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

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ABSTRACT

Glial fibrillary acidic protein (GFAP) is an important clinical biomarker of traumatic brain injury (TBI), yet understanding the nature, timing, and impact of its degraded and modified products is needed for clinical utility. We report distinct GFAP breakdown products (BDPs) and post-translational modifications (PTMs) that are unique to TBI. Mapped PTMs provided fragment and patient-specific citrullination signatures known for destabilizing GFAP filaments. GFAP and its fragments from patients' cerebrospinal fluid (CSF) and serum were sequenced by mass spectrometry (MS), identifying two TBI-specific coproducts covering coil1 (20-26kDa) and coil2 (15-19kDa) in the rod-domain. These coproducts were imbalanced in biofluids with coil1-BDPs enriched over coil2-BDPs detected by label-free quantitative MS and independently confirmed by immunoblot densitometry in twenty-three TBI patients. Ten-day temporal trajectories showed ongoing proteolysis, progressing from large to small fragments, with prolonged elevation of 37/39kDa fragments, and delayed rise of small products. For the first time, profiles of GFAP fragments, but not of uncleaved GFAP, predicted six-month outcome (Extended Glasgow Outcome Scale, GOSE), highlighting proteolytic processing as prognostic biomarker signature. These new TBI-GFAP-BDPs were independently substantiated in a human culture trauma model, offering mechanistic insights and linking biomarker data to injury-induced astrocytopathy. To this end, specific findings include: Coil1-BPDs were fluid-released, while coil2-BDPs remained cellular, shown by epitope-defined coil-specific GFAP antibodies. A new cleavage site between the two coils was identified by selective epitope loss. Coil2's cellular retention could be explained by incorporation of citrullinated coil2-BDPs into non-filamentous aggregates within pathological astrocytes after injury. Trauma-triggered proteolysis involved calpains and caspases in distinct astrocyte injury states documented using inhibitors and live-cell reporters. These novel degradation findings have significant translational relevance for monitoring disease progression in TBI patients and for linking biofluid GFAP fragments to a trauma-inflicted astroglial proteinopathy during neurodegeneration.

INTRODUCTION

Traumatic brain injury (TBI) affects 56 million people worldwide annually; nearly one in five U.S. adults have a lifetime history of TBI, making it the most common neurological disorder [70, 96]. More than half of mild TBI and 85% of moderate-to-severe patients fail to achieve long-term recovery [110, 111]. TBI nearly doubles the lifetime risk (22-29%) for neurodegenerative disease, a public health challenge that grows as populations age [101]. As chronic impairment and post-traumatic neurodegeneration comprise a spectrum of multiple dementia etiologies after TBI, yet their driving pathology and molecular mechanisms are unclear [34, 143]. Defining early-stage TBI pathological proteoforms associated with biomarkers will facilitate risk assessment to capture emerging signatures that may develop into post-TBI neurodegeneration [21, 101].

A pathological hallmark of neurodegenerative diseases is accumulation of toxic proteoforms resulting from maladapted protein homeostasis, including amplified posttranslational modifications (PTMs) and protein degradation [53, 131, 167]. Deranged proteostasis is also a prominent consequence of TBI, triggered by rapid and sustained calcium elevation initiated by membrane disruption and excitotoxicity. Increased proteolysis with rapid postinjury activation of calpains and caspases is well-documented in pre-clinical TBI models [10, 14, 36, 136, 174]. While tau, ß-amyloid and α-synuclein proteinopathies are known in neurodegeneration and TBI, deranged proteostasis plausibly affects many more proteins. Aiming to connect evolving neurodegenerative processes with a diagnostic signature requires understanding the relationship between cell and biofluid proteoforms. While imaging and postmortem studies can confirm advanced proteinopathies, tools to monitor their early initiation after TBI remain underdeveloped. As proteolysis and altered PTMs emerge from early TBI pathophysiology, their study and detection in biomarkers are necessary for evaluating pathogenic conversion and improve recovery prognosis and potentially guide future therapeutic intervention [49, 114, 163].

Two strategies that may capture a pathological conversion of TBI toward neurodegeneration are: 1) linking early-elevated TBI biomarkers to neuropathology [125], or 2) investigating pathological changes in TBI biomarkers themselves. Post-injury calpain and caspase activation is often reported using αII-spectrin breakdown products (SBDPs), but their translation into clinically useful biomarkers is limited due to its ubiquitous expression [97, 128, 133, 147]. In contrast, astroglial intermediate filament glial fibrillary acidic protein (GFAP) is highly brain-enriched and extensively studied as a TBI biomarker: GFAP fluid levels are elevated with intracranial injuries [12, 105, 122]. Circulating fluid GFAP levels rise fast, peak 6-18h postinjury, and then decline [56, 121, 123, 134]. Elevated serum GFAP predicts poor outcome and mortality [95, 124, 134, 165]. GFAP blood testing is FDA approved within a narrow context of use (COU) on injury day [12, 118], and is incorporated into the new National Institute of Neurological Disorders and Stroke (NINDS) TBI classification framework as well as in preclinical biomarker validation studies, reflecting its growing role in translational and clinical TBI evaluation [13, 100, 158]. While well-established as an acute diagnostic biomarker, longitudinal monitoring of altered GFAP proteoforms and their possible use to indicate deranged proteostasis remain open priorities [39, 149, 163].

GFAP breakdown products (BDPs) are in biofluids after TBI and commercial GFAP assays include their detection, yet TBI-specific fragment sequences and their degradation kinetics are poorly defined [56, 102, 178]. Can pathological GFAP-BDP proteolysis be tracked after TBI? What connects GFAP biomarker signals with a neurotoxic proteinopathy? The mechanistic link between biofluid GFAP and its pathological proteoforms in injured astrocytes is unknown, beginning with the puzzle of the acute release of a cytoskeleton-tethered protein [126]. In neuropathology, GFAP upregulation is widely used as a reactive astrogliosis indicator, yet trauma-release occurs faster than de novo expression [60, 103, 145]. GFAP modification, misfolding, and degradation have detrimental consequences for astrocytes causing astrocytopathy [90, 103, 119, 153]. Those consequences are critical because astrocytes are indispensable for intact neurovascular coupling, integrity of the blood-brain barrier, metabolite support, neural network function, synaptic plasticity and cognition [15]. Astrocyte degeneration and their injury states after trauma are just beginning to be acknowledged [56, 109, 175]. The relevance of GFAP proteinopathy in neurodegeneration is underscored by its observed mutations in Alexander disease, an inherited astroglial pathology, where GFAP modification and fragmentation lead to toxic aggregation, Rosenthal fibers, and rise in biofluid GFAP levels [6, 37, 59, 65, 91, 104, 120]. Therefore, this study aims to clarify a critical relationship between GFAP degradation and modification in TBI biofluids and in injured astrocytes in order to improve biomarker interpretation and outcome prognosis.

To bridge these translational gaps, we sequenced and quantified TBI-induced GFAP fragments, then independently validated these fragments and determined their temporal trajectory in a cohort of TBI patients. We show that fragments, not uncleaved GFAP, predict outcome. Exploring mechanistic steps in a unique human trauma culture model, we confirmed trauma-initiated distinct BDPs with citrullination, identified a novel trauma-specific GFAP cleavage site and connected distinct product distribution fates with fluid elevation and

pathological astroglial GFAP aggregation. Together, these findings advance the molecular understanding of GFAP dynamics as neurotrauma biomarker and relate it with pathology relevant to neurodegeneration.

MATERIALS AND METHODS

Aim and study design

The objective of this study was to investigate relationships between GFAP proteoforms in TBI biofluids and in traumatized astrocytes. GFAP proteolysis and key PTMs were investigated in an observational TBI patient cohort. Trauma-induced fragments and proteoforms were quantified using label-free MS and GFAP-BDPs were validated by quantitative immunoblot densitometry and assessed for long-term outcome prognosis. These findings were further explored by mechanistic studies in a human astrocyte culture trauma model. Severity and temporal correlations of GFAP fluid levels with cell death and damage were already previously analyzed [56]. Thus, the current study aim was focused on targeted questions that include: Where and how proteolysis occurs, the fates of trauma-produced fragments, and the phenotypes of traumatized astrocytes accumulating these fragments.

Setting: TBI patients' demographic and clinical data and brain tissue specimen procurement

This prospective observational TBI cohort study included 112 CSF samples and 3 serum samples from 26 consented adult TBI patients admitted to neurocritical care at level one trauma centers at UCLA and at the University of Florida, Gainesville, and a commercial control serum sample (Precision Med; Supplement Tables S1, S2). Patient median age was 40±19 years, 22 subjects were male and 4 were female, and median score on the Glasgow Coma Scale (GCS) was 6 ±3.4. Enrollment occurred between 2018-2023 and included anonymous demographic and clinical parameters (Supplement Table S1). All except two patients had recovery scores on the Glasgow Outcome Scale (GOS) reported both at hospital discharge and by phone interview at six-month postinjury on extended GOS (GOSE). Deidentified human fetal brain tissue from voluntarily aborted pregnancies was donated adhering to stringent ethical regulations. See Declarations for Ethics approval statements on all with consent donated human specimen at the end. Cerebral astrocyte cultures were prepared from donated human fetal cranial specimen aged 16-19.5 gestational weeks.

Immunoprecipitation and mass spectrometry sequencing of GFAP from TBI patients

Mass spectrometry (MS) experiments were performed on six cerebrospinal fluid (CSF) and serum samples from four 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 200mM triethanolamine, pH8.6) followed by heat/ice denaturing and reduction in 1% ß-mercaptoethanol [20, 28, 107]. Eluted material was separated by preparative SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 10% NuPAGE gradient gels in morpholinoethansulfonic acid 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 antibodies (mabs) to GFAP (mab1A64-244, Abbott Diagnostics or mab 3E10, EnCor Biotechnology Inc., Supplement Table S3) to provide reference signals for gel band excision. Bands at 50 and 25kDa were omitted to avoid immunoglobulins (Supplement Fig. S1). Gel bands were fully destained, dried, and then

reduced with 10mM tris(2-carboxyethyl) phosphine in 100mM 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 12h. Peptides were extracted from the gel bands by three rinses of 50% ACN/0.1% formic acid solution at 37°C for 15min 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 (1h 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 45s. Digestion of defined amounts of recombinant human GFAP served as technical controls (Supplement Figure S2). MS data were searched against the human GFAP protein sequence (Uniprot (https://www.uniprot.org/uniprotkb/P14136/entry) via Proteome Discoverer (PD) version 1.4. SEQUEST-engine confirmed peptides were unique to GFAP (not shared by e.g. vimentin, neurofilaments). "High" confidence peptides (green on maps) had at least two peptides filtered using ≤1% false discovery rate (FDR) threshold of cross-correlation "X-corr scores" for peptide matches contingent on ion precursor charge states. Few "medium" confident peptides (yellow) had at least two peptides with ≤5% FDR (Fig. 1; Supplement Table S8). Identified PTMs included acetylation and citrullination that were further validated by: (a) a unique signatures of citrullination (immonium ion at m/z 130.0975 and/or neutral loss of isocyanic acid at 43.0058Da) or lysine (K) acetylation (cyclized immonium ion at m/z 126); (b) a MS2 product of b- and y-ions documented mass increments consistent with modifications (Supplement Fig. S10); (c) a comparison of identified citrullination sites after treatment with peptidylarginine deiminase (PAD) enzyme [67].

Partial chemical cleavage of GFAP for monoclonal antibody epitope mapping

The binding region of mab anti-GFAP clone 5C10 was determined initially by binding to the peptide covering amino acids (aa) 233-261 of the human coil2A sequence using dot blots (AALKEIRTQYEAMASSNMHEAEEWYRSKF). The specific epitope was subsequently identified using partial chemical cleavage at tryptophan, which there is only one in GFAP, aa W256. Recombinant GFAP (33µg) was treated in excess with 1% BNPS-Skatole (100µg; 1mg/ml glacial acetic acid; 3-bromo-3-methyl-2-(o-nitrophenylsulfenyl)indolenine, Sigma, Supplement Fig. S11), an oxidant and brominating agent selectively cleaving proteins at tryptophan residues [57, 155]. An equal volume of distilled water precipitated BNPS-Skatole that was removed by centrifugation (5min at 12,000rpm). The supernatant was lyophilized and the GFAP and tryptophan-cleaved BDPs were separated by SDS-PAGE, blotted followed by mab incubation of 5C10 or 3E10, a coil1 GFAP antibody, and a pan-intermediate filament antibody, IFA (hybridoma supernatant, epitope coil2B aa 358-377: ALDIEIATYRKLLEGEENRI) [79, 130].

Western blotting using scaled densitometry and GFAP calibration

SDS-PAGE was conducted as previously described using 7.5% acrylamide for αII-spectrin, 12% gels for GFAP [56]. Protease inhibitors (cocktail CompleteMini, Roche 1:50) and EDTA (10mM) were added to TBI patient CSF, samples were equilibrated to Laemmli sample buffer, boiled for 5min followed by ice incubation and ß-mercaptoethanol to 1% was added prior to loading 34 µl/lane. The same procedure was followed for astrocyte lysates and concentrated culture media 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 bottom. Proteins were cold transferred at 120V for 3h 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% BSA in TBST overnight at 4°C. Secondary antibodies conjugated with peroxidase (Pierce) were incubated in 5% BSA in TBST for 1h at RT. Enhanced Chemiluminescence (ECL) substrate was applied to washed membranes for 5min at RT (Pico and Femto ECL, Pierce) and multiple exposures were used either of Kodak films or with an iBright imager

(ThermoFisher Scientific). Calibrants were recombinant human full-length GFAP and a 20kDa His-tagged GFAP-BDP (aa 72-217, EnCor Biotechnology Inc.) diluted with 0.5% BSA in TBS at defined concentrations (0.08-2.6ng) that were measured at different exposures (Supplement Fig. S5, Table S4). Densitometry was performed on eight exposures from each blot (2s-20min) after background correction. Subsaturated 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 between two exposures (Supplement Table S4). Band sizes were computed using distances of each molecular weight marker from the gel-front and curve-fit equations.

