study. ***All authors*** *read, reviewed, edited and approved of the manuscript.*

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Competing interests

IBW and **JAL** are inventors of the following patents licensed to BRAINBox Solutions Inc., where **TVM** serves as Chief Scientific Officer (CSO). **GS** is CEO and founder of EnCor Biotechnology Inc., which distributes several monoclonal GFAP antibodies used in this study.
US Patent Issue No.: US 10,557,859 B2 original patent; Inventors I.B. Wanner and J.A. Loo, Issue date: 2/11/2020; 25 claims prior publication 12/20/2018: "Astrocyte traumatome and neurotrauma biomarkers". Applicant Regents of the University of California, Oakland, CA. Licensee: BRAINBox Solutions Inc.
US Issue No.: US 11,249,094 B2 Additional 13 claims in divisional patent 13 Claims, Filed 12/116/2019; Issue date: 2/15/2022; Inventors: Wanner and Loo, UCLA "Astrocyte traumatome and neurotrauma biomarkers". Assignee UCLA; Licensee: Brain Box Solutions Inc.

Supplemental Material

This manuscript includes 15 Supplemental figures and 6 Supplemental tables that are compiled into one PDF file of Supplemental Material.

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Figure legends

Thumbnail: Astrocyte injury states with GFAP proteoforms and fates after TBI.
The infographic depicts four distinct, progressive -and partially coexisting- pathophysiological states of human astrocytes following TBI. Each injury state is characterized by specific cellular morphologies and GFAP proteoforms and their molecular fates. Illustrated are key processes of GFAP degradation and proteinopathy together with ensuing astrocyte dysfunction that jointly offer a mechanistic basis for longitudinal GFAP fluid biomarker signatures. Understanding these astrocytic and cytoskeletal alterations is central to interpreting GFAP as a TBI biomarker and linking it to neurodegeneration. The thumbnail captures the reasoning for a new GFAP TBI COU in Neuro-ICU patient monitoring and recovery prognostication. **(1) Uninjured***, healthy* astrocyte: Intact processes, continuous plasma membrane and fully assembled GFAP filaments. **(2) Mechanoporated***, wounded* astrocyte: Shear-forces and mechanical loading inflict membrane damage, poration and process beading (clasmatodendrosis), triggering calcium influx and ion imbalance. GFAP filaments get stretched and disassemble into GFAP monomers that may even break (black) and are subsequently degraded, initially by calpains into large fragments (gray BDPs), followed by calpain, caspase and other proteases into novel TBI cleavage co-products: sets of coil1-BDPs (red) and coil2-BDPs (purple). Coil1-BDPs are released into fluids through permeable membranes (red), while coil2-BDPs form intracellular aggregates (purple clusters). **(3) Dystrophic (oncotic),** astrocyte: *Swollen* with vacuoles, membrane blebbing and advanced process damage. Accumulated PTM-modified GFAP granules marked by citrullination (green) and acetylation (brown) represent a GFAP proteinopathy newly recognized in TBI. **(4) Necrotic,** *dying* astrocyte: Loss of membrane integrity, membrane rupture and uncontrolled lysis release modified GFAP aggregates associated with astrocyte demise.

Figure 1: TBI patients' GFAP-BDP sequences display variable product endings.
A) TBI patient CSF immunoblots of a 15% gel show GFAP detected by a polyclonal (top) and a monoclonal (clone 3E10, bottom) antibody (postinjury days, i+1 through 1+3) of an 84 year old male sustaining facial fractures, subarachnoid and intraventricular hemorrhage and intraparenchymal hematomas from a fall. Observed GFAP band molecular weights were bands (II.) 36-39kDa, bands (III.) 20.4-23kDa, and bands (IV.) 17-19kDa, missed by clone 3E10.

B) MS peptide maps of immunoprecipitated GFAP breakdown products (BDPs) of four cut out gel bandsets (I.-IV., Suppl. Fig. 1) from six CSF TBI samples of three patients. Percent of fragment sequence coverage (left) by high confidence (green, FDR≤1%) and repeatedly identified medium confidence (yellow FDR≤5%) peptides. Bands (I.) had 359 amino acids (aa) with calculated molecular weight of 41.7kDa and suggested new N- and C-termini of serine 53 (S53) and lysine 411 (K411). Bands (II.) spanned 349 aa from glycine 56 (G56) to leucine (L404) within which 37/38kDa fragments with variable endings between TBI samples were located (Suppl.Fig.1). Bands (III.) contained two GFAP BDPs, 92% covered coil1-containing product of 185 aa, calculated molecular weight of 21.8kDa; 77% covered coil2-containing product of 151 aa and peptides, estimated molecular weight of 17.5kDa. Bands (IV.) 96% covered 181 aa coil1 product with size between 21-21.6kDa -±PTMs; and 77% covered coil2 product of 130 aa with estimated molecular weight of 15-15.3kDa. **C)** Predicted endings of the two sets of novel TBI patients' GFAP-BDPs. Coil1 products N-termini were S53 (III.) and alanine, A55 (IV.) and new C-termini were arginine 239 (R239) C-terminus (III.) and A233 (IV.), both stretching beyond previously described caspase 6 cleavage around VELD225[34]. Coil2 products had consistent N-terminus phenylalanine 261 (F261), see reported caspase 3 cleavage RSKFA↓DLTS[52], and variably ended with K411 (III.) and with R390 (IV.).

Figure 2: Novel GFAP cleavage creates co-products with distinct localization fates in TBI.
Monoclonal antibody (mab) mapping confirms GFAP cleavage into two co-product sets that resided in separate compartments using a human trauma culture model of in vitro differentiated cerebral astrocytes[33]: coil1-containing products were trauma-released and coil2-products remained cell-associated. **A)** Coil1 or coil2 harboring small GFAP-BDPs for antibody epitope alignment. **B)** Binding regions and defined epitopes of GFAP mabs within coil1 and coil2 GFAP-BDPs. Mab 3E10 binding region is in coil1B between aa 196-217 with stutter motif HEEEVRE, aa204-210, as epitope. Binding region of mab 5C10 covers aa 240-260 in coil 2A with aa257-260 as core epitope. Mab GA5[34] binds at the end of coil 2B covering aa 374-390. Mouse GFAP multiclone antibody (4A11, 1B4, 2E1 BD Pharmingen) contains unspecified epitopes in both coils. **C)** Two donor's whole cell lysates (Cells) and concentrated media (Fluid) from control (-) and 2 days post-stretched human astrocyte cultures ("+", 4.8-5.4psi) were probed with mab GFAP antibodies. All clones detected full-length GFAP and large fragments in cells and trauma-released fluids. Mab 3E10 detected 20kDa products in fluids missed small BDPs in cells. BD Multiclone detected 13-28kDa GFAP BDPs in cells and fluids. Mab 5C10 did not detect small fragments. Mab GA5[34] detected 17-28kDa GFAP BDPs in cells, missed small BDPs in fluid.

Figure 3: Inhibition of trauma induced GFAP degradation across compartments and astrocyte injury states' protease activation.
A) Unstretched or stretch-injured human astrocytes were treated with calpain inhibitor calpeptin (C, 13mM), caspase inhibitor Z-VAD-FMK (V, 11mM), both (CV), or no drug (-) for 48 hours. Adherent cells were lysed (Whole Cell lysate, WCL), centrifuged fluid pellet contained lifted

material (Lifted Cell Pellet) and fluids were concentrated (Conditioned medium, CM). *Top:* 2sec exposures for 50kDa GFAP and 42-47kDa BDPs. *Mid:* 1min exposures for 38-25kDa BDP bands. *Bottom:* 20min (WCL, CM) and 5min (Lifted cell pellet) exposures for 17-25kDa BDPs (See Suppl.Fig.7 for 5hr blot and 48h densitometry).
B+C) *Live imaging of distinct cerebral human astrocyte morphotypes and their acute trauma stages defined by membrane leak, calpain and caspase activities during 5-6 hours postinjury.*
B). *Left*: Elongated (*Top*) and bushy (*bottom*) morphotypes and injury shapes on phase contrast. *Right*: Fluorescence images for plasma membrane permeability (5min uptake assay of propidium iodide, [PI+], red), calpain activity (CMAC-tBOC-leucyl-methionine, [CMAC-tBLM], converted substrate, blue) and caspase activity ([NucView488] converted substrate, green). *Top*: Control fibrous astrocytes display intact processes, acutely stretched cells had thinned, frequently beaded clasmatodendrotic processes (arrows). [PI-] astrocytes were calpain active. [PI+], leaky cells presented variable caspase activity (yellow/orange), lacking calpain-activity. *Bottom*: Bushy control and stretched astrocytes were calpain active (blue). The majority of stretched bushy astrocytes were calpain and caspase active (teal). [PI+], leaky bushy cells either retained both activities (yellow/white), or lost these protease activities (red, arrows) displaying necrotic, atrophic morphology. Phase-dense vacuoles were unstained. **C)** Enlarged from B: Individual fibrous (top) and bushy (bottom) astrocytes showing distinct structural and functional features of intact, injured and necrotic astrocyte phenotypes: *Intact* [PI-], calpain[+]; *Wounded*: [PI-/+], cytoplasmic and nuclear caspase[+]; *Dying:* [PI+] nuclear caspase [-/+], calpain[-]. Suppl.Fig.8 for separate fluorescence channels. Scale bars: 100µm.

