Title: Axonal marker neurofilament light predicts long-term outcomes and progressive neurodegeneration after traumatic brain injury

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One Sentence Summary:

Axonal injury after moderate-severe TBI can be quantified using plasma NfL, which predicts longterm functional outcomes and progressive neurodegeneration after injury.

Abstract:

Axonal injury is a key determinant of long-term outcomes after traumatic brain injury (TBI) but has been difficult to measure clinically. Fluid biomarker assays can now sensitively quantify neuronal proteins in blood. Axonal components such as neurofilament light (NfL) potentially provide a diagnostic measure of injury. In the multicenter BIO-AX-TBI study of moderate-severe TBI, we investigated relationships between fluid biomarkers, advanced neuroimaging, and clinical outcomes. Cerebral microdialysis was used to assess biomarker concentrations in brain extracellular fluid aligned with plasma measurement. An experimental injury model was used to validate biomarkers against histopathology. Plasma NfL increased after TBI, peaking at 10 days to 6 weeks but remaining abnormal at 1 year. Concentrations were around 10 times higher early after TBI than in controls (patients with extracranial injuries). NfL concentrations correlated with diffusion MRI measures of axonal injury and predicted white matter neurodegeneration. Plasma TAU predicted early gray matter atrophy. NfL was the strongest predictor of functional outcomes at 1 year. Cerebral microdialysis showed that NfL concentrations in plasma and brain extracellular fluid were highly correlated. An experimental injury model confirmed a dose-response relationship of histopathologically defined axonal injury to plasma NfL. In conclusion, plasma NfL provides a sensitive and clinically meaningful measure of axonal injury produced by TBI. This reflects the extent of underlying damage, validated using advanced MRI, cerebral microdialysis, and an experimental model. The results support the incorporation of NfL sampling subacutely after injury into clinical practice to assist with the diagnosis of axonal injury and to improve prognostication.

INTRODUCTION

Long-term outcomes of traumatic brain injury (TBI) are frequently poor (1). Traumatic axonal injury (TAI) is a key determinant of these clinical outcomes (2, 3). However, validated methods to quantify the extent of axonal injury are lacking in a clinical context. Several proteins are

potential biomarkers of axonal injury after TBI. These include cytoskeletal proteins neurofilament light (NfL) and microtubule associated protein tau (tau), as well as the more the more widely expressed neuronal ubiquitinating enzyme ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1). Proteins whose expression is predominantly astroglial, such as calcium binding protein S100 B (S100B), or glial fibrillary acidic protein (GFAP) may also provide a surrogate readout of axonal damage by quantifying the inflammatory response and non-neuronal tissue damage (4). Neurofilament light has shown diagnostic, monitoring and prognostic utility across several neurological diseases (5). Advanced immunoassays such as single molecule array (Simoa) allow ultra-low concentrations in venous blood to be accurately measured, with levels reflecting cerebrospinal fluid concentrations (6-8). After moderate-severe TBI, NfL is acutely elevated in plasma, sometimes remaining increased years after injury (6, 9). Plasma levels continue to rise post-injury, in contrast to other blood biomarkers which typically peak within days (8, 10). Initial work shows that blood levels of NfL can predict late clinical outcomes, although it is uncertain how much prognostic information is provided when compared to other biomarkers such as GFAP, UCH-L1, tau, and S100B (6, 8, 11, 12). The optimal time to sample NfL after TBI is unclear as the dynamics of its fluctuation over time have not been characterised in detail.

The source of plasma NfL after TBI is also unclear, which limits its diagnostic value. NfL is a type IV intermediate filament that is found within axons. It is present in high quantities within long myelinated axons in the cerebral deep white matter that are susceptible to TAI (13, 14). Hence, plasma levels of NfL may reflect its release from white matter damaged following head injury. One way to assess this possibility is to directly measure levels from extracellular fluid collected from damaged white matter. This has not been done previously but can be achieved using invasive cerebral microdialysis with catheters permeable to neuronal breakdown products. High levels of tau in brain extracellular fluid early post-injury have been demonstrated using this method, with levels correlating with imaging evidence of axonal injury (15). Preliminary microdialysis work with NfL has shown increased levels in peri-contusional and apparently normal-appearing white matter regions that correlate with extracellular tau levels (16). However, NfL concentrations in microdialysate and peripheral venous samples have not been compared. Establishing that plasma and extracellular NfL correlates helps to clarify its origin and the simultaneous quantification of a range of brain injury proteins (e.g. NfL, tau, UCH-L1 and GFAP) in microdialysate will help understand whether it provides unique information.