Antibodies used on TBI CSF and astrocyte samples

All antibodies are tabulated with known epitopes, sources, dilutions and Research Resource Identifiers (RRIDs, Supplement Table S3). A rabbit polyclonal GFAP antibody (DAKO) was used for immunoprecipitation and immunoblotting. For densitometry the antibody was applied initially at a high dilution (1:20,000-150,000) for detecting full-size GFAP and large GFAP-BDPs, followed by a lower dilution (1:5,000-7,000) for detecting small GFAP-BDPs, which were adjusted in subsequent scaling. Mab 3E10 was generated to a recombinant construct containing aa 72-217 of human GFAP, comprising coil1A and 1B. The epitope for 3E10 was localized to aa 196-210 (EIRFLRKIHEEEVRE), using peptide competition with a set of 20 nested aa peptides, each overlapping by 5aa. Additional mabs were 5C10 (EnCor Biotechnology Inc.), 1A64-244 (Abbott Diagnostics), a mixture of three clones (4A11, 1B4, 2E1, BD Pharmingen) and mab GA5 (Sigma [27, 29]). Citrullination studies used clone CTGF-1221 that was kindly provided by Akihito Ishigami (Professor, Tokyo Metropolitan Institute for Geriatrics and Gerontology) to detect citrullinated GFAP (cit-GFAP) at aa sites R270 and R416 [63]. 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 mab against αII-spectrin was used at 1:3,000-1:5,000 dilution (Enzo Life Sciences).

Human cerebral astrocyte trauma model

Human culture trauma model: In vitro differentiated fetal cerebral astrocytes were prepared as previously described [159]. Briefly, cortical tissue was mechanically dissociated, filtered and astrocytes were enriched by Percoll gradient centrifugation. Cells were expanded in DMEM/F12 with 10% fetal bovine serum. 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 compromising membrane integrity that led to delayed cell death [56].

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 5h 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 dithiothreitol, DTT (Calbiochem)), 1% NP40 and 1 tablet/10ml protease inhibitors (Mini Protease Inhibitor Cocktail, Roche). Lysis buffer was incubated for 5min at 4°C, lysates were collected by scraping into low binding tubes and homogenized by 3x5s sonification bursts

without foaming (Branson Digital Sonifier, 90% or constant amplitude duty cycle, output level 5). Culture medium conditioned by astrocytes (CM) 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) and then lysed for 5-10min in 0.5% lysis buffer followed by 3x5s sonication. 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 the original volume to obtain a protein concentration of 1-1.5 µg/ml. All samples were immediately frozen in aliquots at -80°C.

Protease inhibition in the human trauma culture model: Pan-calpain inhibitor calpeptin (13µM Z-Leu-nLeu-H, Millipore/Sigma), a non-toxic cell-permeable peptide aldehyde inhibitor [85, 172]. Pan caspase inhibitor ZVAD-FMK (11µM benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone; Enzo; ThermoFisher) is a cell-permeable, irreversible pan-caspase inhibitor that is effective against a spectrum of caspases (1-10, except 2; Invitrogen) [42]. ZVAD-FMK is reported showing also calpain inhibition after controlled cortical impact to the mouse brain [75]. Inhibitors were applied 10min to cultures after stretching omitting gentamicin in the medium and were incubated with cells for 5, 24 or 48h replenishing drugs every 8-10h. Other tested calpain inhibitors included leupeptin (20µM) and SJA6017 (10µM Calpain Inhibitor VI, Calbiochem) had limited effect, were toxic by either increasing cell permeability as did PD1500606 (100µM, Calbiochem), or by increasing cell death as did MDL28170 (Z-Val-Phe-al, 4µM, Calbiochem) and were not further pursued.

Statistical analysis approaches

GFAP band measurements were assessed for normality by the Shapiro Wilk criterion for Gaussian distribution fit. GFAP and GFAP-BDP measurements deviated from normal distribution, determined by significantly lowered W-values, and were log-transformed replacing zero with half of the lower limit of detection (0.7pg or a concentration of 27pg/ml; Supplement Fig. S6).

***Reproducibility*:** Immunoblot densitometry reproducibility was determined on technical replicates using the coefficients of variation (CV=SD/mean x100, Supplement Table S5).

Covariates: Effect modifiers patient age, race, sex and concurrent body temperature, and their interactions were evaluated.

Model of variance components: Used was a restricted maximum likelihood (REML) linear mixed model (LMM) with patient identifiers (IDs) as random effect to account for repeated measures per patient. Added to the model was injury elapsed days, and knotted spline estimation.

Effect testing: For the effect of age, partial omega squared (ω_p^2) was used to report the proportion of the population variance fixed effects because it adjusts for variable sample size [80]. It was computed using fixed effect statistic (F-statistic) results of the REML-LMM output in SAS/Jmp along with degrees of freedom (df) and its denominator term (DFDen) as follows: (1)

$\omega_p{}^2 = \mathrm{F} * \mathrm{df}_{\mathrm{effect}} - (\mathrm{df}_{\mathrm{effect}}/\mathrm{F} * \mathrm{df}_{\mathrm{effect}}) + \mathrm{DFDen} + \mathrm{df}_{\mathrm{effect}}$. (1) *Age covariate effect formula*

Temporal profiles: Longitudinal means were graphed by spline fit lines from resampled daily CSF draws along with 95% confidence intervals (CI, Figs. 7C, 8). Temporal GFAP and fragment trajectories were evaluated using REML-LMM with age as covariate (Supplement Table S7 and Figure S13). Cohen's D effect sizes on each postinjury day (PID) were computed using adjusted means (Adj.Means) on each PID compared with overall means weighted for sample availability on each day [61, 112] with pooled (SD) from the total random variance component (Supplement Table S8, Supplement Figure S10) [82]. Formula (2):

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

Bivariate correlations: To assess GFAP substrate-product and product-product relationships, log-transformed GFAP band signals were pairwise correlated reporting uncorrected Pearson correlation coefficients (Fig.3, Supplement Fig. S8). Biplots used orthogonal fit for equal variance between GFAP bands and are reported with coefficient of determination (R^2) and Benjamini-Hochberg-based FDR-corrected Logworth, [$-\log_{10}$(p-values)], (Fig. 3). The outlier portion is documented with robust correction defined as percent of datapoints with a distance exceeding 3X the residual SD. Correlation outliers that might affect the correlations across all variables were inspected using the Mahalanobis distance to determine potential multivariate outlier with the threshold being the upper control limit, UCL and calculated using sample size and the number of variables used, by applying robust weighting to discern outliers [54, 83, 152].

Outcome prognosis: The GOSE score was used to assess six-month recovery prediction by longitudinal GFAP profiles. GFAP trajectories are shown for good (GOSE 5-8), and poor (GOSE 1-4) outcomes and were graphed using cubic splines with same curvature fit (λ= 0.28) and 95% CI (Fig. 4). GFAP profiles were assessed for their ability to distinguish good versus poor outcomes using a REML-LMM model with ID as a random and elapsed PIDs (modeled with knotted splines) as a fixed effect and controlling for age. Differences in GFAP adjusted means between good and poor recovery were calculated as described as follows in formula (3).

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

Outcome effect formula

Cohen's D in SD units here uses total residual variant component capturing within-subject variability (Supplement Table S7). Fig. 3D shows adjusted means using above REML-LMM model with standard errors and post-hoc t-statistic and patient ID as random effect and elapsed postinjury time as fixed effect.

Label-free MS analysis: 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 GFAP-BDPs as fixed effect while undetected peptides were excluded (Fig. 1D; Supplement Fig. S3).

RESULTS

Novel trauma-specific GFAP breakdown products in TBI patients and their fluid imbalance

GFAP-BDPs in TBI patients differ from Alzheimers and Alexander Disease fragments

GFAP displays a range of fragments and cytoskeleton proteolysis is increased after TBI [36, 56]. Here, we determined TBI-specific GFAP degradation by sequencing fragments of immunopurified gel-slices (I.-IV.) using MS in six TBI CSF samples (Supplement, Fig.S1). Molecular masses of each size-range were estimated pooling slice-specific high-confidence peptides from each patient and compared with band migration on GFAP immunoblots (Fig.1AB). Bands (I.) contained a 41.7kDa fragment. Bands (II.) migrated between 36-39kDa as shown in GFAP immunoblots and contained peptides covering a calculated mass of 40.6kDa. This slightly higher mass from pooling indicates variable proteolysis across patient's samples. Further, charged GFAP residues may have contributed to slightly faster gel migration [51, 141]. Bands (III.) contained two BDPs of the alpha-helical

rod domain: a coil1 covering 21.8kDa product and a 17.5kDa coil2B product (Fig.1AC). Bands (IV.) included a shorter coil1-BDP (21.3kDa) and a shorter coil2-BDP (15.2kDa). These sequences confirm previously observed 42, 37 and 18-25kDa GFAP-BDP bands and support a consecutive cleavage producing various sized coil1 and coil2 products [56]. None of the small products covered peptides in coil2A, the linker region between them, while these aa were readily detected in larger bands (47, 50kDa) and in full-length recombinant GFAP (Supplement Fig.S2). Peptide absence between the two coil products suggests one or more previously uncharacterized trauma-specific cleavage site(s), which was further investigated in a human trauma model (see below).

Coil1 products had the same N-terminus yet a four-residue larger C-terminus in slice (III.) versus (IV.) These coil1 products extended beyond a prior reported caspase 6 cleavage (at VELD225) associated with Alexander disease [27]. Immunoblots using a polyclonal antibody detected small GFAP-BDPs within these observed molecular masses (Fig.1C). Coil2-products had the same N-terminal phenylalanine (F261) but distinct C-termini, with the longer one ending at K410 (151aa, 17.5kDa in slice III.) and the shorter coil2 ending at arginine (R390, 130aa, 15.2kDA, in slice IV., Fig.1AB). Coli1 mab 3E10 did not detect the 17kDa band confirming its coil2 (Fig.1B, Supplement Fig.S1). Notably, the TBI-associated 15-17.5kDa coil2-products were consistent with an AD-reported caspase 3 cleavage site (aa residues 258-266, RSKFA↓DLTD), but smaller than the 30kDa GFAP fragment identified in Alzheimer's disease (AD) samples [108]. Documenting different GFAP-BDP endings in TBI versus other neurodegenerative diseases underlines conditional specificity in its proteolysis and hence limits extrapolation from recombinant GFAP digests in cell-free systems or after toxin treatments [171].

Imbalance of coil1 and coil2 GFAP breakdown products in TBI patients' biofluids.

As high-confidence green peptide markings show, coil1 had greater coverage than coil2 products. This imbalance was quantified showing peptide frequencies across MS runs with significantly higher coil1 (59±29%) versus coil2 (13±7%) frequencies (Fig.1D). Ion peaks were significantly higher in coil1 over coil2 peptides, further supporting greater coil1 abundance in TBI CSF (Supplement Fig.S2). In serum, TBI had elevated 37kDa and 20kDa bands over control (Supplement Fig.S4A). Immunoprecipitated GFAP from four different TBI serum samples of two patients identified a coil1-product spanning aa51 to R217 (97 residues, 18.2kDa, Supplement Fig.S4). With 59% sequence coverage, the termini could not be confidently identified. Noteworthy, coil2-containing peptides were absent, further underlining a TBI biofluid imbalance between the two coils.

In summary, deep sequencing of GFAP-BDPs in TBI patient biofluids identified proteolytic processing expected to generate equimolar coproducts, yet coil1-products were overrepresented over coil2 products. Prevalence of these GFAP-BDPs was further validated in a cohort TBI patients' CSF.

Temporal GFAP proteolysis and fragment-specific imbalance in neurointensive care TBI patients

Temporal dynamic of GFAP degradation after TBI

MS fragment sequences were validated by independently quantifying matching GFAP bandsets in 23 TBI patients' serial CSF samples over ten postinjury days (PIDs). Scaled immunoblot densitometry allowed standardizing multiple sub-saturated exposures followed by calibration to known dilutions of intact and small BDP recombinant GFAP proteins. This expanded the dynamic range to span four-five orders of magnitude documented by limits of detection and quantification (Supplement Table S4, S5, Fig.S5). Inter-experimental replicates provided technical reproducibility with average CV of 27.8% (Supplement Table S5) [80].

Longitudinal GFAP profiles document substantial variability in summated GFAP ("Total"), full-length and degradation products over ten PIDs across patients expressed by interquartile range (IQR), kurtosis and variance (σ) for each bandset (Fig.2). GFAP levels increased significantly with patient age, as it contributed 23-38% to the residual variance of uncleaved GFAP and three sets of GFAP-BDPs after adjusting for time and donor effects (ω_p^2, Supplement Table S6, Fig.S7). Total GFAP levels peaked on injury day with age-adjusted effect

size of 1.3 SD units above the weighted mean (Cohen's D, p=0.035, Figure 2C). Most bands' daily means exceeded the overall mean on until PID4, and by PID7 onwards means were observed below overall mean documenting an overall GFAP decline in CSF over time. Specifically, mean profiles of uncleaved and large 45-49kDa fragments declined significantly over time, while all other fragments (15-39kDa) had also secondary raises (Fig.2F-H). This distinction supports ongoing proteolysis contributed to the longitudinal GFAP pattern. The two sets of small GFAP-BDPs had wider confidence bands caused by secondary peaks along many undetectable values, prompting further inspection.

Fragment covariation and imbalance of small GFAP-BDPs

Strongest correlation was determined between 50kDa GFAP and 37-39kDa fragments (Pearson coefficient, r=0.82, R^2=0.68, LogWorth=24.3; Fig.3). The 40-42kDa fragments correlated similarly with the 37-39kDa fragments (r=0.73, LogWorth=16.3). These plausibly reflect co-release of substrates and intermediary products. Small BDPs, notably the 15-19kDa bandset had weaker relationships with precursors (Fig.3; Supplement Fig.S8). Further, 15–19kDa BDPs had 28% more undetected values than 20–26kDa BDPs across patients (Fig.3C). Small BDP coproducts covaried also less with one another. This TBI cohort quantification provides further evidence for a selective absence imbalance among small GFAP products supporting MS-observed coil1/2 imbalance. These findings are further explored using a trauma culture model for mechanistic insight (see below). Given both full-size and fragment GFAP species in our dataset, we sought to determine which GFAP proteoforms would result in better outcome prognosis in moderate-severe TBI [134]. This distinction may inform whether uncleaved GFAP or its post-traumatic proteolysis relate better to overall TBI recovery.

Profiles of GFAP fragments predict TBI outcome but not that of full-length GFAP

To investigate how GFAP degradation relates 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 using GOSE. Age-controlled CSF means of 37-39kDa GFAP-BDPs distinguished patients with poor outcome (GOSE 1-4) from those with favorable outcome (GOSE 5-8, Cohen's D: 1.1 Standard deviational units, p=0.02; Fig.4A, Supplement Table S7). Small 20-26kDa BDPs separated good-versus-poor GOSE early postinjury with lower levels in patients with good outcome, while adjusted means overlapped during later PIDs (Fig.4B). Same GOSE-binarized partitioning of full-length GFAP signals led to substantial mean and confidence band overlap between poor and good recovery across ten PIDs. Similar outcome prognosis for same GFAP proteoforms were observed using stricter partitioning of favorable outcome (GOSE7+8), indicating the findings were independent of the way GOSE scores were dichotomized (Supplement Fig.S9). In summary, longitudinal profiling of GFAP degradation products informs on temporal proteolysis and provides greater prognostic resolution than full-length GFAP.