Figure 4: Destabilizing citrullination and acetylation PTMs are signatures of TBI GFAP.
A) Map shows GFAP coil1/2 BDPs with posttranslational modified aminoacids from MS of 6 TBI CSF samples. Citrullinations (green) included a novel citrullination site at arginine 188 (R188), and acetylations (brown) PTMs were validated by signature ions and ion mass shifts in MS2 spectra (Suppl. Figure 6). *Here identified citrullinations are prior described in cell-free peptidylarginine deiminase digests[28]. Arrows: Neo-epitopes R270 and R416 for citrullinated-GFAP specific antibodies cit-R270-GFAP, mab CTGF-1221[36] used in B and Fig.5.
B) *Left*: TBI patient-specific citrullinated GFAP-BDP signatures in nine CSF samples from five TBI patients using two citrullinated-GFAP antibodies. Besides fragments, larger modified GFAP bands were present. *Right*: Unmodified GFAP detected by cloil1 mab3E10 (top) or a rabbit polyclonal GFAP (bottom) show different signal patterns and amounts as citrullinated GFAP on same samples (dilutions and exposures given below blots, antibodies see Suppl.Table3).

Figure 5: Trauma induced citrullinated GFAP formed aggregated clusters in pathological human astrocytes
A) Immunofluorescence of coil2 GFAP (mabGA5,red) and citrullinted GFAP (cit270-GFAP, green) shows trauma-enhanced accumulation of non-filamentous citrullinated GFAP clusters at 5 and 24 hours post-stretching (right) versus control astrocytes (left) astrocytes. **B)** Unstretched

astrocyte (left) and three trauma-pathological astrocyte morphotypes (right) after stretch-injury: reactive astrocyte with thick, hypertrophied processes and bright GFAP with somatic fine-granular cit-GFAP (yellow); astrocytes with fanning-out processes display cit-GFAP sprinkles around GA5 signals (arrows); dystrophic, oncotic astrocytes with swollen somata and malformed or missing processes display non-filamentous GFAP and cit-GFAP aggregates (arrowheads; Suppl.Fig.10E, and polyclonal GFAP signals, F for cell borders. Scale bars: 50 µm.

Figure 6: TBI CSF pattern of GFAP proteoforms share acute peaks while secondary profiles show unique GFAP degradation.

A) GFAP immunoblot of 16 serial CSF samples over four postinjury days from a 40 year old white male TBI patients with admission GCS 3 and good recovery (GOSE score 8). Strongest GFAP and fragment signals were six hours postinjury and secondary peaks of GFAP (37-50kDa) occurred on PID two, three and four. **B)** GFAP immunoblot shows seven serial CSF samples from PID4-10 of a 48 year old Hispanic male TBI patient with admission GCS10 and GOSE6 outcome. Bands 37-50kDa were stronger on earlier days while 15-26kDa BDPs variably increased on PID5-8.

C-H: *TBI patients' 10 PID longitudinal profiles of GFAP total* ***(C),*** *uncleaved* ***(D)*** *and degradation BDP sets* ***(E-H).*** Graphed are log-transformed GFAP CSF trajectories measured on repeated exposures of subsaturated scaled and calibrated (colored lines; 102 observations, 24 TBI patients; densitometry approach see Suppl.Fig.11; Suppl.Table5). Added are estimated resampled mean cubic splines with 95% confidence bounds, interquartile range (IQR, log ng/ml), kurtosis and variance (σ: log(ng/ml)^2). **D, E, H)** Total, full-length GFAP and 15-19kDa BDPs were significantly higher on injury day than on other PIDs ($p<0.05$; Linear mixed model, controlling body temperature and age, Suppl.Table6, Suppl.Fig.13). 15-19kDa BDPs had large dispersion due to high or undetected bands. Calpain-generated 37-39kDa GFAP fragments trended high on injury day ($p=0.06$) and displayed above mean signals until PID3, around mean signals on PID4 and 5 and below mean signals thereafter. **G)** Small GFAP BDPs sized 20-26kDa had pronounced secondary highs and largest dispersion (highest IQR, variance and kurtosis).

Figure 7: Correlation between sets of GFAP proteoforms in TBI patients' CSF.

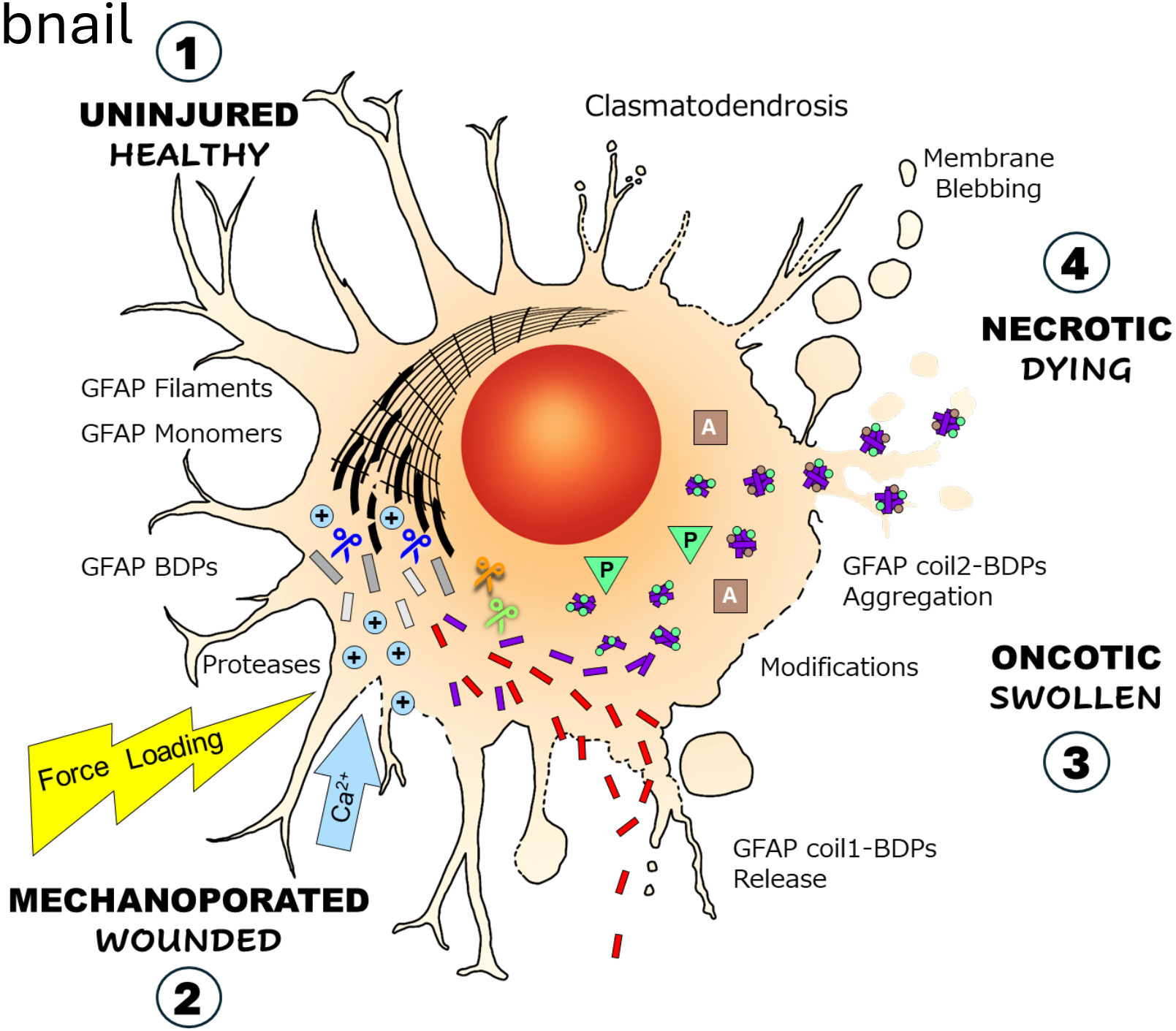
A) Multivariate Pearson's correlation matrix shows correlation coefficients between uncleaved GFAP and all BDP sets measured in same TBI CSF samples. **B)** Bivariate graph of full length (50kDa) GFAP over calpain cleavage products (37-39kDa) with orthogonal fit for equal error weights, R^2 and FDR-corrected Logworth ($\log_{10}$p-values, false discovery rate corrected). **C)** Bivariate graph of GFAP BDP co-products show greater missingness among 15-19kDa versus 20-26kDa bandsets (circled, 28% or 15 more zeroes; orthogonal fit, FDR-corrected Logworth).

Figure 8: Dynamic GFAP proteolysis differentiates poor from good TBI patient outcome not full-length GFAP profiles

Shown are trajectories of GFAP molecular weights over 10PIDs partitioned by good recovery (GOSE 5-8) versus poor outcome (GOSE1-4) with mean cubic splines and 95% confidence bands

(24 patients; 102 observations). **A)** Calpain generated 37-39kDa GFAP BDP trajectories differed in outcome by 1.1SD unit (p=0.02, adjusted for elapsed time and age effects; subject ID as random effect). **B)** Small GFAP-BDPs (20-26kDa) showed 0.9SD mean higher levels in patients with poor recovery over time than those with good recovery with trends influenced by age and missingness. **C)** Full-length, 50kDa GFAP profiles overlapped over time lacking differentiation between good versus poor outcome.