Diffusion tensor imaging (DTI) provides another way to assess the relationship between plasma NfL and axonal injury within the brain. Fractional anisotropy is widely used as a measure of axonal injury that: (a) correlates with histopathological evidence of TAI (17); (b) provides prognostic information about long-term clinical outcomes; and (c) predicts the pattern of progressive white neurodegeneration indicated by brain atrophy (3, 18). Hence, if plasma NfL originates from damaged axons, levels should correlate with diffusion imaging measures of TAI in the brain and might also predict the extent of post-traumatic neurodegeneration as quantified by white matter atrophy (6).

Plasma NfL could also originate outside the brain and it remains unclear how specific increased levels are to TBI. Mutations in the NEFL gene, which encodes NfL, cause the hereditary peripheral neuropathy Charcot Marie Tooth disease and blood NfL levels can be elevated in both chronic (19) and acute peripheral neuropathies (20). This is important as TBI often occurs alongside peripheral nerve injury, which can potentially increase NfL. This has been an issue for other potential blood biomarkers in TBI. For example, S100B has substantial expression in adipose tissue as well as astrocytes, confusing the interpretation of high levels when significant soft tissue injury is present (21). Hence, comparing NfL levels in groups of patients with TBI and non-TBI trauma is necessary for a complete understanding of the specificity of NfL as a measure of traumatic axonal injury in the central nervous system.

Experimental injury models of TBI can be used to test the relationship between blood biomarkers and controlled injuries, providing ground truth validation. The controlled cortical impact (CCI) model provides a reproducible means to control the extent and distribution of injury, and to examine the associated histopathological effects (13, 22, 23). High field magnetic resonance imaging can be used to provide diffusion imaging measures aligned to human studies, allowing histopathological effects of injury to be compared with neuroimaging measures of axonal injury and blood biomarker levels of NfL. Previous experimental work on blood NfL in TBI is limited but suggests a relationship between plasma NfL levels and neurofilament-immunoreactive axonal swellings indicative of injury (24, 25). A dose-response relationship of NfL to injury has not previously been directly established.

The multicentre prospective BIO-AX-TBI study (NCT03534154) and aligned experimental injury model were conducted to: (a) investigate the optimal timing of axonal injury fluid biomarker assessment after single moderate-severe TBI; (b) assess how these relate to radiological measures

of axonal injury and clinical outcomes; and (c) compare plasma biomarker levels against brain extracellular fluid in humans and ground truth histopathology in an experimental injury model (Figure 1) (26). We tested the following specific hypotheses in relation to NfL: (a) levels will increase after TBI and this will be detectable in plasma; (b) specific changes in NfL will be seen after TBI when compared to patients with extracranial injuries but no TBI; (c) plasma NfL will correlate with levels in extracellular fluid on cerebral microdialysis; (d) plasma NfL will correlate with diffusion MRI measures of axonal injury, specifically reduced FA; (e) plasma NfL concentrations will relate to histopathological evidence of axonal injury in the CCI model; (f) plasma NfL will predict white matter atrophy over time; and (g) plasma NfL will predict clinical outcomes at six and twelve months post-injury.

Fig. 1. Study design. (A) BIO-AX-TBI patient recruitment and retention. Univ: university; Hosp: hospital. (B) Assessments: typical patient pathway (top), centres performing additional advanced repeated neuroimaging and longitudinal blood biomarkers (middle), CT is performed clinically at all sites, and invasive cerebral microdialysis at two sites (lower). (C) Experimental injury model at London site.

RESULTS

Demographics, clinical characteristics and outcomes of patients and healthy controls

One hundred ninety-seven patients were recruited after moderate-severe TBI from eight major trauma centres (Supplementary Table 1). Median age at injury was 47 years (IQR 30) with 77% (152) male (Figure 2A,B). 74% (146) underwent MRI, with a median age of 44 (IQR 30) of whom 80% (116) were male. Injuries were high energy in 56% (110), comprising falls from more than three metres or collision at more than 30 km/h (Supplementary Table 2). 91% (180) of injuries were accidental, with 47% (93) road-traffic related. Acute loss of consciousness was definite or suspected in 75% (147) of patients and 69% (136) had definite or suspected retrograde amnesia. The cohort included a range of severities within the moderate-severe classification, with prehospital Glasgow Coma Scale (GCS) 3 to 8 in 45% (89) (Figure 2C).