Citrullinated GFAP-BDPs show a TBI patient signature distinct from that of classic GFAP

The uneven fluid presence of stoichiometrically equimolar generated coproducts was measured by label-free MS comparison of coil1 and coil2 peptide abundances and by immunoblot densitometry of small GFAP-BDPs supporting selective coil2 fluid deficits. We then investigated whether TBI-associated GFAP modifications may be present in CSF possibly associated with fragment disparity. Acetylation and citrullination promote selective destabilization and aggregation and associate with neurodegeneration [24, 69, 84, 103, 157]. Notably, citrullination has a destabilizing effect on intermediate filaments, is reported in rodent injury models and citrullinated GFAP is observed in chemically stressed cell lines and cell-free systems [64, 67, 84, 126, 166]. To our present knowledge, GFAP-PTMs have not been characterized in human TBI patient samples.

Validated mapping of citrullination and acetylation in GFAP fragments from TBI patients

We analyzed mass spectra of coil1/2 GFAP-BDPs for acetylation and citrullination to identify PTM signatures in TBI, to support biomarker assay development and to detect cytoskeleton-destabilizing events and proteinopathies. MS-detected acetylated and citrullinated peptide fragments were validated through presence of signature ions and neutral mass losses (Supplement Table S8). Three lysine acetylations were validated in coil1, and four citrullinated arginines in coil1, including a potential novel citrullination site in coil1 (R79), and three citrullines in coil2, which were all consistent with observed mass deviations in MS2 spectra (Fig.5A, Supplement Fig.S10, Table S8). Additional five citrullinations detected in this TBI CSF samples set is validated reported as in vitro modifiable GFAP sites citrullinated by PAD treatment [67]. These GFAP-BDP acetylations and citrullinations in TBI-CSF were robust, as they were repeatedly detected.

TBI patients have different citrullinated GFAP signatures

Citrullination was further analyzed using site-specific citrullinated GFAP antibodies (Supplement Table S3, epitopes see Fig.5A) [63]. Two cit-GFAP antibodies with neoepitopes in coil2 gave patient-specific signals distinct from those of classic GFAP (Fig.5B). Cit-GFAP signals strongly detected 13-16kDa and 26-30kDa GFAP-BDPs compared to larger proteoforms and clearly distinct from the band signal proportions of unmodified GFAP detected in same samples (Fig.5B right). Large GFAP-BDPs were detected by CTGF-1221, as it is reported with a second epitope (R416) to the more c-terminal region located outside of coil2, whereas cit270-GFAP is directed solely to R270 within coil2. Additional citrullinated bands above 50kDa are likely pathological GFAP proteoforms, possibly ubiquitinated or multimeric forms [115, 166]. Notably, these patterns had substantial patient-to-patient variation that highlight individual differences in proteolysis and PAD activities during TBI progression. These preliminary observations support an interest in including modified GFAP for future TBI patient molecular profiling.

To further investigate trauma-induced GFAP proteolysis and the disparity between coproducts, citrullination, and their association with injured astrocytes, we employed a human culture trauma model for mechanistic insights.

Mechanistic exploring of GFAP proteolysis and modification in a human astrocyte trauma model

A series of epitope-defined GFAP antibodies was used to map coil1 and coil2 products for tracking their destiny and defining GFAP cleavage site after trauma. Inhibitors and reporters then documented calpain and caspase contribution to traumatic GFAP degradation and activity in injured human astrocytes. Lastly, citrullinated GFAP aggregates identified pathological astrocytes providing an explanation for selective fragment retention.

Trauma-generated GFAP coproducts are destined to different locations

Primary fetal human cerebral astrocytes were in vitro differentiated on elastic membranes and were reproducibly injured by abrupt pressure-pulse stretching [159]. GFAP filaments are disassembled, degraded and released in this trauma model [56]. To identify coil-specific BDPs, mabs with defined GFAP epitopes were probed on immunoblots of WCL ("cells") and concentrated CM ("fluid") of unstretched controls and stretched cultures from the same donors (Fig.6, Supplement Table S3). All clones detected full-length GFAP and large fragments but differed in small BDPs [72]. Clone 3E10 detected a 20kDa GFAP-BDP in astrocyte CM that increased after trauma, yet failed to detect it in cells (Fig.2C, red). Clone GA5 detected an epitope at the end of coil2, beginning of the GFAP tail [29]. GA5 detected a set of 17-28kDa GFAP-BDPs in cells but not in fluids (Fig.2C, purple). A cocktail of three mabs served as positive control, proofing GFAP-BDPs in cells and fluids from same donors (Fig.2C, blue). Thus, following mechanical trauma of human astrocytes, coil1 products were preferentially released, while coil2 products were selectively retained within cells. This new human trauma model insight supports the enriched presence of coil1 BDPs in biofluids of TBI patients.

Mapping a novel GFAP trauma cleavage site

Clone 5C10 detected full size and large GFAP-BDPs in cells and fluids of same donors, but no fragments smaller than 40kDa in either compartment. This suggests that 5C10 recognized an epitope that was destroyed during trauma proteolysis of GFAP (Fig.2C, yellow). To pinpoint the 5C10 epitope, we performed a chemical-mediated partial digest of GFAP using BNPS-skatole that cleaves after tryptophan residues as there is only a single tryptophan in GFAP (W256) [57, 155]. BNPS-skatole treated GFAP produced a N-terminal 34.6kDa and a C-terminal 20.5kDa fragment and 5C10 bound avidly to the C-terminal fragment on western blots (Supplement Fig.S10). The identity of the C-terminal 20.5kDa BNPS-skatole fragment was confirmed using anti-IFA, a pan-intermediate filament antibody to coil2B [79, 130]. 3E10 coil1 antibody bound the N-terminal 34.6kDa BNPS-skatole fragment not detecting the C-terminal BNPS-skatole fragment. This concludes that the sequence following tryptophan W256 from tyrosine 257 to tyrosine 260 (aa YRSKF) is an important component of the 5C10 epitope. As 5C10 does not detect small GFAP-BDPs after trauma, this also informs of the trauma-induced cleavage site in this region. It may likely include elements of the neighboring caspase 3 cleavage site, (aa YRSKFA↓DLTD). By MS identified TBI-patient detected coil1 sequence extended no further than R239 and the beginning of coil2 products were consistently observed F261 pinning the cleavage within this area. This unique antibody mapping data identified a novel trauma-cleavage upstream of F261 combined with potential caspase 3 activity downstream of it (Fig.6AB).

To determine the in rodents characterized contribution of TBI activated calpains and caspases in producing small GFAP-BDPs, we used suitable inhibitors and live-imaging reporters in the human trauma model [11, 36, 76].

Generation of small GFAP-BDPs involves calpains and caspases

Human traumatized astrocytes were treated with calpain inhibitor calpeptin (C), or caspase inhibitor ZVAD-FMK (V), and GFAP-BDPs were analyzed in WCL from adherend cells, fluids, and pelleted lifted cells and debris. αII-spectrin with its calpain and caspase-defined fragments (SBDPs[139, 151]) served as positive control, as they were expressed in differentiated human fetal astrocytes (Supplement Fig. S11) as reported in human astrocytes [74]. Both calpain (145/150kDa) and caspase (120kDa) SBDPs increased two days after stretch-injury in cells, debris, and fluids and decreased with respective inhibitors (Supplement Fig.S6). The 120kDa caspase-SBDP was enriched in detached material of demised cells. Note, both calpain-SBDPs were observed as double-band in WCL, yet a single 150kDa band was found in lifted cells, and a single 145kDa band was detected in fluid. These findings suggest that αII-spectrin calpain fragments originated intracellularly and had distinct compartment preferences after trauma alike what we found for GFAP-BDPs.

Two days post-stretching, GFAP-BDPs were elevated in cells, fluid and lifted material (Fig.7A, Supplement Fig.S12A). Calpeptin reduced 35-38kDa and smaller BDP bands. ZVAD-FMK effectively inhibited 19-26kDa BDPs. Combined inhibitors had an additive effect, confirming that both calpains and caspases were involved in human GFAP degradation after trauma. GFAP-BDPs were present in smaller amounts in unstretched samples, also inhibitor-sensitive, consistent with baseline cytoskeleton turnover [126]. Undigested GFAP initially (5h postinjury) accumulated in the cytoskeletal-soluble WCL samples (Supplement Fig.12B). At two days with calpeptin inhibition 50kDa GFAP increased in WCL of cells and lifted material indicating accumulated substrate (Fig.7A, Supplement Fig.12C). This suggests filament disassembly as an early trauma consequence followed by calpains digesting GFAP monomers. This data provides the first evidence of endogenous trauma-inflicted calpain and caspase digestion of human GFAP going beyond prior rodent work [171].

Given the limited knowledge of human astrocyte injury states relative to the expanding literature on GFAP as TBI biomarker, we next used live protease activity reporters combined with a membrane integrity dye and fixed citrullinated GFAP immunofluorescence to characterize astrocyte injury phenotypes. These microscopy pathophysiology studies aim to further characterize human traumatized astrocytes in our trauma model that previously linked GFAP fluid elevation to percent cell wounding [56].

Acute astrocyte injury states related to membrane wounding and proteolysis

Human astrocyte pathophysiology after stretch-injury is shown using live-fluorescent calpain and caspase reporters together with PI to assess membrane permeability. These differentiated human astrocytes displayed two morphotypes, fibrous elongated cells and bushy, star-shaped cells, resembling white and gray matter astrocyte morphologies [3] (Fig.7B). Under control conditions, both types showed calpain but rarely caspase activity, consistent with fetal neocortical expression of calpains along with low calpastatin [138]. Five hours post-stretching cultures showed a mix of astrocytes with leaky and intact plasma membranes documenting dynamic membrane damage. Injured fibrous astrocytes lacked calpain activity and frequently displayed beaded, clasmatodendrotic processes with caspase activity (Fig.7BC) [32, 56]. Injured bushy astrocytes were simultaneous calpain and caspase activ, lost radiating processes and had blebbing with phase-dark vesicles and vacuoles devoid of enzyme activity (Supplement Fig.S13). Acutely pyknotic cells, defined by shrunken phase-dense morphology, had bright PI-stained nuclei and either no active protease, or active caspases, indicating progression towards integrity loss, early necrosis or a transition from apoptosis to secondary necrosis. Thus, we identified a novel pathological phenotype of acute degenerating dystrophic human astrocytes exhibiting both active protease activity and membrane wounding hours after trauma.

These initial images document concurrent injury phenotypes and proteolysis states in human traumatized astrocytes illustrating a spectrum of early vulnerability. This model offers an opportunity for future temporal proteolysis analyses and associated aberrant proteoforms. For this initial study, we examined GFAP proteoforms by coil2-specific antibodies in traumatized astrocytes.

Citrullinated GFAP in traumatized astrocytes detects pathological aggregates

Distinguishing GFAP proteoforms in traumatized astrocytes is critical, as typically the prominent reactive astrogliosis dominates astrocyte documentation after insults. As our findings thus far indicate, coil2-GFAP-BDPs preferred cellular residence, hence we chose two coil2-specific antibodies, cit270-GFAP detecting citrullinated coil2 and 5C10 to coil2/tail of classic GFAP alongside a polyclonal GFAP antibody for comparison after confirming specificity of cit270-GFAP signals (Supplement Fig.S14A). Stretch-trauma cultures had visibly more cit270-GFAP signals than controls at 5 and 24h postinjury (Fig.8). Cit270 stained clusters, speckles and large granules but did not stain cytoskeletal fibers as shown by staining for unmodified GFAP. Cit-GFAP aggregates and GFAP filaments showed distinct distributions. Cit-GFAP stained either cells negative for classic GFAP, or excessively bright stained cells. Increased GFAP antigenicity is reportedly associated with filament disassembled GFAP after acute wounding, calcium ionophore-treatment, or calpain activation in astrocytes [56, 86]. Morphotypes with cit-GFAP granules included reactive, shrunken or swollen dystrophic astrocytes devoid of processes (Fig.8B, Supplement Fig.S14D). Cit270 signals were also peppered around astrocyte processes seemingly extracellular, as phase-contrast images delineated astrocyte shapes (Fig.8B, Supplement Fig. S14E). Thus, mechanical injury-induced GFAP citrullination and filament breakdown identified pathological astrocytes hosting dysfunctional GFAP. This finding provides evidence for coil2-associated aggregation that could contribute to its selective cellular retention and underrepresentation in TBI patients biofluids. It further establishes cit270-GFAP as a proteinopathy marker of pathological GFAP aggregation associated with human traumatized astrocytes and new tool to study astrocytopathy and astrocyte degeneration relevant to the pathophysiology of TBI.

In conclusion, TBI patients show GFAP destabilization by citrullination and acetylation, and trauma-specific proteolysis, producing unique small coproducts with distinct release behaviors. The observed coil-specific BDP fluid imbalance in fluids is driven by citrullination-modified GFAP aggregation, highlighting a GFAP proteinopathy in pathological astrocytes. Notably, CSF trajectories of GFAP proteolysis, not the uncleaved protein release, predict outcome, underscoring the diagnostic value of monitoring postinjury processes including proteostasis disruption for TBI patient evaluation.

DISCUSSION

This study identifies novel TBI-specific GFAP proteolysis as a dynamic process generating proteoforms with distinct patient-specific longitudinal trajectories. The trauma-derived small GFAP-BDPs directly document an association between previously in neurotrauma unrecognized proteinopathy in pathological astrocytes with GFAP as biofluid marker. These results suggest that disrupted proteostasis may contribute to the underlying TBI pathophysiology and could play a role in shaping patient recovery or progressing neurodegeneration, warranting further studies beyond the conventional view of GFAP as TBI biomarker.

Acute trauma pathophysiology explains early GFAP vulnerability in injured astrocytes

Mechanical trauma, e.g. whiplash or blunt impact, shears brain microstructures causing diffuse axonal injury and astroglial clasmatodendrosis, membrane wounding that can lead to protracted cell loss [32, 44, 56, 144]. This mechanoporation of plasma membranes triggers rapid release of full-length GFAP and its fragments [7, 56, 88]. High-speed biomechanical force or strain can stretch intermediate filaments to disassemble or even break strands thereby exposing the rod-domain making it susceptible to proteolysis [17, 41, 126, 135]. Non-filamentous GFAP is documented minutes after stretch-injury in mechanoporated human astrocytes [56]. Thus, filament-disassembly is closely linked to membrane permeability that disturbs ion balance and threatens astrocyte viability [56]. Ion imbalance is followed by water influx causing membrane blebbing and astrocyte oncosis (swelling) [25, 52]. With progressive energy depletion, cell integrity may fail resulting in early necrosis. Added stressors such as reactive oxygen species, acidosis and hypoxia further impair mitochondrial function ultimately shift cell death pathways from apoptosis to necrosis [5, 19, 30, 52, 94, 99]. These processes may contribute to secondary GFAP release in TBI patients reflecting cytotoxic edema and delayed astrocyte loss [22, 89, 154, 156, 162, 177].