Thumbnail

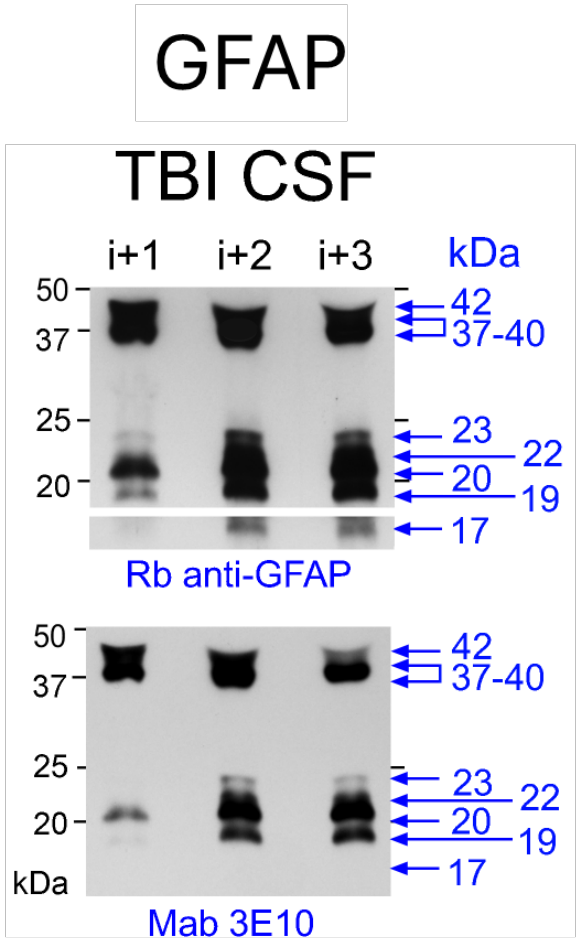


Symbol key

- GFAP Filaments
- GFAP Monomers +/- break
- Large GFAP-BDPs (42-49kDa)
- Calpain GFAP-BDPs (37-39kDa)
- Small GFAP Coil1-BDPs
- Small GFAP Coil2-BDPs
- GFAP Aggregates
- Calcium
- Peptidyl deiminases, PADs
- Citrullination
- Acetyltransferases
- Acetylation
- Calpain
- Caspase
- Endogenous Trypsin activity