Fig. 2. Demographics, Clinical Characteristics and Outcomes. (A) Age and sex of patients after traumatic brain injury (TBI), healthy controls (HC) and with non-TBI-trauma (NTT). (B) Relationship of age at injury to hospital admission duration and mortality (red:died, blue:survived). (C) Acute Glasgow Coma Scale (GCS) after TBI. (D) Pupillary assessment in emergency department (ED). N: normal; Uni dil.: unilaterally dilated; Bilat dil:

bilaterally dilated. (E) Marshall classification of most severe CT in first day. (F) MRI pathologies subacutely (ie. at ten days to six weeks). (G) Glasgow Outcome Score Extended (GOSE) at six and twelve months post-TBI.

Acute CT (Figure 2E) identified parenchymal contusions in 66% (130), subarachnoid haemorrhage in 60% (118), subdural haematoma in 57% (113) and extradural haematomas in 22% (44). Diffuse axonal injury was reported on CT in 18% (35). Raised intracranial pressure was present in 29% (58) of patients and was persistent/refractory in 16% (31). Neurosurgical evacuation or decompression were performed in 34% (66) of patients.

Subacute MRI was performed, between ten days to six weeks post-injury in 71% (140) of patients (Figure 2F), a time-window chosen to ensure stabilisation of dynamic diffusion changes. Parenchymal contusions were present in 85% (119), with subarachnoid haemorrhage in 21% (30), subdural haematomas in 68% (95), extradural haematomas in 4% (6), oedema in 27% (38) and microhaemorrhages in 69% (97). Group-wise lesion maps were hand-drawn for each scan to be accounted-for in the imaging analysis (Supplementary Figure 1).

During the study period, 7% (13) of patients died, at a median of 11 days post-injury. Functional outcomes were assessed over a year (Figure 2G). All patients had a known outcome at six or twelve months post injury: specifically, the GOSE was available in 94% (185) at 6 months and 82% (161) at 12 months. At six months, 29% (58) of patients made a good recovery, defined as GOSE score 7 or 8. Six individuals were in a vegetative state (GOSE 2) at 6 months, and were unchanged at 12 months. By 12 months post-injury 32% (62) of patients made a good recovery.

To assess the blood biomarker specificity, 25 patients hospitalised with non-TBI traumatic (NTT) extracranial injuries were recruited (for injury details see Supplementary Table 3). These patients, who had no head injury history, had a median age at injury of 41 (IQR 29) of whom 92% (23) were male. NTT patients had fluid biomarker assessment twice in the first ten days post-injury, aligned to the TBI group. The groups did not differ significantly in age or sex.

Healthy volunteers were assessed across all the sites, comprising 128 individuals with a median age of 39 (IQR 27) of whom 78% (61) were male. 95% (121) underwent MRI and 75% (96) had blood biomarker assessment. This group was slightly younger ($W = 10784$, $P = 0.028$) and more female ($X2$ 9.10, $df = 1$, $P = 0.003$) than the TBI patients. However, there was no significant age difference between patients and healthy controls who underwent MRI. To contextualise MRI atrophy rate assessments after TBI within normal life-course change, 30 healthy controls with a

median age of 40 (IQR 27), of whom 70% (21) were male, were imaged twice. Neither sex nor age differed significantly between the main and longitudinal healthy control groups. (Supplementary Table 1).

Blood biomarker profiles after traumatic brain injury

Injury markers including plasma NfL were serially assessed on two occasions within ten days of injury, subacutely at ten days to six weeks, six and twelve months (Figure 3). Plasma NfL was significantly elevated post injury (Figure 3A,B), increasing over time and peaking subacutely, around 20 days post-injury (median peak NfL 507.3 pg/ml, IQR 728.2). NfL remained elevated at six and twelve months post-injury compared with healthy controls (both $P<0.001$, $W=387/431$, respectively, Figure 3C). 85% (45/53) of TBI patients had abnormally high NfL at six months (i.e. > 2 standard deviations from the healthy control mean). 52% (13/25) remained elevated at twelve months post-injury (Figure 3C, Supplementary Table 4). Age at injury was not a significant determinant of NfL level at 6 or 12 months using linear regression.