Citrullination and proteolysis relate to GFAP proteinopathy and impaired astrocyte function

Assembled GFAP filaments are essential for astrocyte morphology, mechanical resilience, ATP release and other key functions [126]. Calcium ionophores induce a rapid, transient increase in GFAP immunocytochemical signals in rat astrocytes due to filament unwinding and epitope exposure [86]. Trauma-induced unfolding and associated calcium influx could similarly expose a concealed proteolytic cleavage site within the GFAP rod. Calcium elevation catalyzes GFAP proteolysis by rapidly activating calpains through conformation change leading to partial GFAP cleavage, consistent with their limited proteolytic activity [7, 23, 31, 35, 38, 71, 86, 93, 106, 126, 127, 161]. Calpain cleavage also primes caspase activity hence both enzymes generate small GFAP-BDPs [47, 93]. Baseline calpain activity is reported as part of the ubiquitin-proteasome, autophagy-lysosomes, and cytoskeleton-remodeling systems, modulated by endogenous calpastatin, consistent with observing active calpain in unstretched astrocytes [50, 129, 138, 142].

Stretching causes focal mechanical loading, producing a range of astroglial injury states varying further by differences between gray- and white-matter morphotypes, indicated by distinct calpain and caspase activities after trauma. Caspase activity in clasmatodendrotic astrocytes parallels astrocyte damage in rat astrocytes after excitotoxic injury, further linking cytoskeleton remodeling and caspase-mediated breakdown [1]. As cytoskeleton integrity is necessary for structural resilience and metabolic competence, GFAP proteolysis may exacerbate astrocyte vulnerability as is the case in reduced GFAP expression after injury [88, 160].

Emerging studies show that pathological GFAP proteoforms critically undermine astrocyte function, compromise glutamate buffering, neurovascular unit integrity, neural metabolism, network activity and synaptic plasticity that could exacerbate neurodegeneration [15, 68, 91, 146]. Citrullinated GFAP fragments are main

constituents of Rosenthal fibers that lead to progressive neurodegeneration in Alexander disease [27, 29, 37, 55, 91, 103, 132]. Similar destabilizing proteoforms are reported after mechanical stretching, hypoxia and chemical insults induced by calcium-dependent PADs elevating GFAP citrullination in astrocytes and Müller glia [4, 137, 166]. Taken together with our observation of trauma-driven cit-GFAP aggregates in pathological astrocytes, the data jointly suggest that trauma-induced GFAP proteoforms could be a potentially harmful proteinopathy.

Uniqueness of traumatic injury-produced GFAP breakdown products via coil2A sensitivity

Cleavages that produce TBI-associated GFAP-BDPs are distinct from established protease sites in other neurodegenerative diseases. A caspase 6 GFAP cleavage ($VELD_{225}$-VAKP) is described in mouse culture models of Alexander disease, splitting GFAP in linker2 [9, 27, 29]. Here we identified longer coil1-products with a TBI-cleavage between residues 240-260. GFAP-BDPs reported in Alzheimer's disease are generated by a caspase 3 cleavage at $_{258}$RSKFA↓DLTD [108]. Our biochemical studies identified ($_{257}$YRSKF$_{261}$) as likely cleavage region flanking this caspase 3 site generating the new N-terminus of the coil2-product, yet coil2 was shorter than the reported AD GFAP-BDPs. The shorter TBI coil1-product end is not explained by this cleavage. Coil2A appears particularly sensitive to proteolysis after TBI and our findings suggest calpains and caspases are involved, though other proteases, including possible endogenous trypsin-like activity, could generate the new observed termini (LTAALKEIR↓T; R239). Endogenous trypsin activity is reported after brain injury inflicting astrocyte compromise and adverse inflammatory effects [45, 169]. Joint proteases can account for the observed sequence gap within coil2A between the two product sets, warranting further studies.

Structural evidence for protease vulnerability of the TBI cleaved region

Intact GFAP molecules anneal by α-helical coiled-coil interactions forming parallel dimers, antiparallel tetramers and ultimately 10nm diameter units that grow in length to mature filaments [72]. Computer modeling and structural data show that the entire rod segment of vimentin, closely related to GFAP, is a continuous α-helix [46]. 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 are therefore thought to represent non-helical linkers. The Alexander disease-associated GFAP cleavage is found within one such non-helical region, "linker12". Notably, the region containing the 5C10 epitope and caspase3 site is historically referred to as "stutter1", where a hydrophobic heptad repeat is replaced by a single hendecad sequence, disrupting the packing and thereby rendering is more susceptible to proteases [148].

Pathophysiological significance and biomarker implications of new GFAP-BDPs

TBI proteolysis of GFAP produces coproducts with unequal abundance in patient biofluids due to differences in release or susceptibility to further degradation or both. We show preferred coil1-release and selective coil2-retention as non-filamentous cit-GFAP aggregates in traumatized human astrocytes while an in vivo study documents strongly upregulated citrullination in the contused rat brain localized to astrocytes [84]. Thus, cit-GFAP aggregates associate with an astrocyte pathology that we link with GFAP as TBI biomarker.

TBI CSF samples carry both coproducts with coil2-BDPs either absent or elevated. One scenario could involve adverse events that may cause glial integrity loss with release of otherwise retained proteoforms. Astroglial membrane lysis during cytotoxic edema could account for such uncontrolled necrotic or oncotic events [25, 52]. On one hand, coil1-GFAP fluid may be 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 increasing astrocyte demise. Thus, coil- and citrullination-specific GFAP antibodies can serve as pathological astrocyte markers and as tools for tailoring assay development in TBI [43, 145, 153]. New possibilities for priorities of future research on present needs include (1) to examine cit-GFAP-aggregates in postmortem brains in the context of post-traumatic neurodegeneration, after TBI, BLAST, or chronic traumatic encephalopathy to investigate traumatic astrocytopathy; and (2) to further develop an association of GFAP biomarker profiles with disrupted proteostasis and neurodegeneration [62, 125, 153].

Relevance of GFAP-BDPs for assays design and interpretation

GFAP testing after TBI is already incorporated into large multisite clinical studies for acute patient assessment using commercial antibody-based platforms [12, 13, 77]. However, beyond a general specificity for GFAP it is not clear what proteoforms are being measured, as this information is typically proprietary or unknown and it is presumed that total released GFAP is captured. The distinct release and modifications of coil1 and coil2 products can add insight for design and interpretation of GFAP assays.

Potential for new GFAP 'context of use' in TBI monitoring

Our sequence-defined GFAP degradome and connected cytological data support possible future clinical applications of GFAP in longitudinal TBI patient monitoring. Three interrelated potential COU areas are considered: *(1) Longitudinal monitoring*; (*2) Astrocyte degeneration and secondary injury; (3) Outcome prognosis*.

(1) Longitudinal GFAP monitoring and influence of age

Tracking GFAP degradation kinetics offers a longitudinal window for TBI patient monitoring during intensive care and for early clinical trial endpoints [2, 11, 40, 58, 76, 98, 136, 174]. GFAP-BDPs dominate in circulation, while CSF carries both, uncleaved GFAP and BDPs [56, 122, 178]. Temporal GFAP profiles with injury day peak (uncleaved and large GFAP-BDPs) are shared with other rapidly degrading TBI biomarkers including UCH-L1, S100β, brain lipid binding protein, also called FABP7, neuron-specific enolase and SBDPs [56, 163]. These biomarkers have different half-lives, yet share primary injury-related peaks reflecting on the extent of acute mechanoporation, force-triggered filament breakage and calcium-driven initiation proteolysis [56, 87, 92, 121, 128, 139].

Secondary elevation of GFAP-BDPs, particularly of small BDPs involving caspase activation, is seen in a subset of TBI patients. This pattern aligns with previous reports of delayed serum GFAP peaks in a subset of older TBI patients with high CT burden, poor outcome, high mortality and pathophysiological features of more severe TBI [134]. Age-related increases in baseline GFAP and neurofilament light, two intermediate filaments with pronounced trauma-associated fragmentation, are both linked to reduced clearance or ongoing atrophy [26, 33, 48, 140, 151, 176]. Elderly suffering a TBI clear CSF less efficiently through decreased CSF pulsation, ependymal cilia vulnerability, and disrupted cerebral blood flow affecting biomarker levels [18, 73, 168]. Thus, GFAP-BDP monitoring could add insights on secondary TBI pathophysiology.

(2) Detecting astrocyte pathology to inform on secondary injury

Patient-specific secondary GFAP-BDP release during injury progression. Neuroimaging reports contusions expanding into peri-lesional tissue attributed to cytotoxic edema already stated above [8, 113, 150, 173]. Cytotoxic edema is characterized by microstructural changes of astrocyte swelling and subsequent integrity loss driven by ion dysregulation and metabolic depletion [22, 154, 156]. These interrelated mechanisms may underlie the variable secondary increases in cell death-associated BDPs observed in a subset of TBI patients. Consistent with this interpretation, progressive astrocyte degeneration is quantified in autopsy brains of TBI patients and shows reduced astrocyte numbers between acute (<6h) and subacute (>6hrs to 3PID) postmortem intervals in cerebral white matter and hippocampus [89]. Thus, GFAP-BDP profiling may indicate secondary adverse postinjury events after TBI.

(3) GFAP-BDP trajectories for TBI outcome prognosis

Elevated GFAP is recognized as prognostic marker after severe TBI [56, 78, 81, 117, 124, 134]. Our findings indicate that timecourse of GFAP degradation -not uncleaved GFAP or large fragments- best distinguish poor versus good 6-months outcome. Being robust to the way the score is dichotomized matters, as defining "good" recovery is inherently personal [164]. The results suggest that ongoing injury-driven GFAP proteolysis offers prognostic insights compared to initial impact-driven uncleaved and end-clipped GFAP levels. This aligns with broader clinical knowledge that early injury progression rather than initial status, shape recovery [16, 170]. Current FDA-approved GFAP testing focuses on a narrow admission window and therefore misses this critical dynamic as longitudinal monitoring is not part of the present FDA approved COU [78, 165]. While GFAP assays may include detecting BDPs, there is no proteoform differentiation.

This study has several limitations

This clinical cohort was relatively small, and some patients had incomplete longitudinal data, due to staffing and logistical constraints during the Covid pandemic. Statistical power was limited but steps to remedy this included truncating at 10PIDs, avoiding imbalanced variance, and use of adequate effect size types. Cohen's D was used to predict good versus poor outcomes, as AUC modeling was inadequate for simultaneously including elapsed postinjury time. Technical restrictions limited serum MS sequencing. CSF collection in TBI patients undergoing ventriculostomy offers unique biomarker insights yet does carry a small risk of infections [66, 116]. Donated fetal brain specimen limited comparison across donors and conditions. Live-fluorescence reporters limit the use of high-intensity illumination or repeated imaging yet enabled simultaneous monitoring of proteolysis and cell integrity. Despite these challenges, all key measurements were comprehensive across samples using adequate quantification standards and unbiased approaches. Importantly, findings were cross-validated using independent experimental approaches. As this is a discovery-focused study, rigorous orthogonal validation corroborates and supports the reliability of the results. Certainly, larger standardized clinical TBI patient cohorts and translational preclinical studies across centers are critical for future validation to extend the here reported findings [158].

CONCLUSION

Translational significance and future directions

Collectively, our data position GFAP-BDPs as molecular indicators of disrupted proteostasis and astrocyte pathology. Future work will aim to link cit-GFAP proteoforms with astrocytopathy in human brain tissue after TBI and associated neurodegenerative conditions. Additional serial fluid measurements of GFAP-BDPs may support individualized patient monitoring in relation to physiological parameters and secondary complications in neurocritical care. By connecting GFAP fluid signatures to its latent proteinopathy this work contributes to a translational framework for TBI phenotyping that could help to advance TBI patient care.

ABBREVIATIONS

Traumatic brain injury (TBI), glial fibrillary acidic protein (GFAP), mass spectrometry (MS), breakdown products (BDPs), cerebrospinal fluid (CSF), Extended Glasgow Outcome Scale (GOSE), posttranslational modifications (PTMs), αII-spectrin breakdown products (SBDPs), context of use (COU), National Institute of Neurological Disorders and Stroke (NINDS), Glasgow Coma Scale (GCS), Institutional Review Board (IRB), Glasgow Outcome Scale (GOS), SDS polyacrylamide gel electrophoresis (SDS-PAGE), mouse monoclonal antibodies (mabs), acetonitrile (ACN), liquid chromatography-tandem MS (LC-MS/MS), full spectra (MS1), higher-energy collisional dissociation (HCD; MS2), Proteome Discoverer (PD), false discovery rate (FDR), peptidylarginine deiminase (PAD), amino acids (aa), Tris-buffered saline with Tween (TBST), room temperature (RT), Enhanced Chemiluminescence (ECL), propidium iodide (PI), Whole cell lysate (WCL, "cells"), Conditioned medium (CM, "fluid"), Research Resource Identifier (RRID), restricted maximum likelihood (REML), linear mixed model (LMM), patient identifier (ID), partial omega squared (ω_p^2), coefficients of variation (CV), fixed effect statistic (F-statistic), degrees of freedom (df), denominator term (DFDen), confidence intervals (CI), postinjury day (PID), adjusted means (Adj.Means), Alzheimer's disease (AD), CMAC-tBOC-leucyl-methionine (CMAC-tBLM), interquartile range (IQR).

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

For deidentified TBI patient's CSF and serum collection with metadata, adult patients (>18 years of age) were enrolled at two Level 1 trauma centers, in Los Angeles, California, at the UCLA Ronald Reagan Hospital Neuro-Intensive Care Unit, and in Gainesville, Florida (UF Health Shands Hospital, University of Florida). The human subjects' protocols included local IRBs that approved informed consent procedures at UCLA under Chair Paul Vespa and at the University of Florida Dept of Anesthesiology under Dr. Steven Robicsek. The Study "Biochemical markers for traumatic brain injury tissue and data bank" was approved by the Institutional Review Board (IRB) of the University of Florida with the IRB# 201703310. Patients met inclusion criteria if they were 18 years old with a non-penetrating head injury and had a GCS <8 requiring the placement of an intraventricular catheter. Patients were excluded if they had a history of preexisting end-stage organ disease or severe psychiatric illness. At UCLA, the Office for Human Research Protection (OHRP) approved the informed consent protocol for the Brain Injury Research Center study with the BIRC-IRB# 10-0929.