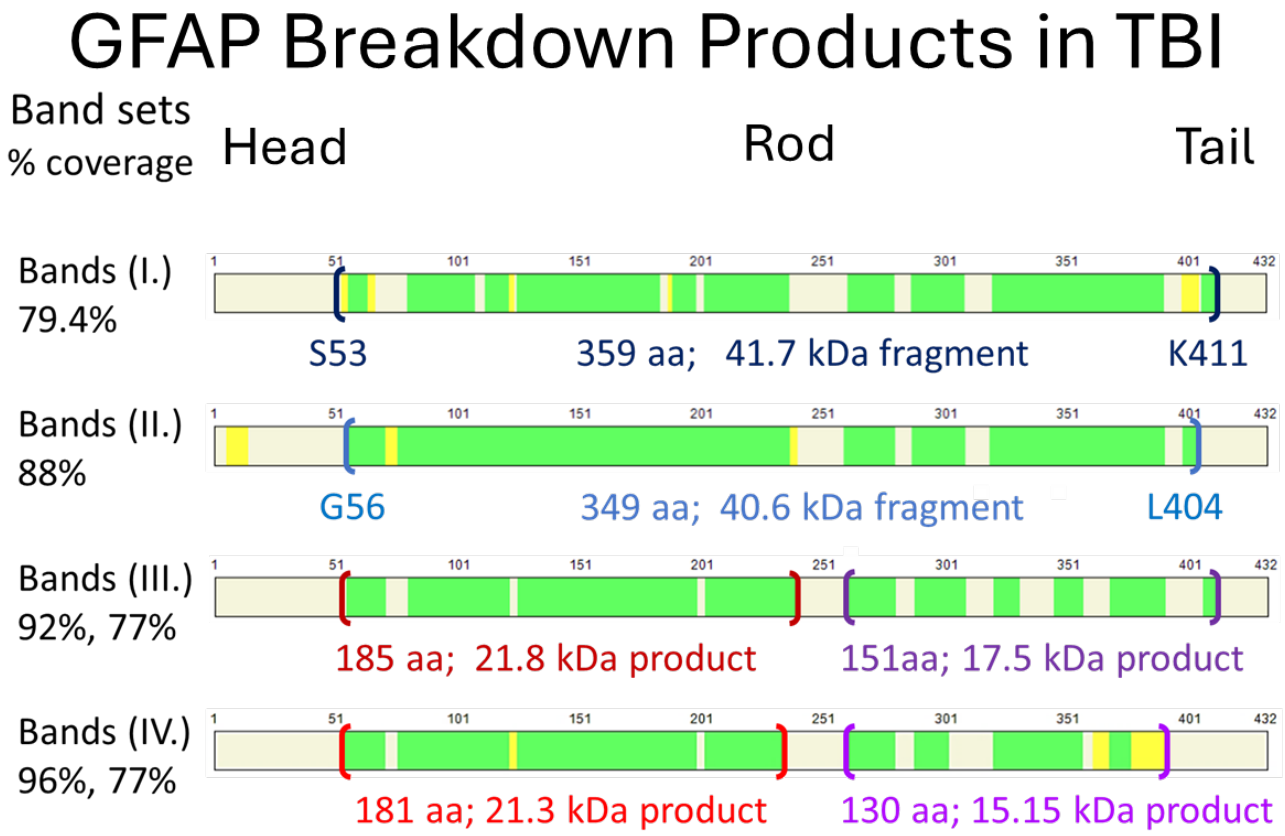
Figure 1

A



15% gel, 56ul CSF

B



C

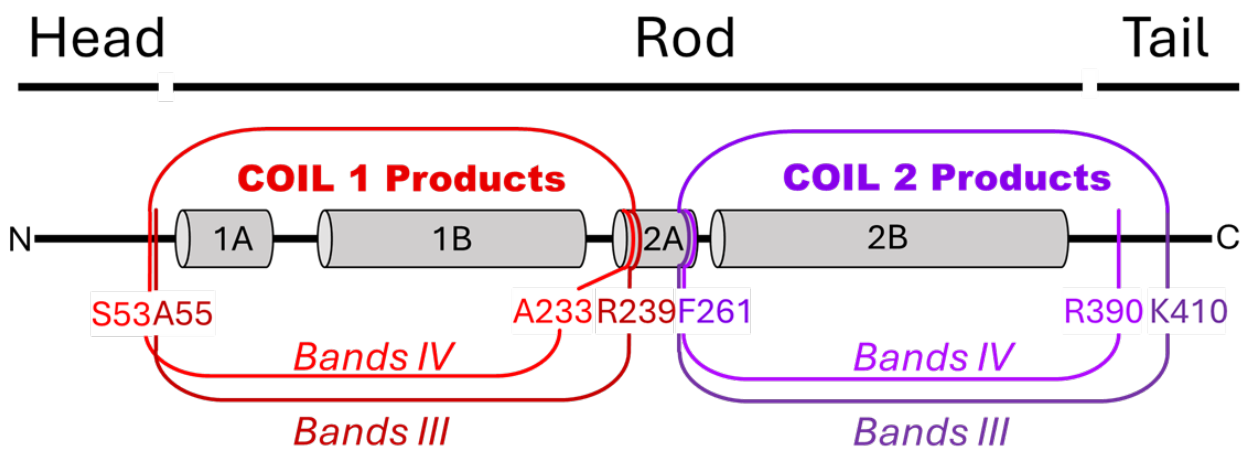


Figure 2

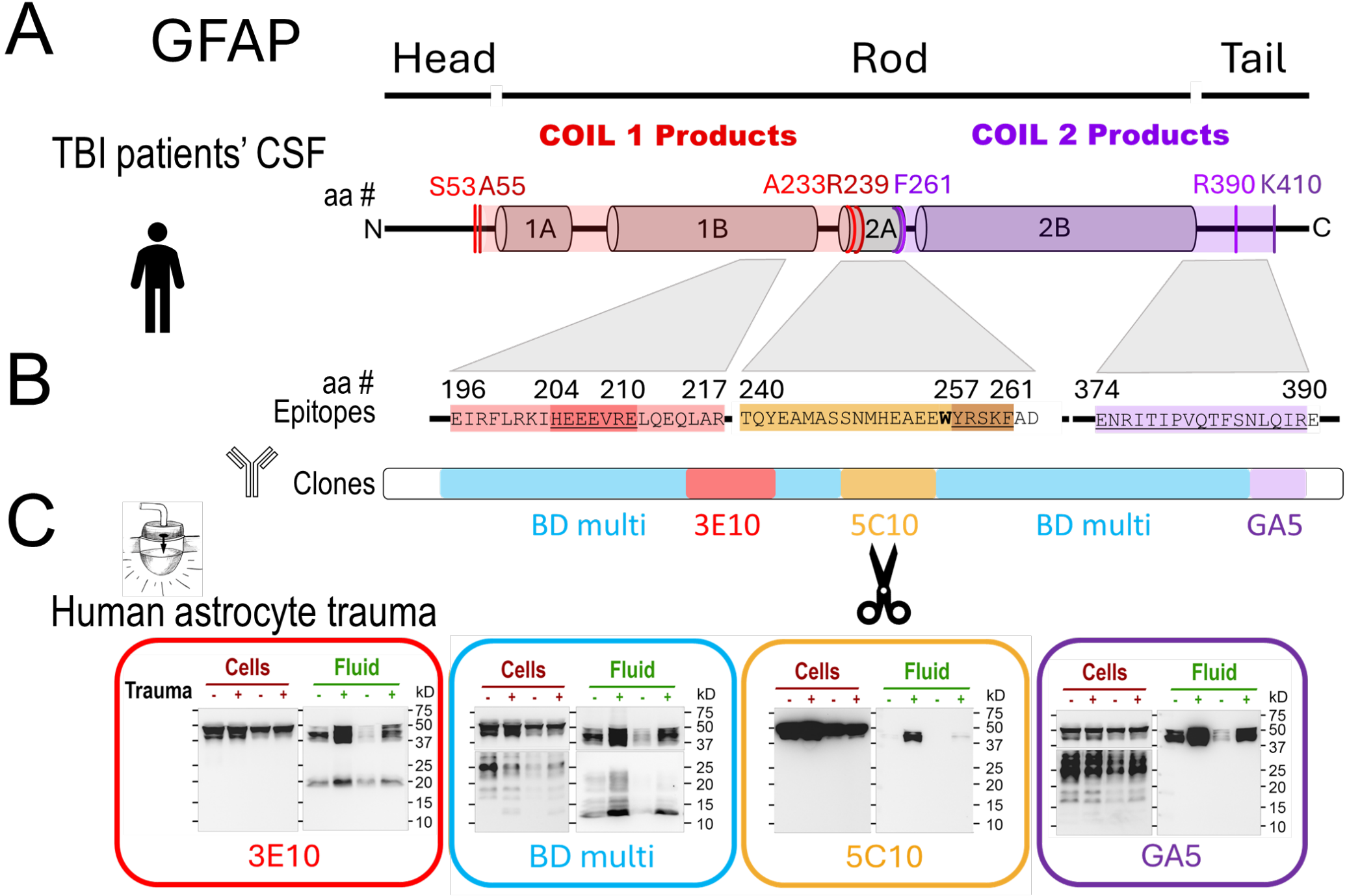

A
GFAP
Head
Rod
Tail
TBI patients' CSF
COIL 1 Products
COIL 2 Products
aa #
S53 A55
A233 R239 F261
R390 K410
N
1A
1B
2A
2B
C
B
aa #
196 204 210 217 240 257 261 374 390
Epitopes
EIRFLRKIHEEEVRELQEQLAR
TQYEAMASSNMHEAEEWYRSKFAD
ENRITIPVQTFSNLQIRE
Clones
BD multi
3E10
5C10
BD multi
GA5
C
Human astrocyte trauma
Trauma
Cells
Fluid
kD
75
50
37
25
20
15
10
3E10
BD multi
5C10
GA5

Figure 3 (A)

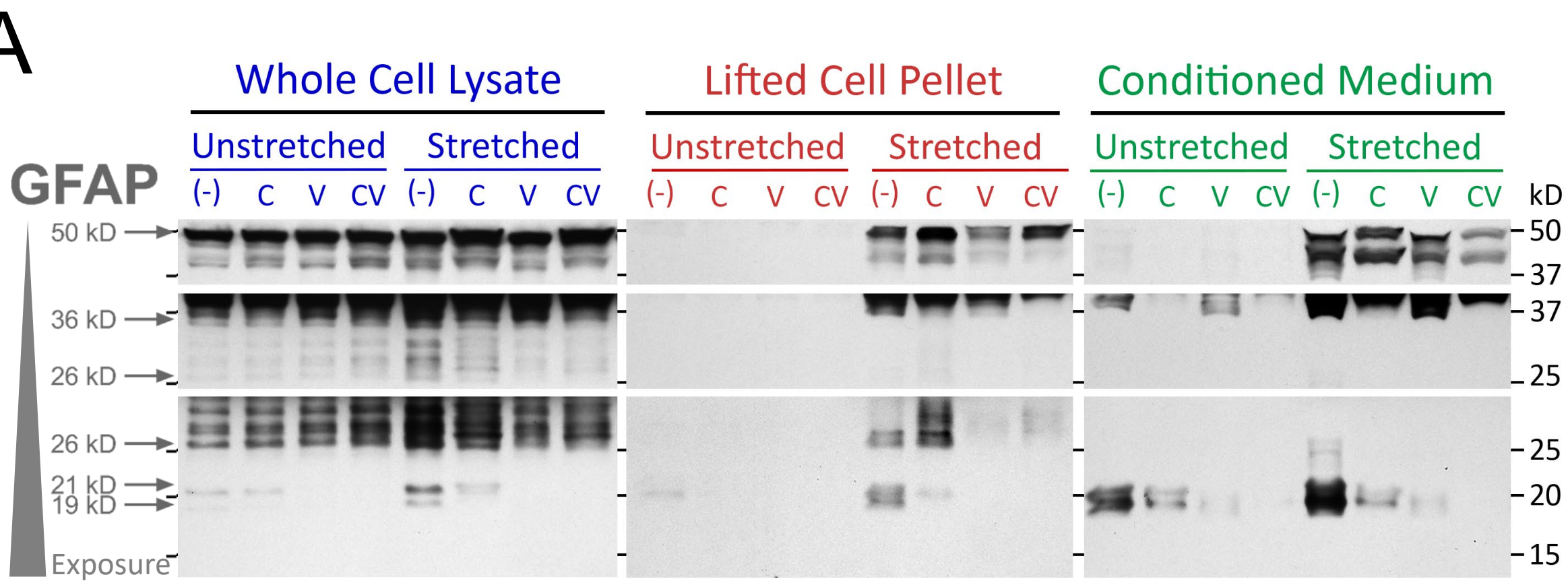

A
Whole Cell Lysate
Lifted Cell Pellet
Conditioned Medium
Unstretched
Stretched
Unstretched
Stretched
Unstretched
Stretched
GFAP
(-) c v cv (-) c v cv
(-) c v cv (-) c v cv
(-) c v cv (-) c v cv
kD
50 kD
36 kD
26 kD
26 kD
21 kD
19 kD
Exposure
50
37
37
25
25
20
15

Figure 3

B

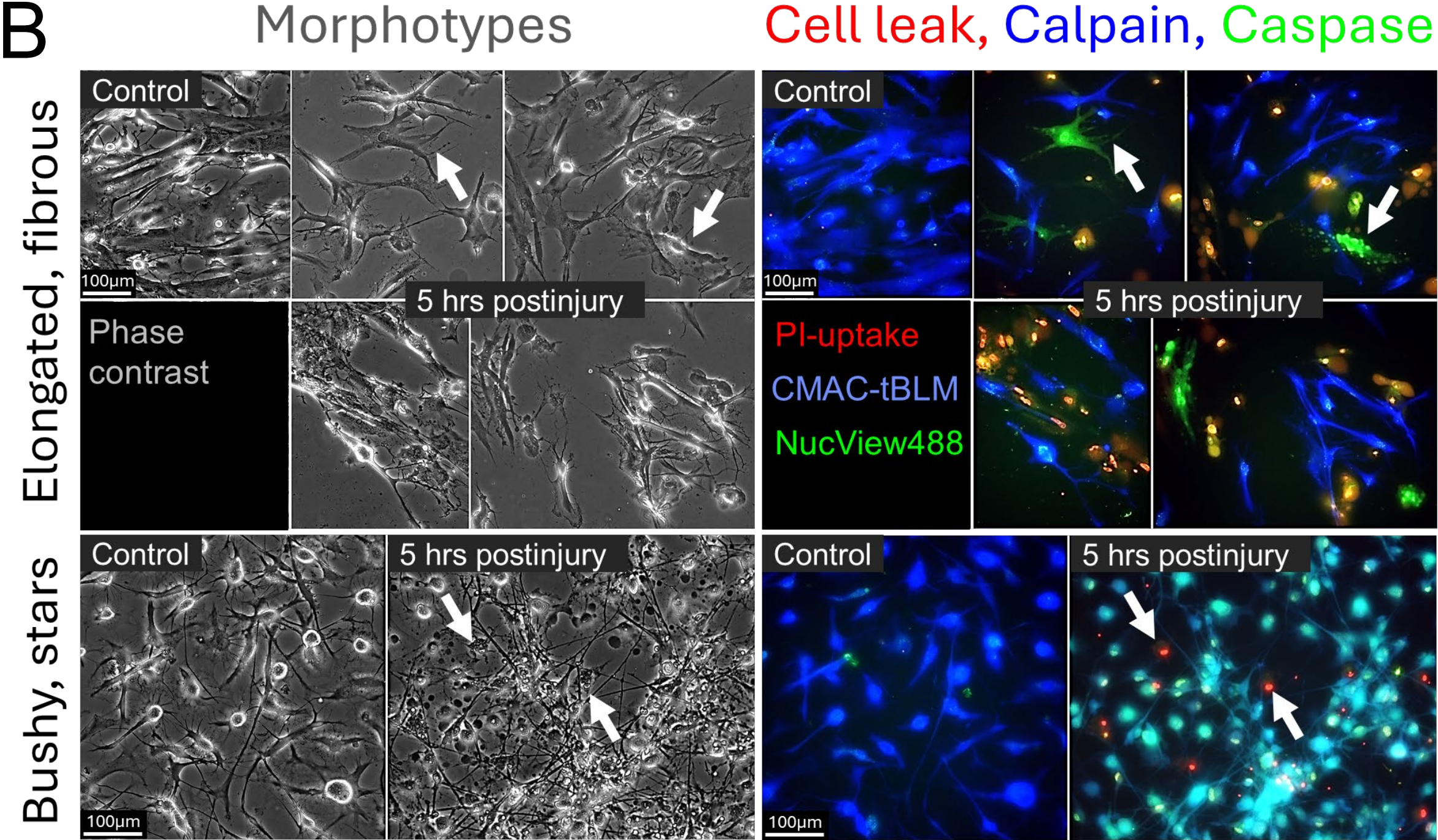

Morphotypes
Cell leak, Calpain, Caspase
Elongated, fibrous
Bushy, stars
Control
5 hrs postinjury
Phase contrast
100µm
PI-uptake
CMAC-tBLM
NucView488

Figure 3(C)

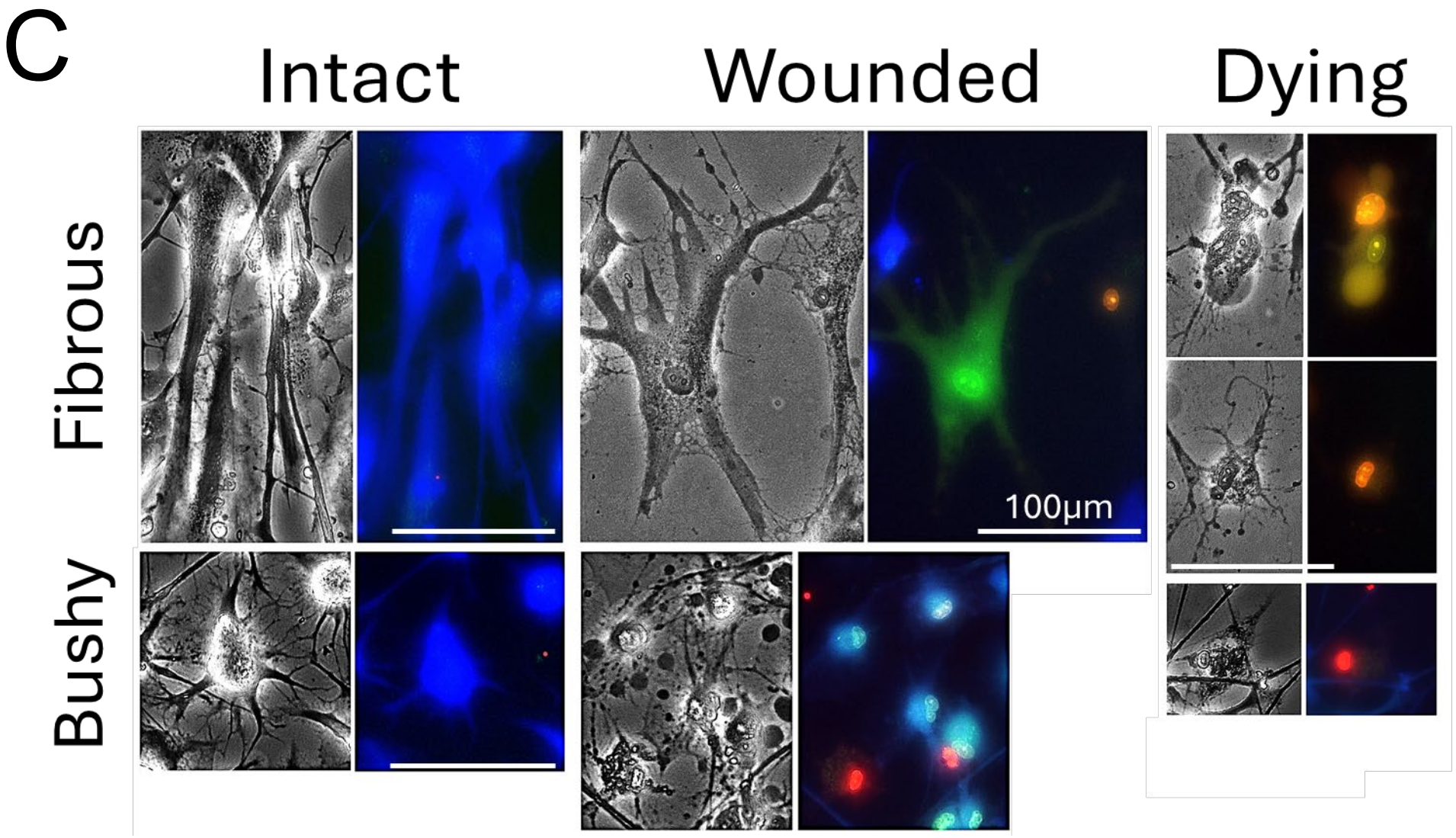
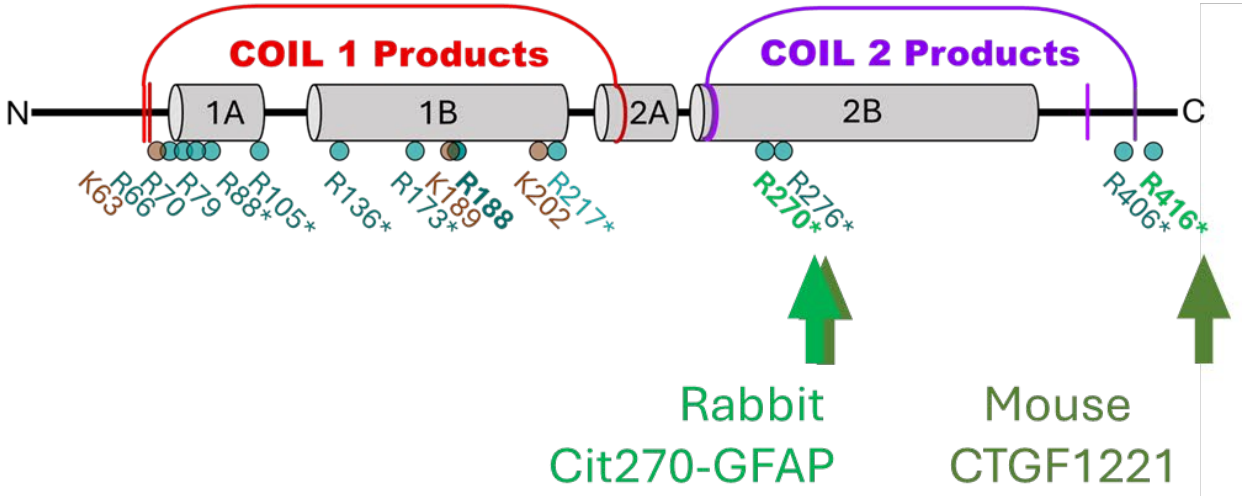