The UCLA Office for Protection of Research Subject (OPRS) declared IRB exemption status for deidentified pre-shelved fetal brain specimen donation per HS-7 exemption amendment. Deidentified human fetal brain tissue from voluntarily aborted pregnancies was donated adhering to stringent ethical regulations. Tissue was preshelved at the UCLA Translational Pathology Core Laboratory (Sara Dry, Chair of Pathology and Laboratory Medicine). Consent was established through UCLA Dept of OB-GYN (Angela Chen, Division Chief of Family Planning). Signed informed consent occurred after decision on elective termination, complying with US Public Law 103-43 and NIH policy, ensuring no incentive (The Public Health Service Act, 42 U.S.C. § 289g-2).

CONSENT FOR PUBLICATION

This manuscript contains no individual personal information, so individual consent for publication is not applicable.

AVAILABILITY OF DATA AND MATERIAL

The datasets supporting the conclusions of this article are included within the article and its additional files with file names listed under Additional material. The CSF GFAP and BDPs longitudinal quantification dataset of 24 TBI patients is freely accessible through the Open Data Commons for Traumatic Brain Injury repository (ODC-TBI), under the Wanner lab space with data dictionary, the unique persistent identifier, the hyperlink to this dataset is provided here: https://odc-tbi.org/lab/dataset?labid=387&datasetid=1432.

Mass spectrometry raw data of GFAP fragments' peptide sequence PD files and label-free peptide/ion quantification of four TBI patients (six CSF samples) are publicly accessible at the Center for Computational Mass Spectrometry (CCMS) ProteoSAFe (Proteomics Software Environment) web server as a MassIVE (MS Interactive Virtual Environment) file folder provided here:

https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=460f951a666a4b599df2bfa37fa33fbd.

Figures and Supplements

This manuscript includes a powerpoint file with eight figures called '*Figures-GFAP-trauma-Wanner'*. A supplement is included, which contains 14 Additional Figures (labeled Figures S1-S14) and eight Tables S1-S8 that are compiled into one pdf file called '*Supplement-GFAP-trauma-Wanner'*. Further, complete and uncropped immunoblot images of the figures are provided in *'GFAP-uncropped blots-Supplementary-Wanner'*.

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 **IBW** and **JAL**, 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: **IBW** and **JAL**, UCLA "Astrocyte traumatome and neurotrauma biomarkers". Assignee UCLA; Licensee: Brain Box Solutions Inc.

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Semel Institute IDDRC and Brain Injury Research Center, BIRC, UCLA, Los Angeles, CA, USA. Facilities supplies for cell culture and microscopy work.

AUTHORS' 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, assisted in writing the manuscript and offered biochemical guidance. **GS** also conducted antibody binding and chemical cleavage studies and wrote the structural part of the discussion. **BME** provided data interpretation and manuscript revision. **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, manuscript organization and contributed to written content and 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 final manuscript.*

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FIGURE LEGENDS

Thumbnail: Astrocyte injury states with distinct GFAP proteoforms and fates after TBI
The infographic summarizes four distinct coexisting progressive injury states of human astrocytes after traumatic injury, each defined by unique astrocyte morphologies and GFAP proteoforms and distributions. Key pathways of GFAP post-translational modification, degradation and aggregation are linked to astrocyte dysfunction and underpin fluid biomarker signatures. The illustrated steps provide interpretation linking astrocytopathy with GFAP biomarker profiles to support Neuro-ICU patient monitoring. ***(1) Uninjured,*** healthy astrocyte: Intact plasma membrane and assembled GFAP filaments. ***(2) Mechanoporated,*** wounded astrocyte: Mechanical forces cause membrane poration and process beading (clasmatodendrosis), leading to calcium influx and filaments disassembly into monomers (black) and fragments. Proteolytic degradation is initiated by calpains into large fragments (gray), followed by caspases and other proteases into coil1-BDPs that are readily fluid-released (red) and coil2-BDPs that form PTM-decorated aggregates (purple). ***(3) Dystrophic,*** swollen (oncotic) astrocyte shows vacuoles, membrane blebbing and advanced process damage with accumulated PTM-modified GFAP granules marked by citrullination (green) and acetylation (brown) that represent a trauma-related GFAP proteinopathy. ***(4) Necrotic,*** dying astrocyte with integrity loss membrane rupture and lysis, releasing modified coil2-BDPs as markers of irreversible cell loss.

Fig. 1 Variable endings and abundance of small GFAP-BDPs in TBI patients' CSF
A) Sequences of GFAP breakdown products (BDP) with peptide maps and percent coverage of mass spectrometry (MS) of immunoprecipitated GFAP of four cut out gel bandsets from six CSF TBI samples (I.-IV., Supplement Fig.S1). Shown are high confidence peptides (green, FDR$\leq$1%) and repeatedly identified medium confidence peptides (yellow FDR$\leq$5%). ***Bands (I.)*** had 359 amino acids (aa) with calculated molecular weight of 41.7kDa and suggested new N- and C-termini of serine 53 (S53) and lysine 411 (K411). ***Bands (II.)*** spanned 349aa from glycine 56 (G56) to leucine (L404) within which 37/38kDa fragments with variable endings between TBI samples were located (Supplement Figure 1). ***Bands (III.)*** contained two GFAP-BDPs, a 92% covered coil1-containing product of 185aa with calculated molecular weight of 21.8kDa; and a 77% covered coil2-containing product of 151 aa and peptides with estimated molecular weight of 17.5kDa. ***Bands (IV.)*** contained a 96% covered 181 aa coil1 product with size between 21-21.6kDa -±PTMs; and a 77% covered coil2 product of 130aa with estimated molecular weight of 15-15.3kDa. **B)** GFAP immunoblots TBI CSF samples of one and three postinjury days (PIDs) detected by a polyclonal (top) and a monoclonal (clone 3E10, bottom) antibody. The TBI patient was an 84-year-old male sustaining facial fractures, subarachnoid and intraventricular hemorrhage and intraparenchymal hematomas from a fall. Observed GFAP molecular weights were *bands (I.)* 40-42kDa, *(II.)* 36-39kDa, *(III.)* 20.4-23kDa, and bands *(IV.),* 17-19kDa detected only by the polyclonal antibody. **C)** Predicted endings of small GFAP-BDPs in TBI. ***Coil1 products*** had N-termini serine S53 and alanine A55 and C-termini were arginine R239 and alanine A233. ***Coil2 products*** had consistent N-terminus phenylalanine F261, and ended with either lysine K411 or arginine R390. Different endings were sequenced from different bands of excised gel slices as indicated by the brackets. **D)** Label-free MS quantification documents peptide detection frequency of coil1 (59± 29%) and coil2 (13±7%) product peptides in eleven MS runs of Bands (III) and (IV.). The difference of peptide detection frequency between the two coils was significant, using mixed model testing ($p<0.0004$; with patient ID as random effect two-tailed T-test with unequal variances, $p=0.00038$).

Fig. 2 Longitudinal GFAP proteolysis in TBI patients' CSF with shared peaks and individual profiles
A) GFAP immunoblot of serial CSF samples over four postinjury days from a 40-year-old male TBI patients (admission GCS 3, GOSE 8) displays highest GFAP and fragment levels at six hours with additional peaks on PIDs 2-4. **B)** GFAP immunoblot shows serial TBI CSF samples over PIDs 4-10 (a 48-year-old male TBI

patient; admission GCS 10, GOSE 6) shows over time declining levels of 37-50kDa and later variably increasing 15-26kDa BDPs. Calibrants: Human recombinant full-length GFAP (50kDa) and his-GFAP-BDP (25kDa).**C-H:** Ten-day profiles of GFAP total **(C),** uncleaved **(D)** and fragment sets **(E-H)** in TBI CSF of 23 TBI patients (102 samples) and resampled mean cubic splines with 95% CI bounds. Graphed are log-transformed calibrated concentrations of subsaturated band densities (calibration curves and scaled exposure densitometry see Supplement Table S4, Fig.S5). Total GFAP, uncleaved, large and small fragments (45-49; 15-19kDa) had significant injury day peaks shown with Cohen's D effect size on injury day versus overall-times adjusted means (linear mixed model, controlled for random donor and age variance, see Supplement Fig. S7, Table S6 for age covariance). Means of 37-39kDa GFAP fragment levels were above mean until day 3, around mean on days 4-5 and below mean thereafter. Small GFAP-BDPs had larger dispersion shown by IQR, kurtosis and variance (σ) due to either high or absent signals yet had mean secondary elevations on later days.

Fig. 3 Correlation between GFAP uncleaved, fragments and coproducts in TBI CSF
A) Pearson's correlation matrix lists coefficients between uncleaved GFAP and all BDP sets measured in 23 TBI patient's 102 CSF samples. **B)** Bivariate plot of full length (50kDa) GFAP substrate and calpain cleavage products (37-39kDa) using orthogonal fit (R^2 with equal error weights) and FDR-corrected Logworth ($\log_{10}$p-value). **C)** Bivariate plot of GFAP-BDP coproducts correlated weaker due to more undetected 15-19kDa versus 20-26kDa bands (circle, 28% 15 more undetected). See Supplement Fig. S8 for all GFAP species bivariate correlations.

Fig. 4 Longitudinal GFAP proteolysis predicts TBI patient outcome, not full-length GFAP
Compared are trajectories of GFAP bandsets over 10 PIDs among TBI patients with good recovery (GOSE 5-8) versus those with poor outcome (GOSE1-4), shown with mean cubic splines and 95% CI bands adjusted for smoothness (21 patients; 95 observations). **A)** Profiles of calpain-derived GFAP fragments (37-39kDa) trajectories differentiated between the two recovery groups by an effect size of 1.1SD (p=0.02, Cohen's D adjusted for elapsed time and age effects; subject ID as random effect). **B)** Profiles of small GFAP-BDPs (20-26kDa) showed 0.9SD nonsignificant difference trend between the two groups. **C)** Full-length, 50kDa GFAP profiles overlapped over time between groups lacking prognosis of patient outcome. **D)** Model-adjusted GFAP means with standard errors are shown documenting differences in GFAP fragment levels between TBI patients with good versus poor recovy. See Supplement Table S7 for Cohen's D for effect size of all GFAP species; see Fig. S9 for alternate GOSE partitioning showing similar trends.

Fig. 5 Citrullination and acetylation PTMs are signatures of TBI patients' GFAP fragments
A) MS mapping of posttranslational modifications in GFAP coil1/2-BDPs from six TBI CSF samples. All citrullinations (13, green, including a novel citrullination site at arginine R188), and acetylations (3, brown) were validated by signature ions and mass shifts in MS2 spectra (Supplement Table S8, Fig. S10) with a subset being further confirmed by prior described cell-free peptidylarginine deiminase (PAD) digests, indicated with "*" [67]. Arrows indicate neo-epitopes R270 and R416 for citrullinated-GFAP antibodies [63]. **B)** *Left*: TBI patient-specific citrullinated GFAP-BDP signatures in nine TBI CSF samples show strong BDP signals with rabbit cit-R270-GFAP, and additional larger molecular weight signals using mab CTGF-1221. *Right*: Unmodified GFAP detected by cloil1 mab3E10 (*top*) or a rabbit polyclonal GFAP (*bottom*) from same samples document proportional differences in unmodified GFAP signals (antibody specifics see Supplement Table S3).

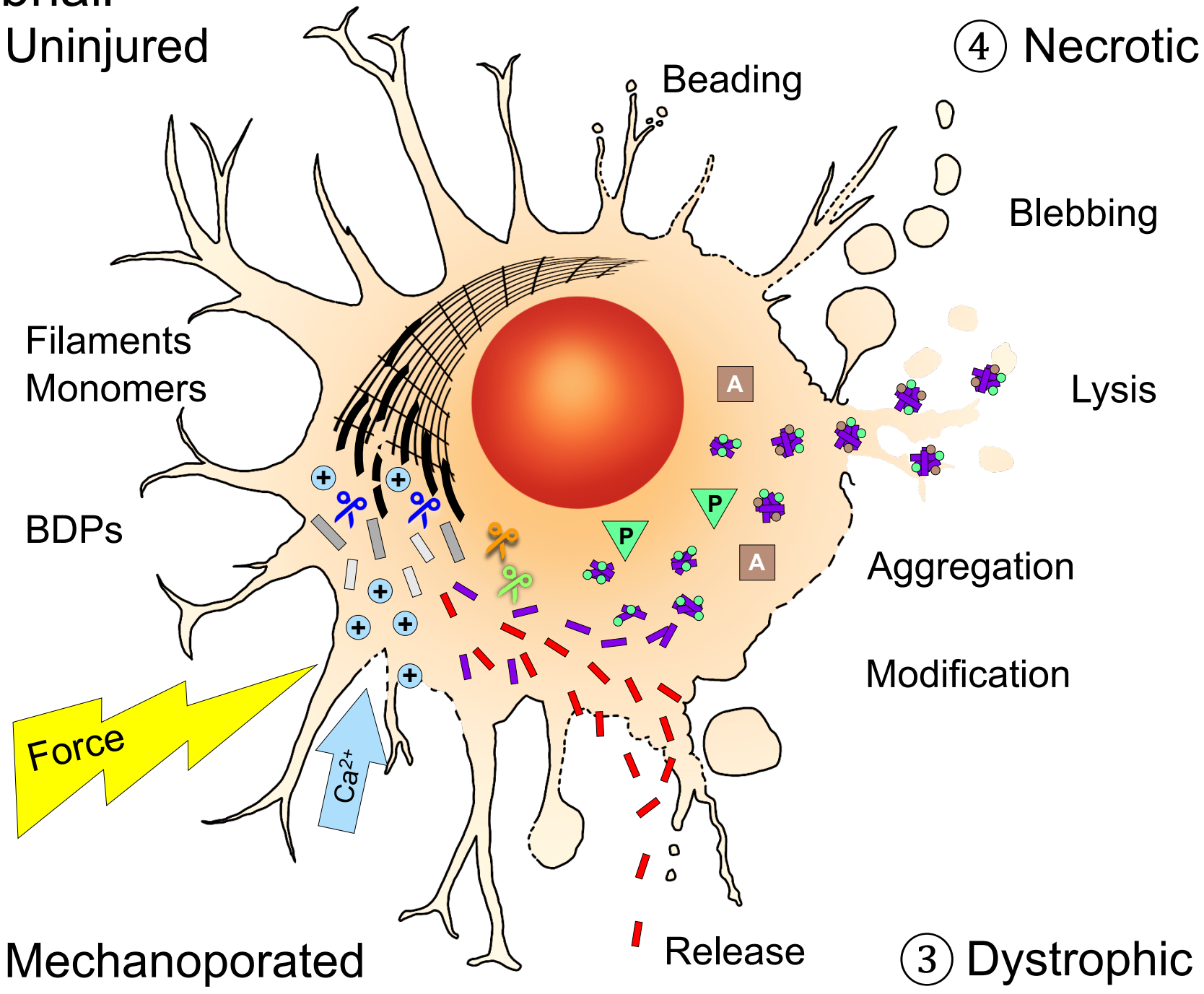
Fig. 6 Novel GFAP cleavage creates coproducts with distinct localization after trauma
Monoclonal antibody mapping confirms MS findings (Fig.1) of GFAP cleavage into two coproduct sets residing in separate compartments after stretch-injury of human cerebral astrocytes [159]: coil1- products were trauma-released whereas coil2-products remained cell-associated. **A)** MS derived coil1/2 sequences for alignment with used antibody epitopes. **B)** Binding regions and defined epitopes of antibody clones within GFAP-BDPs. Mab 3E10 targets coil1B (aa196-217) with stutter motif HEEEVRE (aa204-210) as epitope; 5C10

targets coil 2A (aa240-260) with core epitope (aa257-260) deciphered see Supplement Fig.S10). GA5 binds at the end of coil 2B (aa 374-390) [27]. BD multiclone (4A11, 1B4, 2E1) detects both coils. **C)** Immunoblots of 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) probed with antibodies from B (color-coded). All clones detected full-length GFAP and large fragments in cells and fluids. Small GFAP-BDPs were in coil-specific different compartments 3E10 detected 20kDa products in fluids but not cells. BD Multiclone detected intracellular 13-28kDa GFAP-BDPs in astrocytes and small bands in extracellular fluids. 5C10 did not detect any small fragments. GA5 detected 17-28kDa GFAP-BDPs in cells, but not in fluids.