Figure 4

A



- 13 Citrullinated arginine residues
- 5 Acetylated lysine residues

B

Citrullinated GFAP Unmodified GFAP

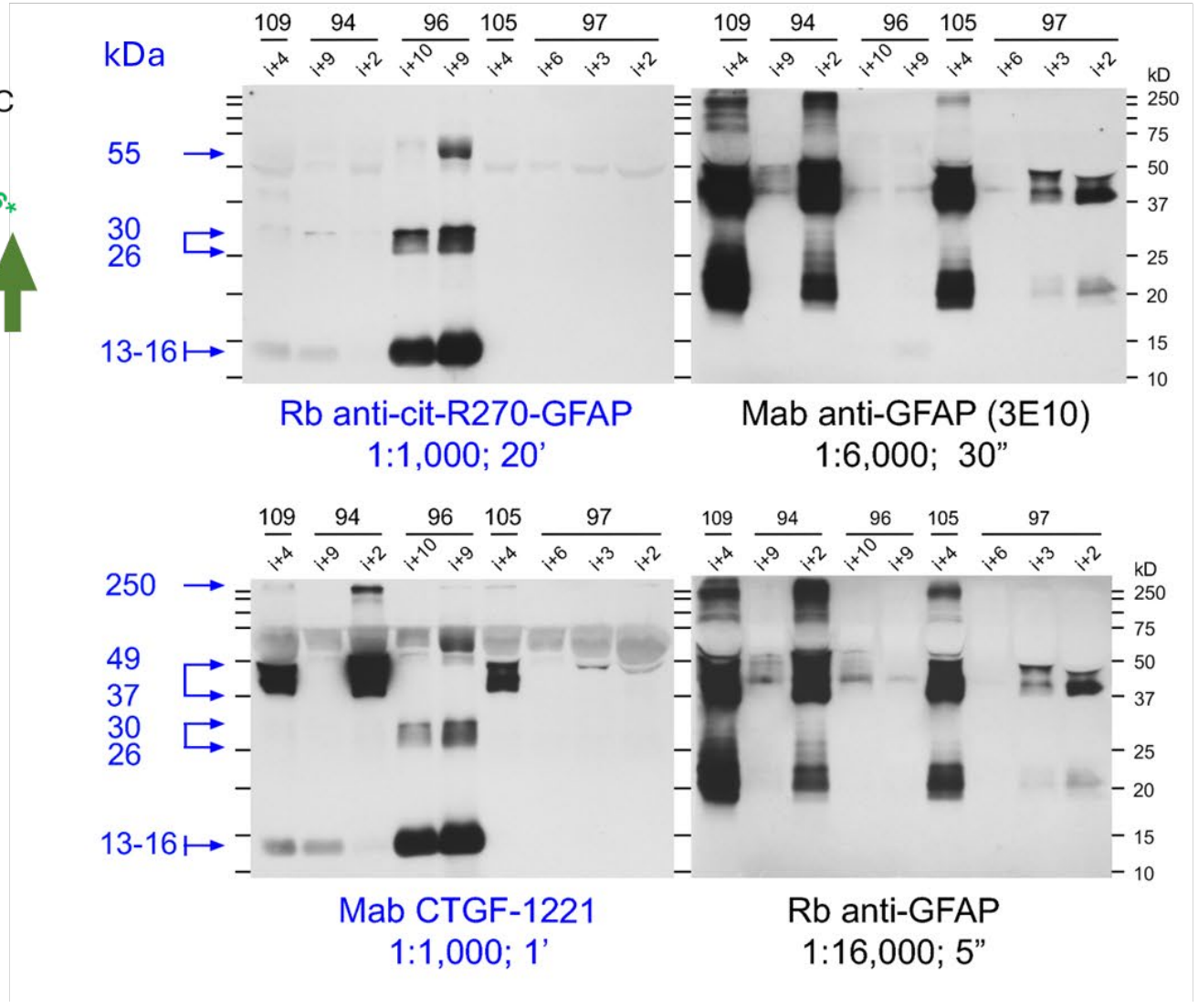


Figure 5

A

Control

Trauma

Unstretched

5 hrs post-stretch

24 hrs post-stretch

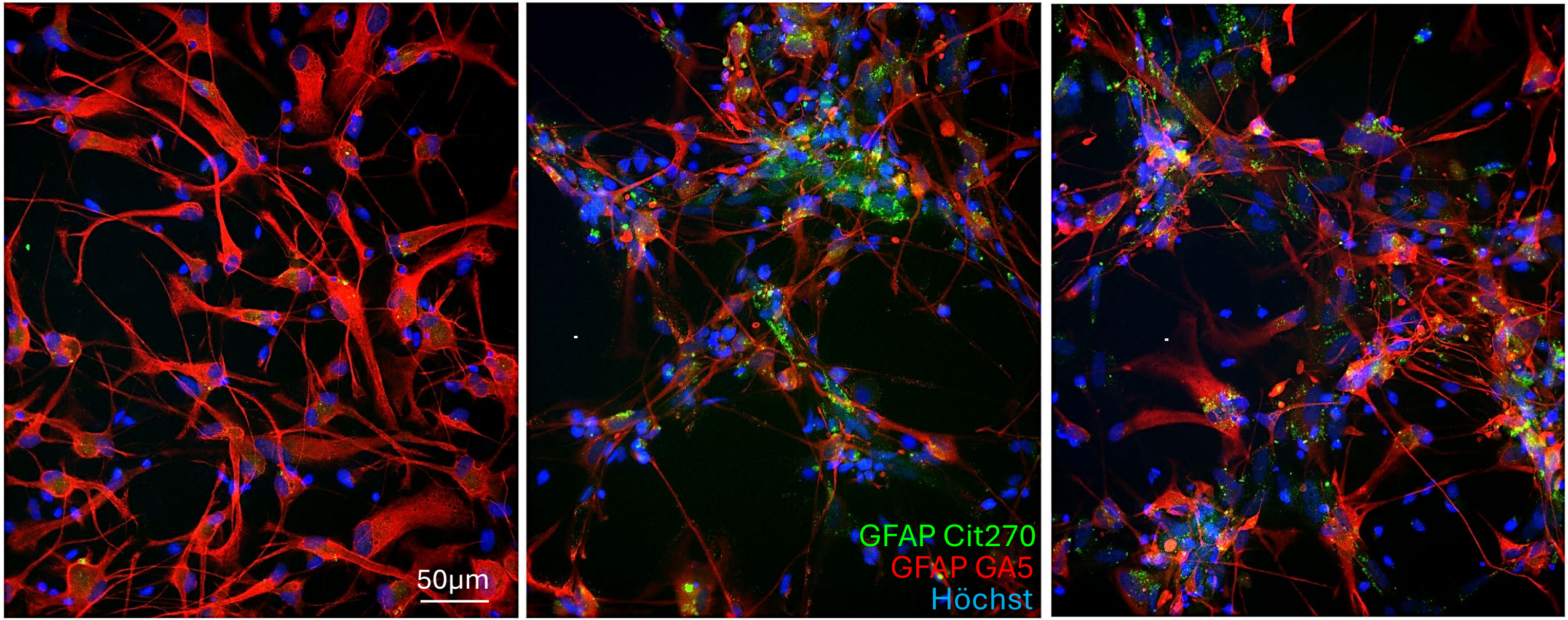


Figure 5 (B)

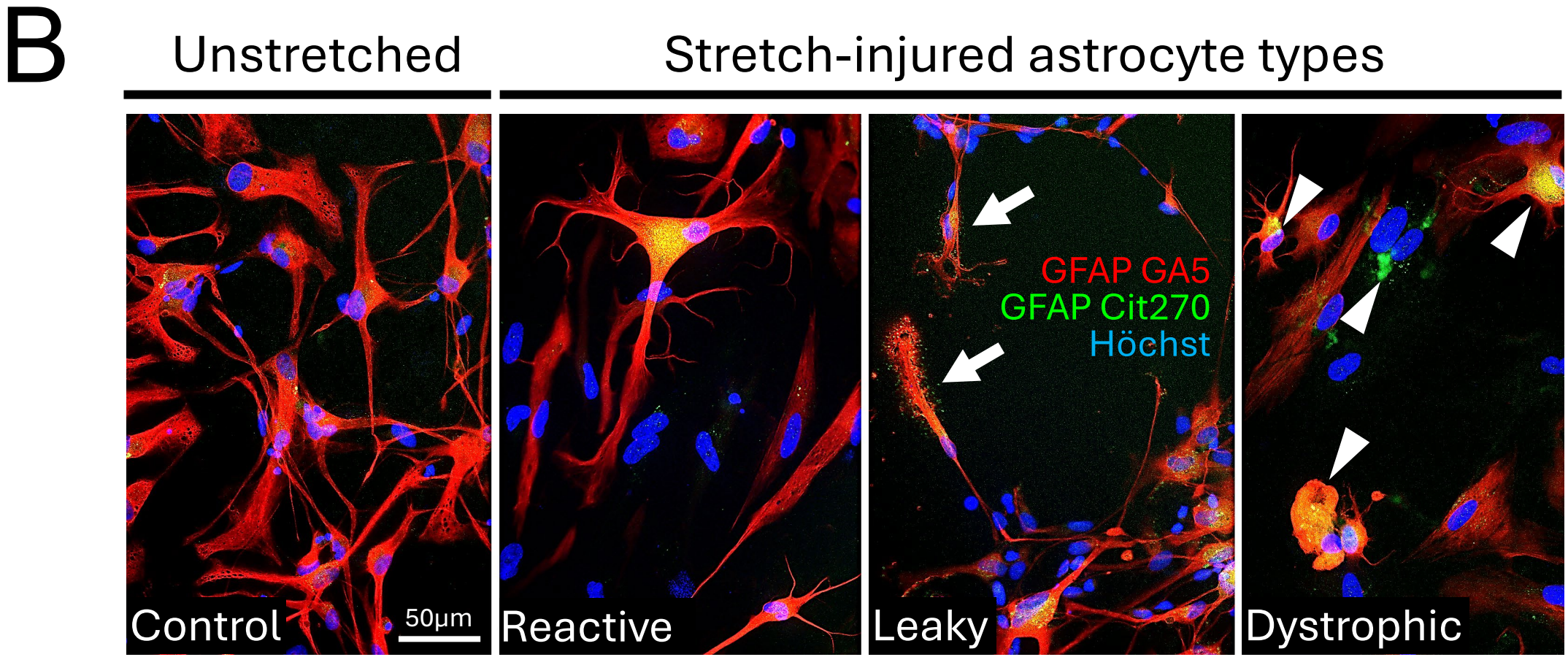
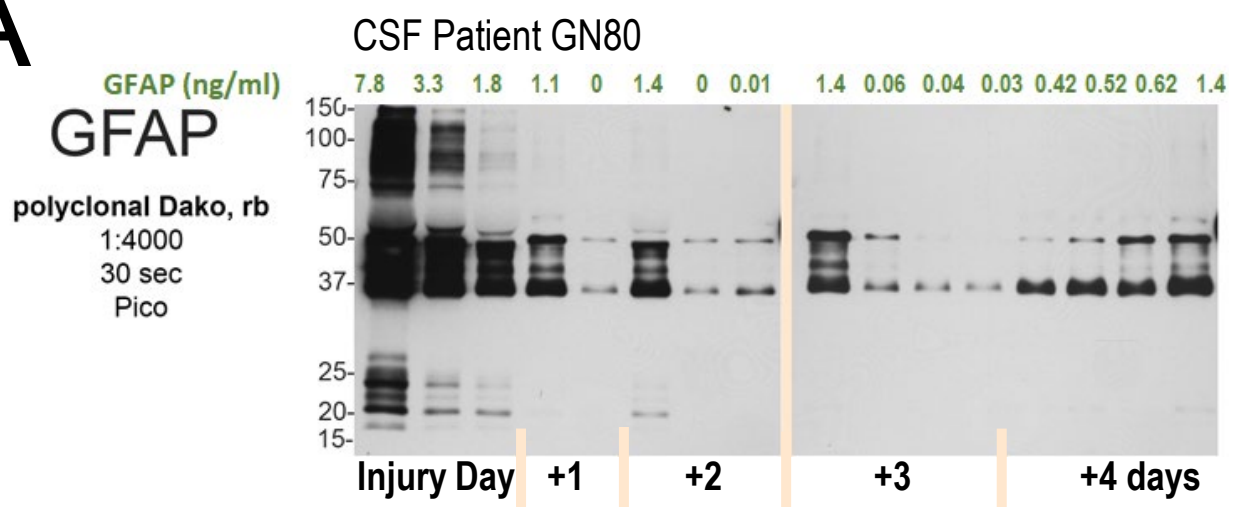
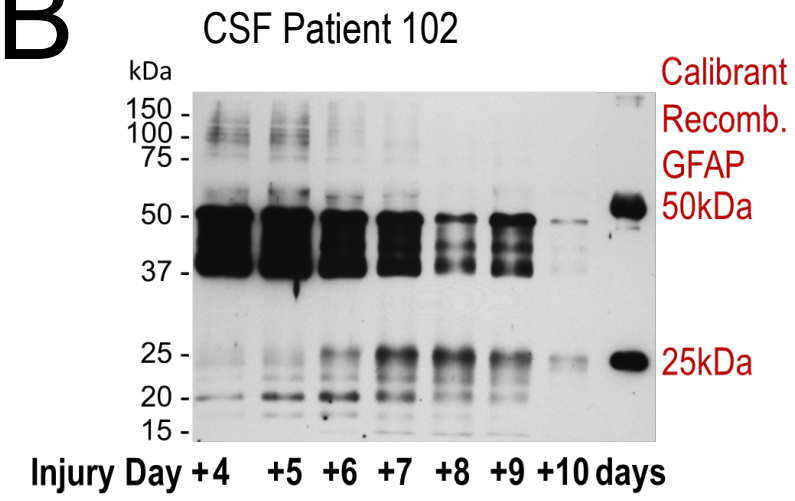

B
Unstretched
Stretch-injured astrocyte types
GFAP GA5
GFAP Cit270
Höchst
Control
50µm
Reactive
Leaky
Dystrophic

Figure 6

A


CSF Patient GN80
GFAP (ng/ml)
GFAP
polyclonal Dako, rb
1:4000
30 sec
Pico
7.8 3.3 1.8 1.1 0 1.4 0 0.01 1.4 0.06 0.04 0.03 0.42 0.52 0.62 1.4
150
100
75
50
37
25
20
15
Injury Day
+1
+2
+3
+4 days

B


CSF Patient 102
kDa
150
100
75
50
37
25
20
15
Calibrant
Recomb.
GFAP
50kDa
25kDa
Injury Day +4
+5
+6
+7
+8
+9
+10 days

C

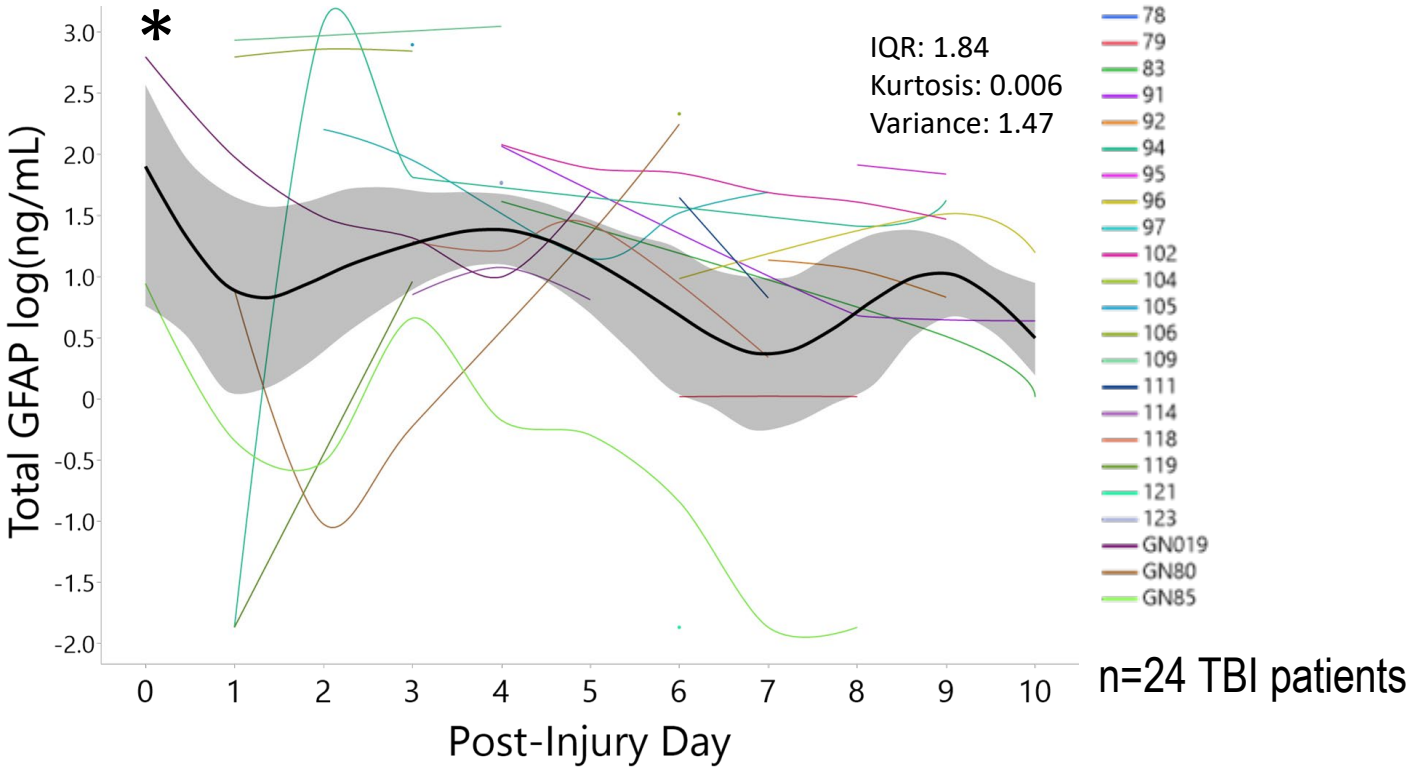

*
IQR: 1.84
Kurtosis: 0.006
Variance: 1.47
Total GFAP log(ng/mL)
3.0
2.5
2.0
1.5
1.0
0.5
0
-0.5
-1.0
-1.5
-2.0
0
1
2
3
4
5
6
7
8
9
10
Post-Injury Day
78
79
83
91
92
94
95
96
97
102
104
105
106
109
111
114
118
119
121
123
GN019
GN80
GN85
n=24 TBI patients