Fig. 7 Calpain and caspase activities in compartmental GFAP degradation and astrocyte phenotyping
A) GFAP degradation was analyzed at 48h postinjury across adherend cells, released pool and lifted material with or without calpain and caspase inhibitors. Unstretched and stretch-injured human astrocytes were treated with single or combined inhibitors followed by analysis of lysed adherent cells, concentrated fluids and pelleted lifted material. Calpain-inhibition shows 35-38kDa band reduction in all compartments along with accumulation of uncleaved GFAP in cellular fractions. In contrast, 19-26kDa BDPs were sensitive to both inhibitors across fractions (Quantified in Supplement Fig.S12). **B+C)** Live imaging shows human astrocyte morphotypes and injury stages defined by membrane permeability with calpain and caspase activity during 5-6 hours postinjury. Elongated (*Top*) and bushy (*bottom*) morphotypes had distinct morphologies. Control astrocytes had intact processes with calpain activity (converted substrate CMAC-tBOC-leucyl-methionine, blue). Stretched cultures were mixted population of cells with intact membranes [PI-] or with leaky membranes [PI+] that included dystrophic astrocytes bearing no, thin or beaded clasmatodendrotic processes. Fibrous dystrophic astrocytes were calpain-inactive and caspase-active even when pyknotic (green/yellow). Bushy dystrophic astrocytes displayed calpain and caspase activity (tiel) with a subgroup being membrane permeable, while other integrity compromised bushy cells were devoid of these protease activities that included necrotic cells (red only). Phase-dense vacuoles were unstained (Supplement Fig. S13). Scale bars: 100 µm.

Fig. 8 Trauma induced citrullinated GFAP aggregates in pathological human astrocytes
A) Immunofluorescence of coil2 GFAP (GA5, red) and citrullinated GFAP (cit270-GFAP, green) shows abundant accumulation of non-filamentous citrullinated GFAP clusters at 5 and 24h post-stretching (*right*) compared to hardly any cit-GFAP in controls (*left*). **B)** Unstretched astrocytes (*left*) and three pathological astrocyte morphotypes (right) after stretch-injury: reactive astrocyte with thick, hypertrophied processes, bright cytoskeletal GFAP and fine-grained cytosolic cit-GFAP (yellow); astrocytes with fanning-out processes surrounded by cit-GFAP sprinkles (arrows); dystrophic astrocytes with swollen somata, malformed, cryptic or missing processes bearing various sized cit-GFAP aggregates in absence or presence of non-filamentous or wavy GFAP (arrowheads; see Supplement Figure S14 for individual channels and phase images). Scale bars: 50 µm.

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

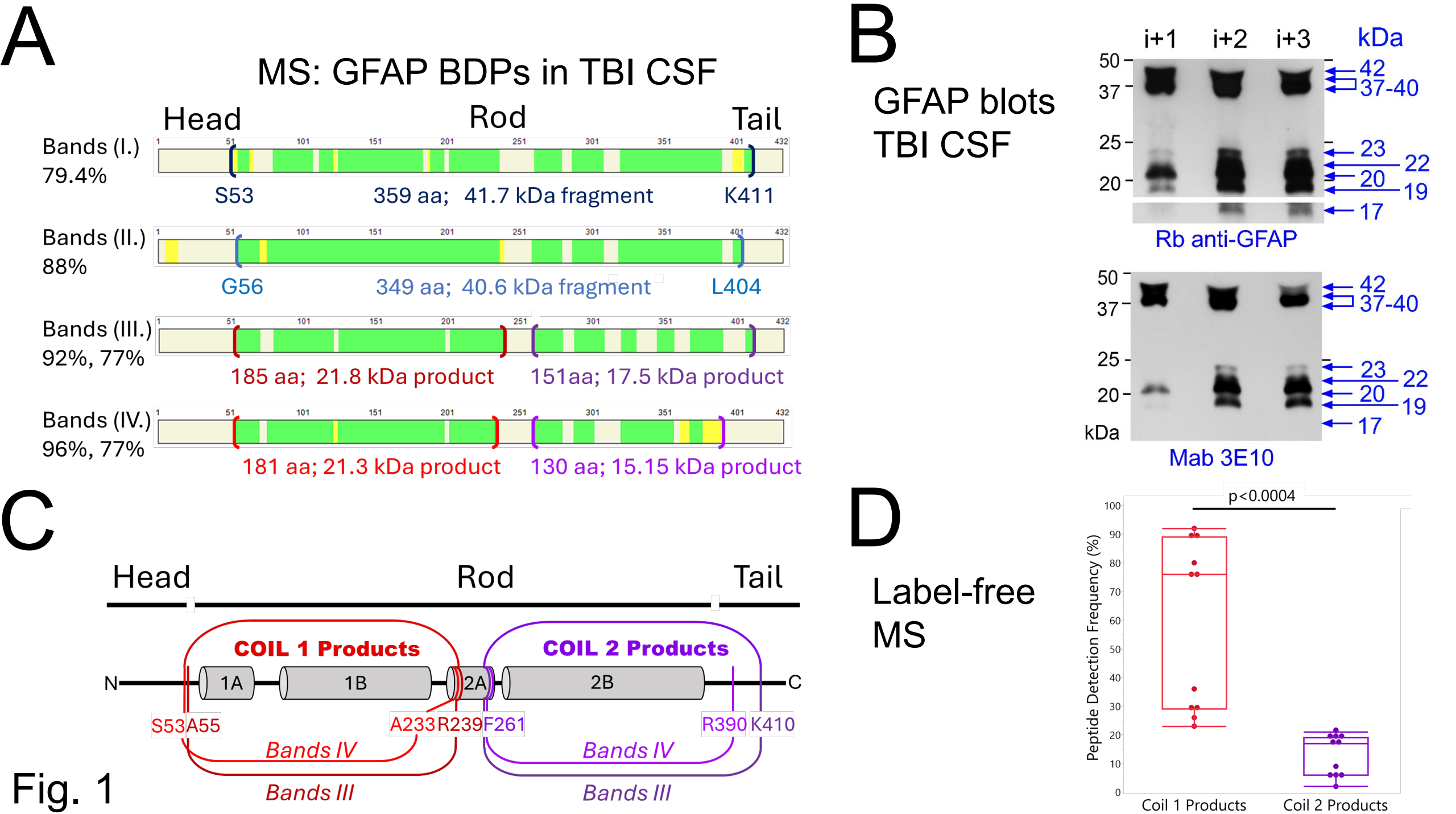

A
MS: GFAP BDPs in TBI CSF
Head
Rod
Tail
Bands (I.)
79.4%
S53
359 aa; 41.7 kDa fragment
K411
Bands (II.)
88%
G56
349 aa; 40.6 kDa fragment
L404
Bands (III.)
92%, 77%
185 aa; 21.8 kDa product
151aa; 17.5 kDa product
Bands (IV.)
96%, 77%
181 aa; 21.3 kDa product
130 aa; 15.15 kDa product
B
GFAP blots
TBI CSF
i+1
i+2
i+3
kDa
50
37
25
20
42
37-40
23
22
20
19
17
Rb anti-GFAP
Mab 3E10
C
Head
Rod
Tail
COIL 1 Products
COIL 2 Products
N
1A
1B
2A
2B
C
S53
A55
A233
R239
F261
R390
K410
Bands IV
Bands III
D
Label-free
MS
p<0.0004
Peptide Detection Frequency (%)
Coil 1 Products
Coil 2 Products

Fig. 1

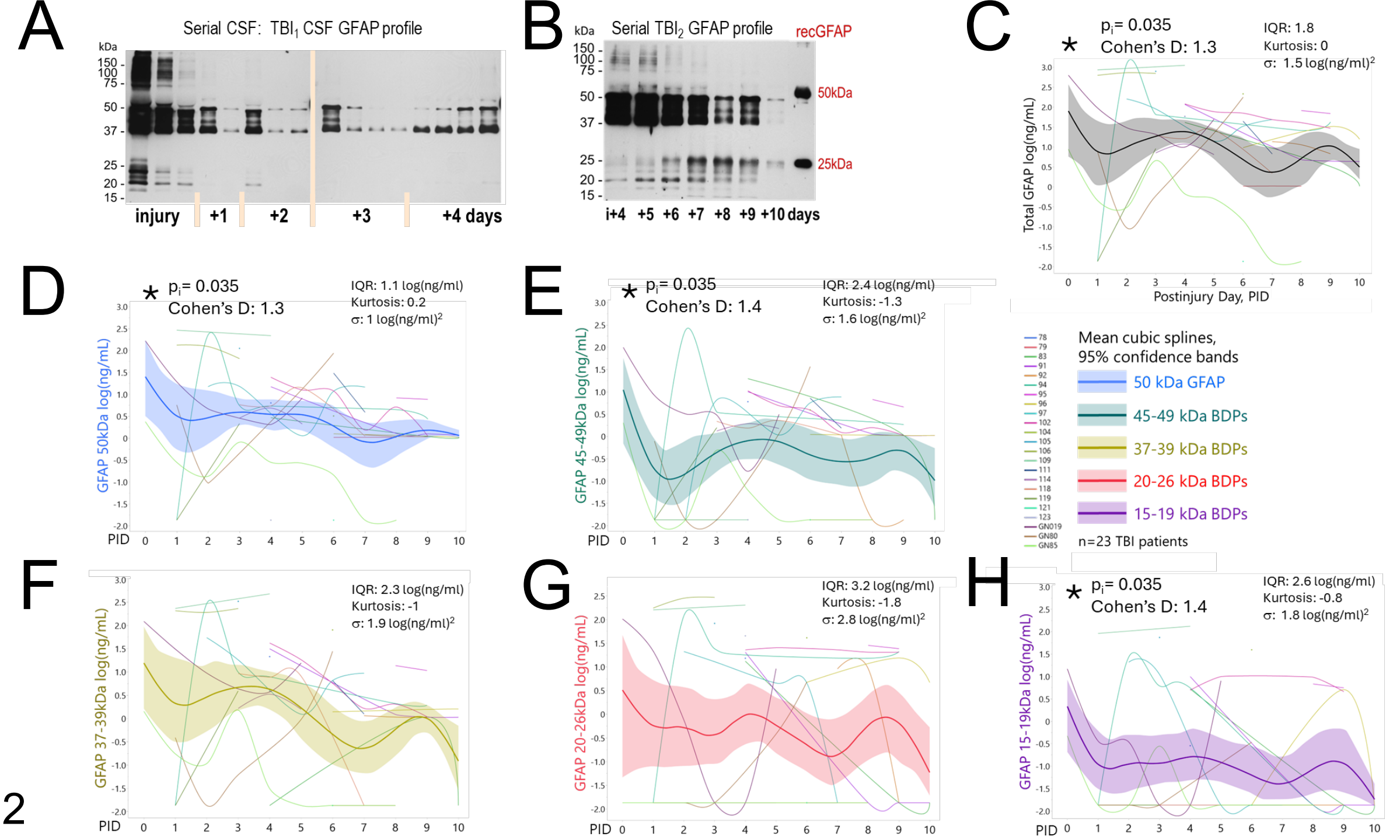

A
Serial CSF: TBI1 CSF GFAP profile
kDa
150
100
75
50
37
25
20
15
injury
+1
+2
+3
+4 days
B
Serial TBI2 GFAP profile
recGFAP
50kDa
25kDa
i+4 +5 +6 +7 +8 +9 +10 days
C
* pi= 0.035
Cohen's D: 1.3
IQR: 1.8
Kurtosis: 0
σ: 1.5 log(ng/ml)2
Total GFAP log(ng/mL)
Postinjury Day, PID
D
* pi= 0.035
Cohen's D: 1.3
IQR: 1.1 log(ng/ml)
Kurtosis: 0.2
σ: 1 log(ng/ml)2
GFAP 50kDa log(ng/mL)
PID
E
* pi= 0.035
Cohen's D: 1.4
IQR: 2.4 log(ng/ml)
Kurtosis: -1.3
σ: 1.6 log(ng/ml)2
GFAP 45-49kDa log(ng/mL)
PID
Mean cubic splines,
95% confidence bands
50 kDa GFAP
45-49 kDa BDPs
37-39 kDa BDPs
20-26 kDa BDPs
15-19 kDa BDPs
n=23 TBI patients
F
IQR: 2.3 log(ng/ml)
Kurtosis: -1
σ: 1.9 log(ng/ml)2
GFAP 37-39kDa log(ng/mL)
PID
G
IQR: 3.2 log(ng/ml)
Kurtosis: -1.8
σ: 2.8 log(ng/ml)2
GFAP 20-26kDa log(ng/mL)
PID
H
* pi= 0.035
Cohen's D: 1.4
IQR: 2.6 log(ng/ml)
Kurtosis: -0.8
σ: 1.8 log(ng/ml)2
GFAP 15-19kDa log(ng/mL)
PID

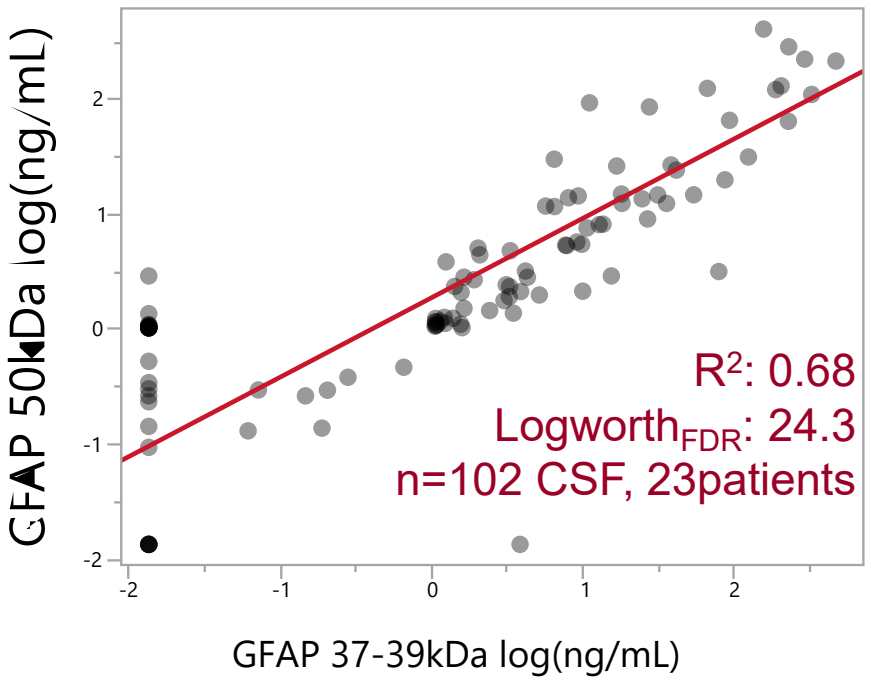
Fig. 2

A

Pearsons Correlation Coefficients

	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

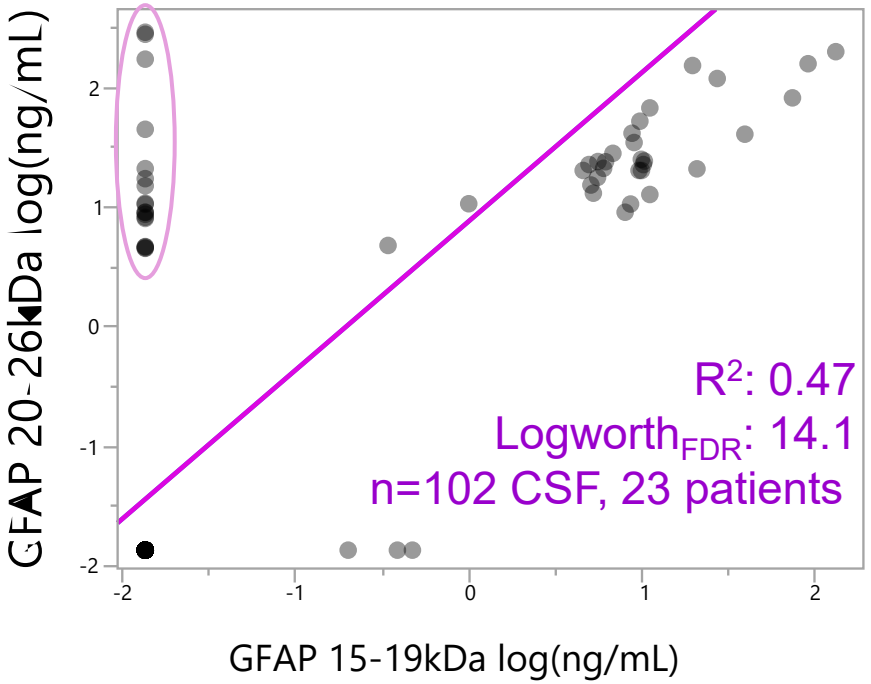


Fig. 3

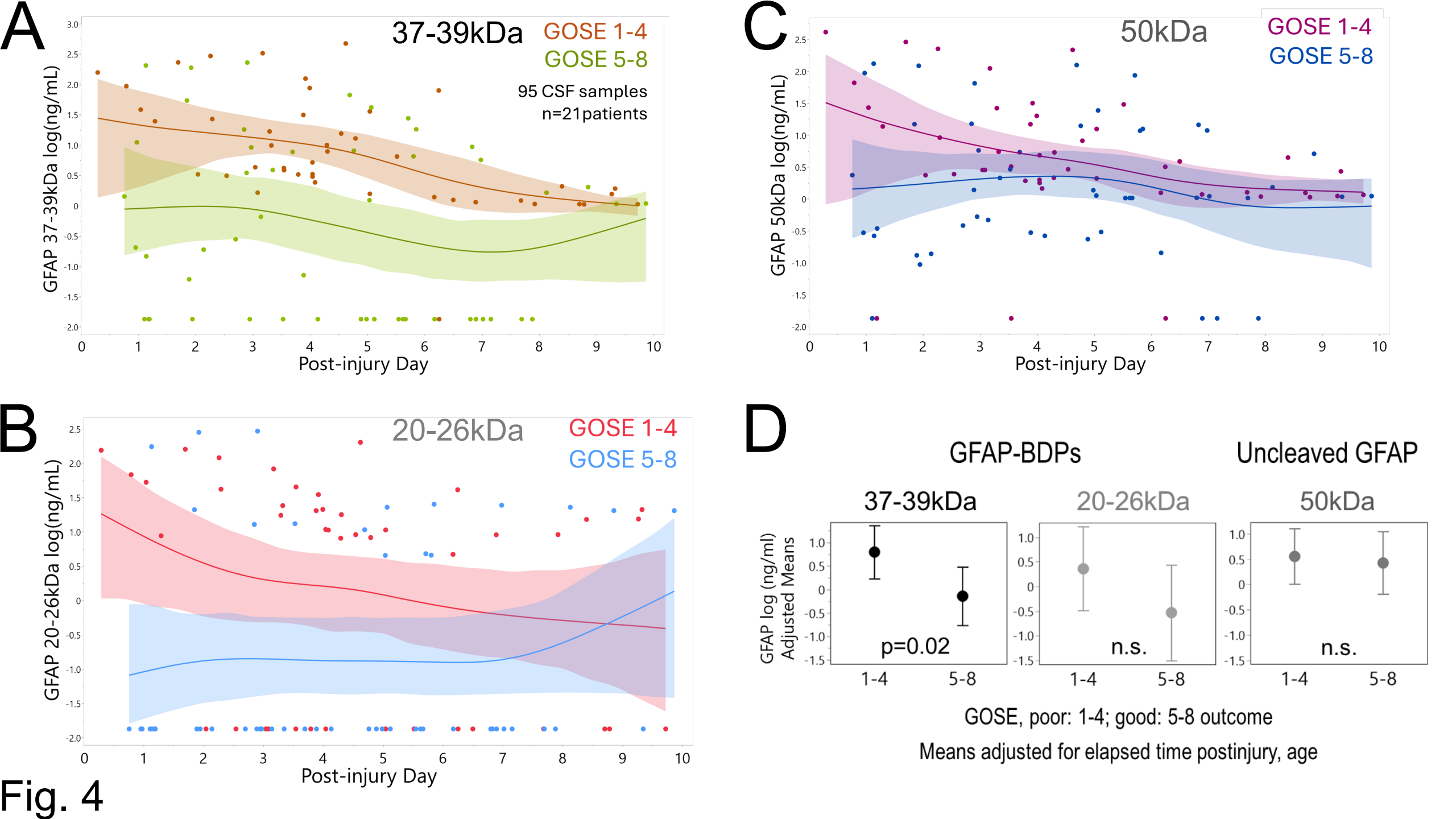
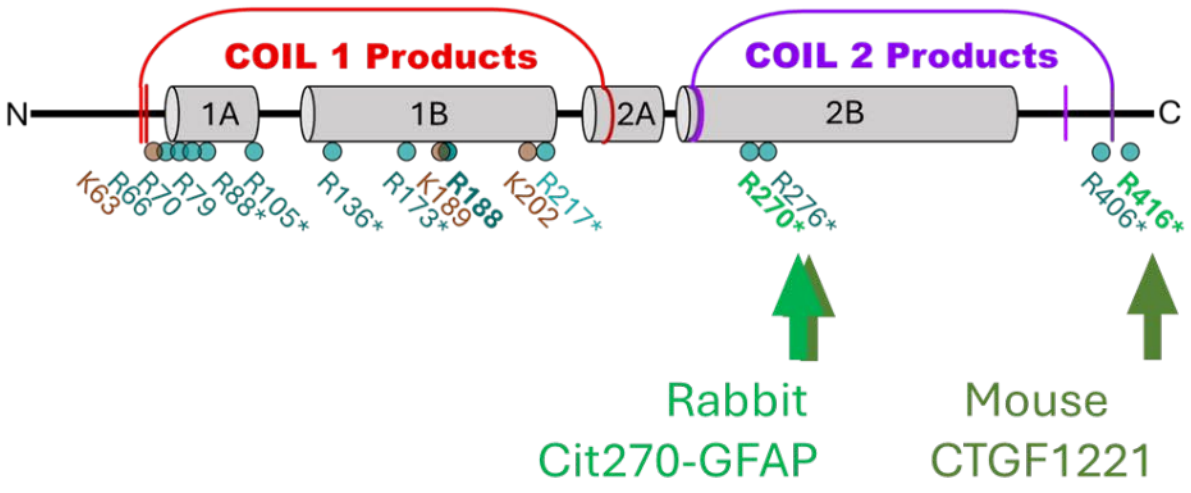

A
37-39kDa
GOSE 1-4
GOSE 5-8
95 CSF samples
n=21patients
GFAP 37-39kDa log(ng/mL)
Post-injury Day
C
50kDa
GOSE 1-4
GOSE 5-8
GFAP 50kDa log(ng/mL)
Post-injury Day
B
20-26kDa
GOSE 1-4
GOSE 5-8
GFAP 20-26kDa log(ng/mL)
Post-injury Day
D
GFAP-BDPs
Uncleaved GFAP
37-39kDa
20-26kDa
50kDa
GFAP log (ng/ml)
Adjusted Means
p=0.02
n.s.
n.s.
1-4
5-8
GOSE, poor: 1-4; good: 5-8 outcome
Means adjusted for elapsed time postinjury, age