Figure 6 Continued

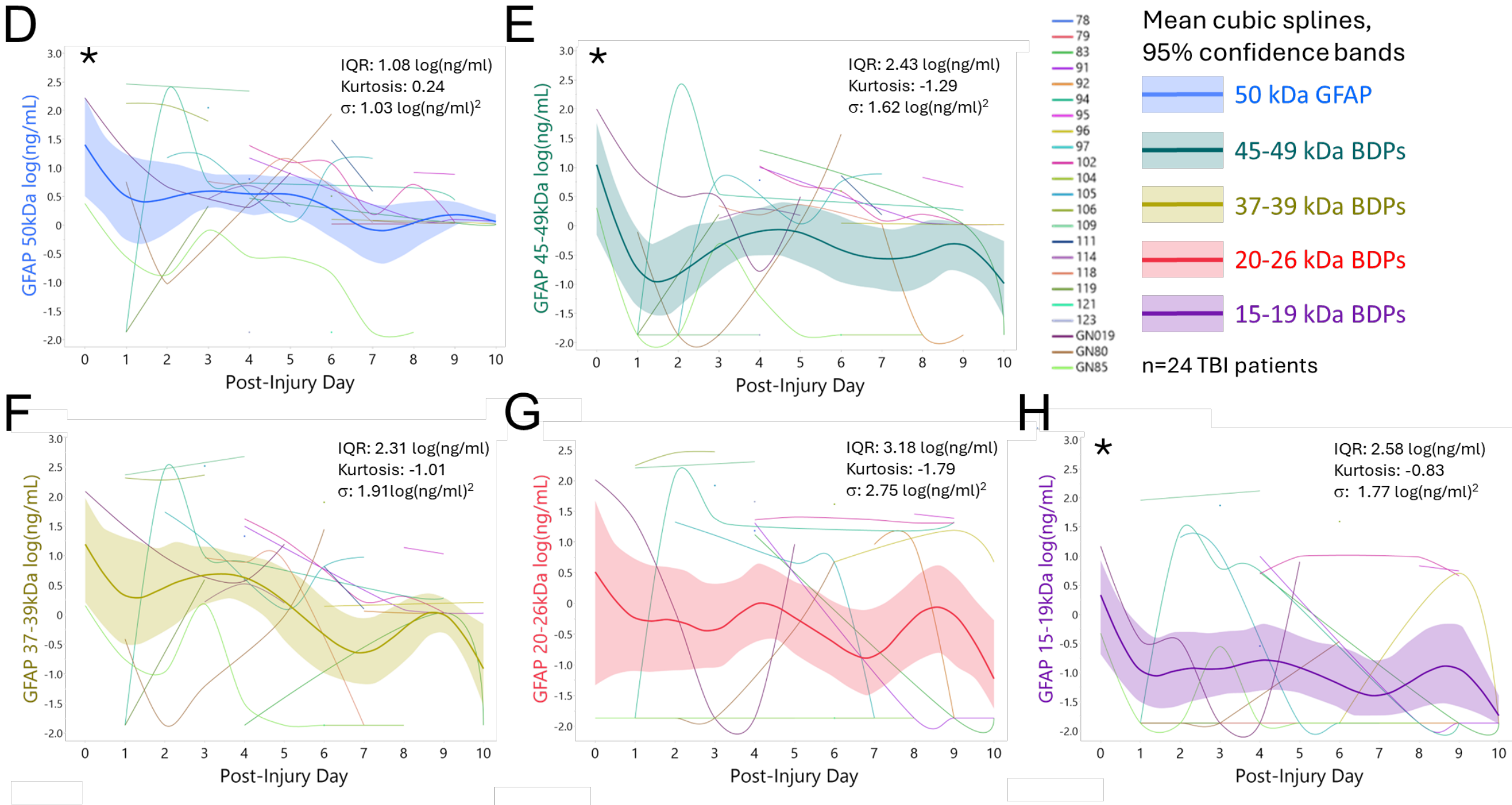


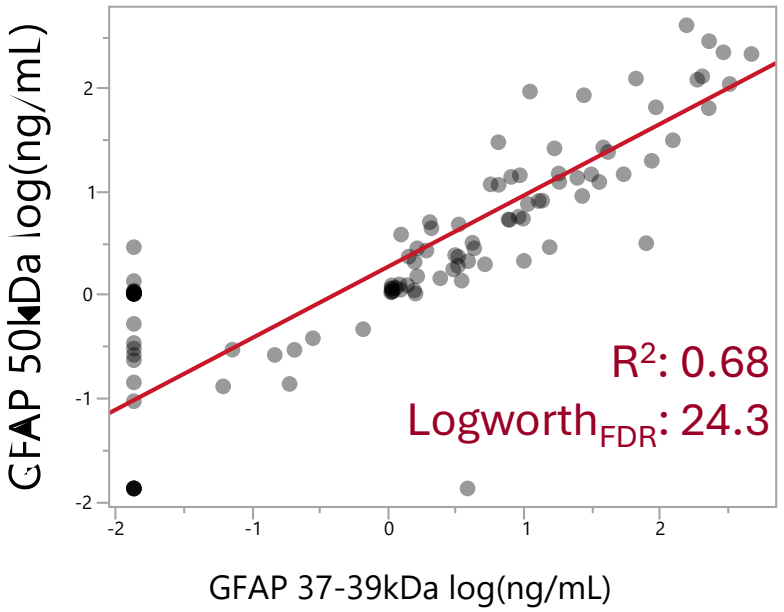
Figure 7

A

Pearsons correlation coefficients between GFAP proteoforms

	45-49kDa	40-42kDa	37-39kDa	20-26kDa	15-19kDa
50kDa	0.575	0.637	0.822	0.604	0.529
45-49kDa		0.596	0.589	0.413	0.422
40-42kDa			0.726	0.549	0.363
37-39kDa				0.714	0.567
20-26kDa					0.685
15-19kDa					

B



C

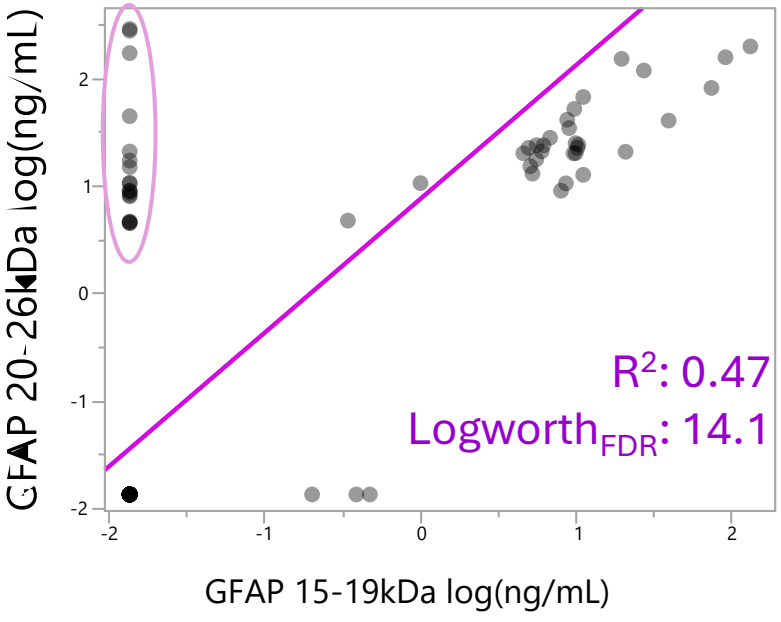
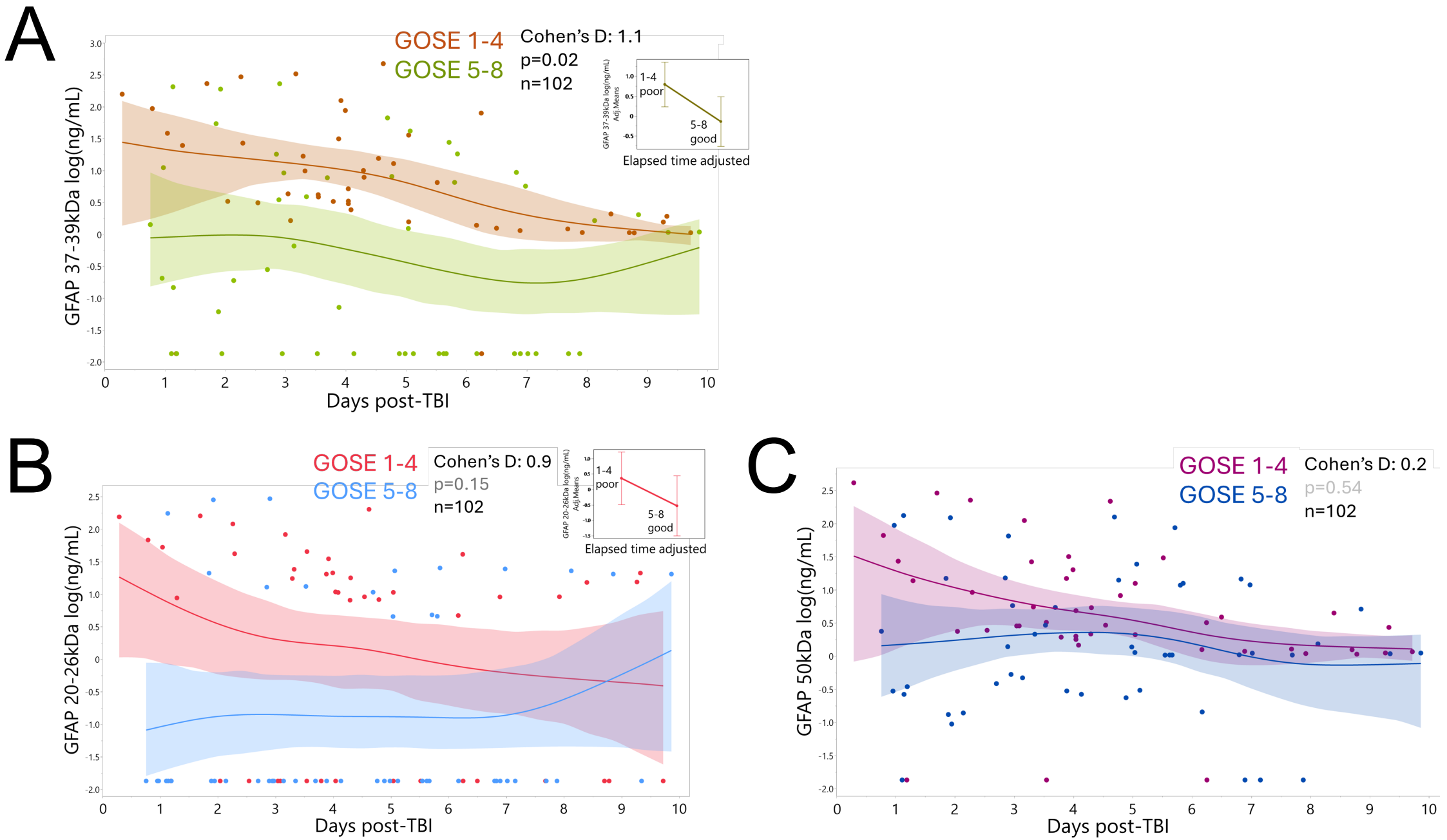


Figure 8


A
GOSE 1-4
GOSE 5-8
Cohen's D: 1.1
p=0.02
n=102
1-4
poor
5-8
good
Elapsed time adjusted
GFAP 37-39kDa log(ng/mL)
Days post-TBI
B
GOSE 1-4
GOSE 5-8
Cohen's D: 0.9
p=0.15
n=102
1-4
poor
5-8
good
Elapsed time adjusted
GFAP 20-26kDa log(ng/mL)
Days post-TBI
C
GOSE 1-4
GOSE 5-8
Cohen's D: 0.2
p=0.54
n=102
GFAP 50kDa log(ng/mL)
Days post-TBI