Fig. 4

A



13 Citrullinated arginine residues

5 Acetylated lysine residues

B

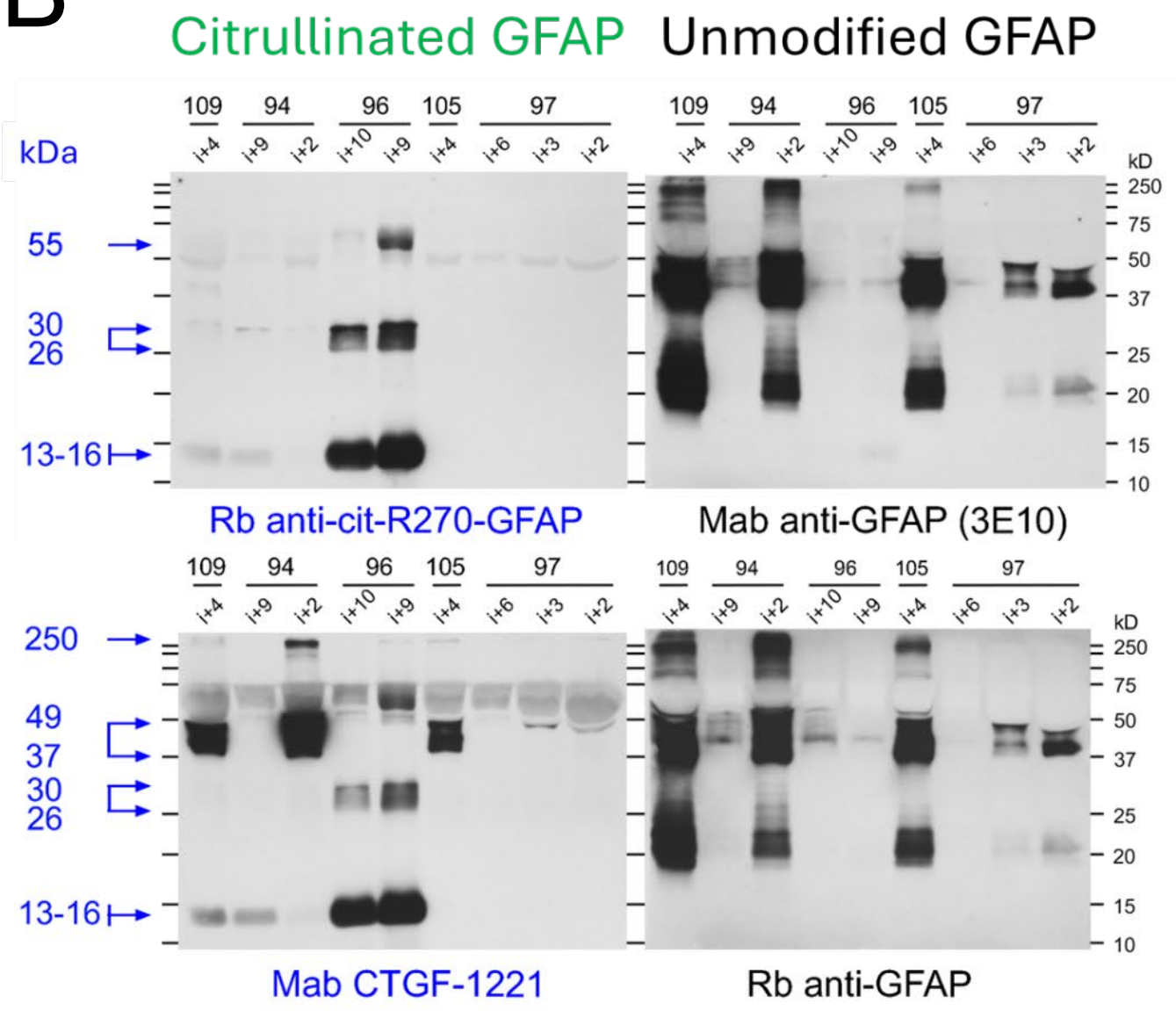


Fig. 5

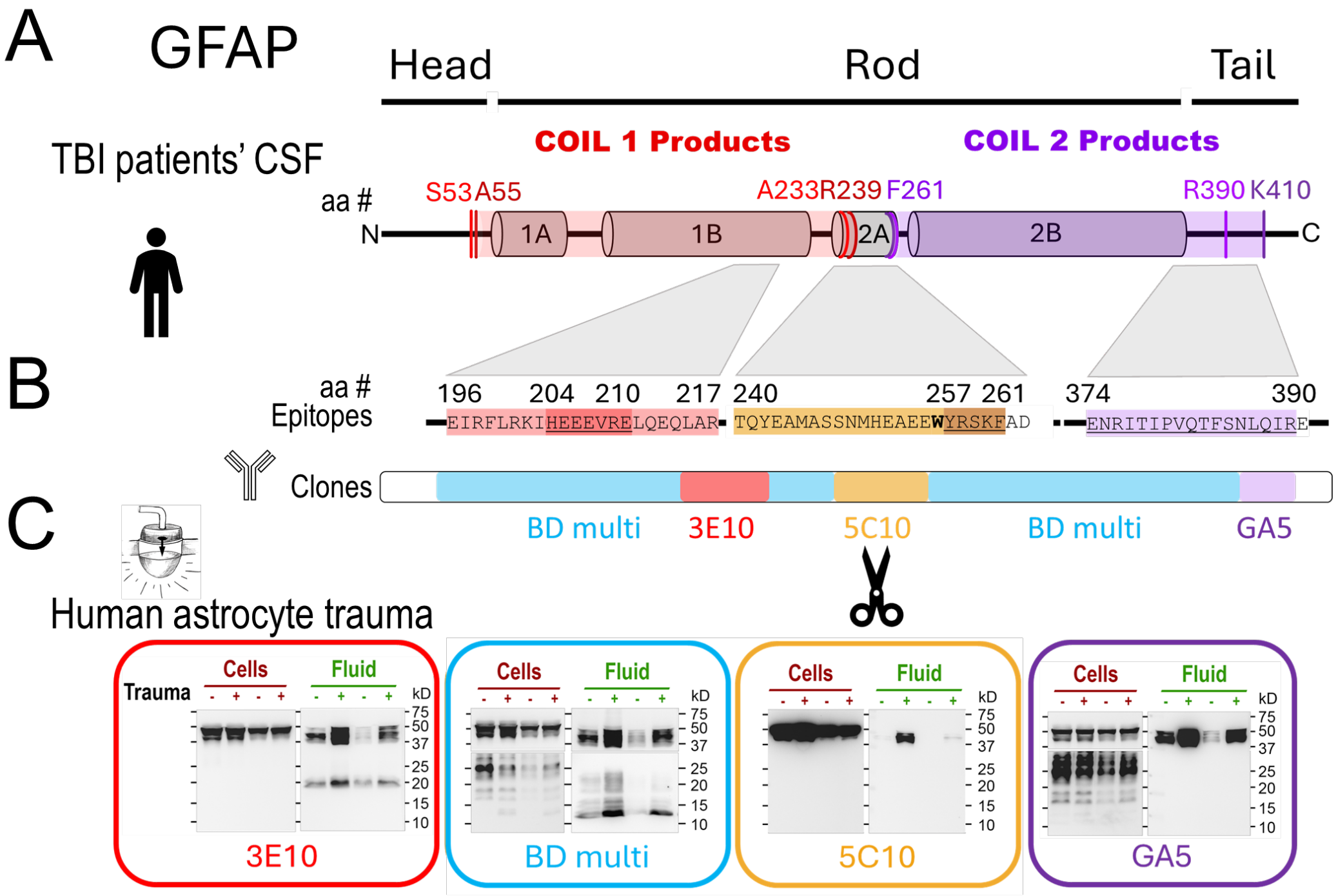

A
GFAP
Head
Rod
Tail
TBI patients' CSF
COIL 1 Products
COIL 2 Products
aa #
S53A55
A233R239
F261
R390
K410
N
1A
1B
2A
2B
C
B
aa #
196
204
210
217
240
257
261
374
390
Epitopes
EIRFLRKIHEEEVRELQEQLAR
TQYEAMASSNMHEAEEWYRSKFAD
ENRITIPVQTFSNLQIRE
Clones
BD multi
3E10
5C10
BD multi
GA5
C
Human astrocyte trauma
Trauma
Cells
Fluid
kD
75
50
37
25
20
15
10
3E10
BD multi
5C10
GA5

Fig. 6

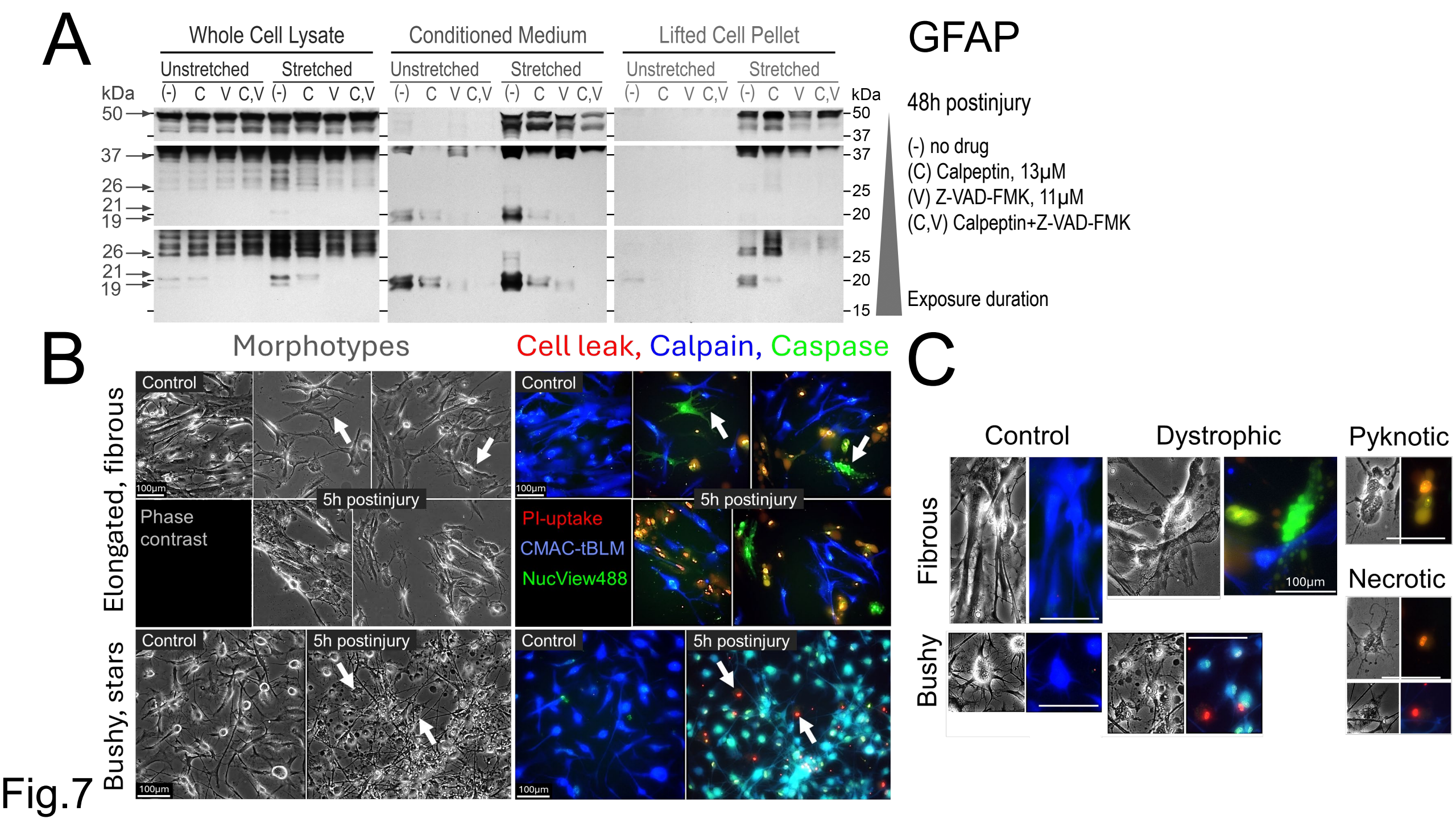
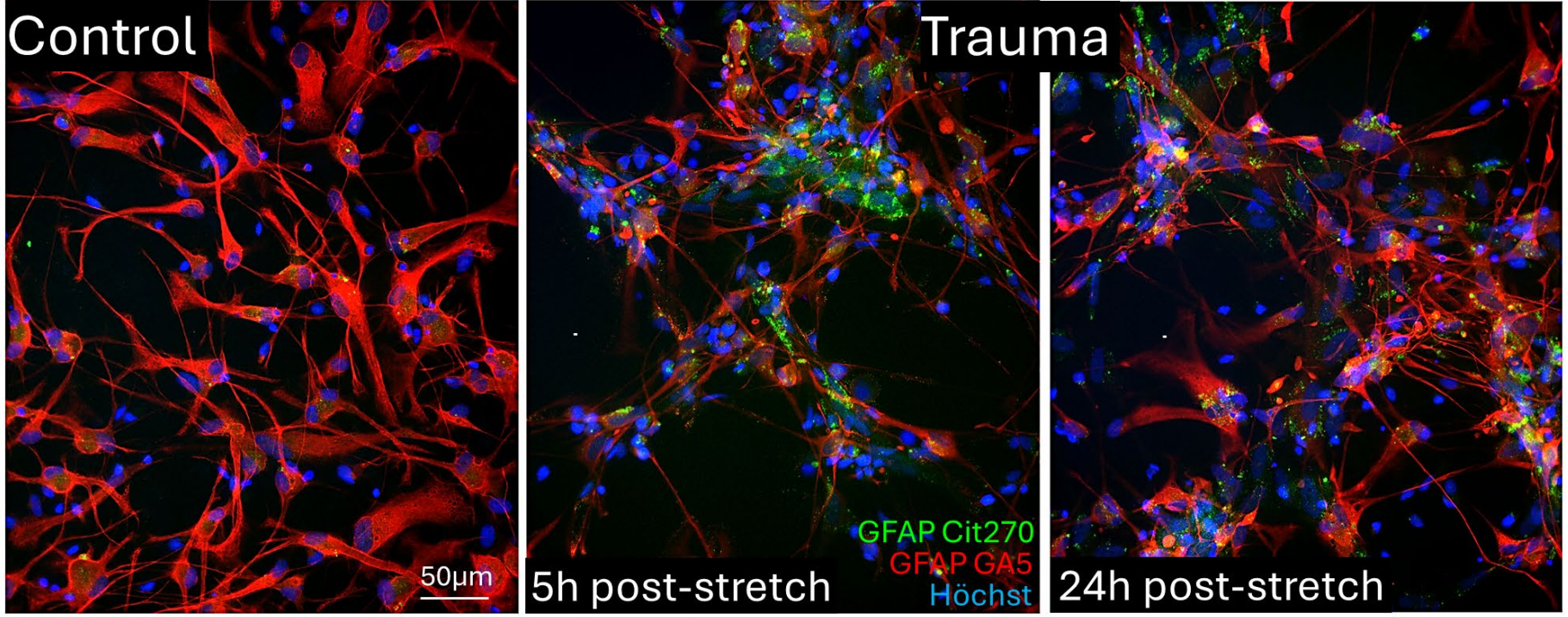

A
GFAP
Whole Cell Lysate
Conditioned Medium
Lifted Cell Pellet
Unstretched
Stretched
kDa
(-) C V C,V
50
37
26
21
19
25
20
15
48h postinjury
(-) no drug
(C) Calpeptin, 13μM
(V) Z-VAD-FMK, 11μM
(C,V) Calpeptin+Z-VAD-FMK
Exposure duration
B
Morphotypes
Cell leak, Calpain, Caspase
Elongated, fibrous
Bushy, stars
Control
5h postinjury
Phase contrast
PI-uptake
CMAC-tBLM
NucView488
100μm
C
Control
Dystrophic
Pyknotic
Necrotic
Fibrous
Bushy
100μm

Fig.7

A


Control
Trauma
50µm
5h post-stretch
GFAP Cit270
GFAP GA5
Höchst
24h post-stretch

B

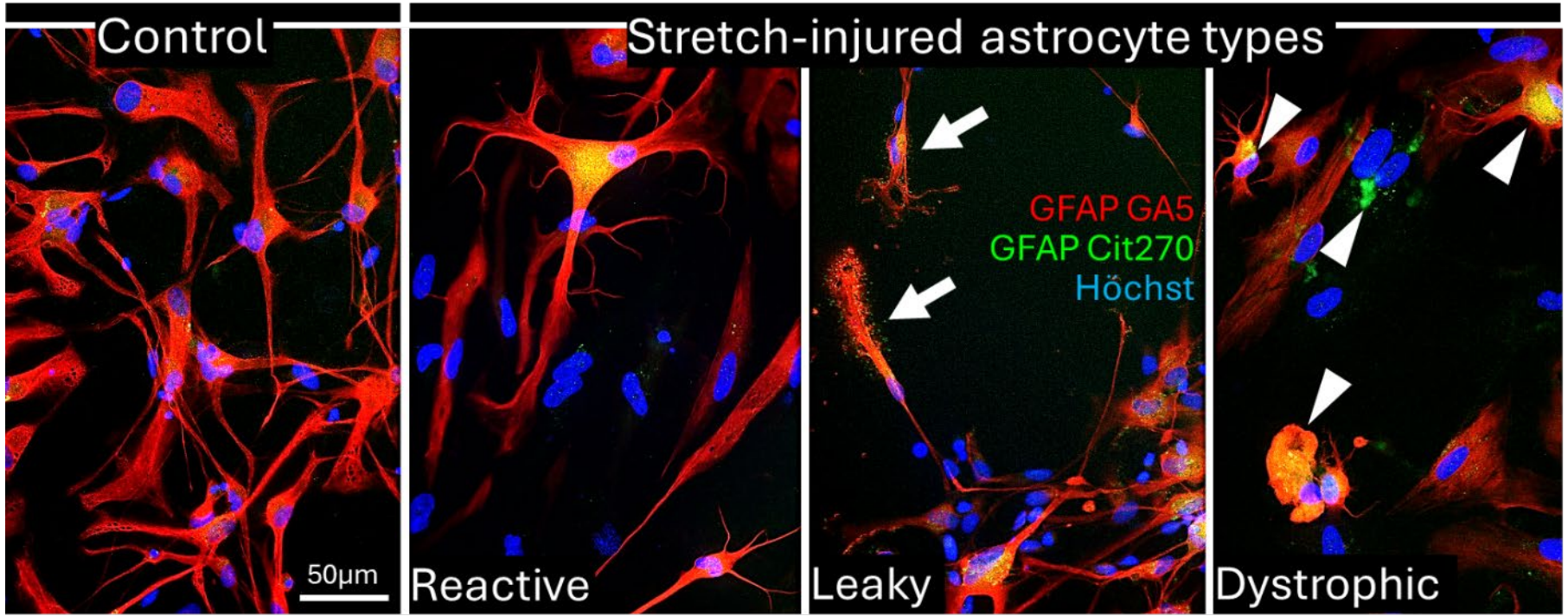

Control
Stretch-injured astrocyte types
50µm
Reactive
GFAP GA5
GFAP Cit270
Höchst
Leaky
Dystrophic

Fig. 8