TBI patients also had significant elevations of plasma total tau, serum S100B, plasma UCH-L1 and GFAP compared with healthy controls. Levels peaked within the first day of injury (median peak for tau 7.0pg/ml; S100B 215.1pg/ml; UCH-L1 97.6pg/ml; GFAP 12,590.5 pg/ml). These biomarkers normalised following the subacute visit except for plasma GFAP, which remained chronically raised at twelve months ($W=691$, $P=0.041$; Supplementary Table 4). 34% (18/53) of patients had raised plasma GFAP at six, as did 32% (8/25) at twelve months. Age at injury modestly contributed to GFAP levels at six (adjusted $R2$ 0.27, $P < 0.001$) but not twelve months post injury. Patients with raised plasma GFAP at six months were significantly older than those with normal levels ($W=134$, $P=0.001$).

Similar plasma levels of NfL and tau were seen in patients with and without visible pathology on CT. There were no significant differences in peak NfL or tau when comparing Marshall grade I patients (n=23, no lesions on scan) with Marshall grades II-VI (n=168, encompassing all diffuse / mass lesions). In contrast, higher peak concentrations of UCH-L1 ($W=910$, $P=0.033$), S100B ($W=776$, $P=0.0027$) and GFAP ($W=779$, $P=0.005$) were seen in patients with Marshall grades IIVI versus grade I. Similarly, there were no significant differences in peak NfL between patients with lesions on MRI (n=125) compared to those without lesions (n=18). Higher concentrations of tau ($W=1518$, $P=<0.001$, UCH-L1 ($W=1428$, $P<0.001$), S100B ($W=1469$, $P<0.001$) and GFAP ($W=1484$, $P<0.001$) were observed in patients with lesions on MRI. Where lesions were present

on MRI, peak blood concentrations of NfL, tau, UCH-L1, S100B and GFAP all correlated with lesion volume (all P<0.001, ρ=0.50, 0.52, 0.52, 0.53, 0.45 respectively).

Patients with no history of TBI (n=25) showed lower concentrations of all blood biomarkers that the TBI group (all P values <0.001). Non-TBI patients did show some increased plasma NfL and UCH-L1 compared to healthy controls, but with there were no differences in tau, GFAP or S100B (Figure 3, Supplementary Table 4). There was a small difference in age between TBI and control groups, but this had no significant effect on the results (see Supplementary Materials - Sensitivity Analysis, Supplementary Table 5).

Fig. 3. Blood biomarker trends in TBI, non-TBI trauma and healthy controls. (A) Trends of blood NfL, tau, UCH-L1, GFAP and S100B concentrations after TBI. (B) Local polynomial (LOESS) regression curves modelling concentrations in the first 40 days post-TBI . (C) Group comparisons of biomarkers in healthy controls (HC), TBI patients and patients with non-TBI traumatic injuries (NTT) at different timepoints.

NfL, tau and UCH-L1 levels are highly correlated between plasma and brain microdialysate

Eighteen patients underwent cerebral microdialysis and simultaneous plasma sampling within the first ten days of injury (median age 34 years, 78% male, Supplementary Table 6). Microdialysis was initiated a median of 21 hours post-injury (IQR 25), continuing for a median of 6 days in total (IQR 4.75). This allowed blood levels to be compared with brain extracellular fluid sampled from white matter potentially affected by traumatic axonal injury. Frontal white matter without evidence of focal brain injury was sampled, from the right hemisphere in 83% (15) and left 17% (3). The microdialysis group had a mortality rate of 28% (5) within a month of injury.

NfL, total tau, UCH-L1 and GFAP concentrations were significantly higher in brain extracellular fluid than plasma (all P<0.001 Figure 4B, Supplementary Table 7). Median NfL, tau and UCH-L1 levels were around 100 times higher in microdialysate than plasma, whereas GFAP was around four times higher (see individual trajectories, Supplementary Figure 2). Linear mixed effects modelling accounting for time since injury and patient variability showed a strong relationship between blood and brain extracellular fluid concentrations for NfL (P=0.001, t=3.3, AIC=-128.4), total tau (P=0.003, t=2.9, AIC=57.8) and UCH-L1 (P<0.001, t=4.8, AIC=19.7) but not GFAP (P=0.76).

Fig. 4. Cerebral microdialysis evidence of axonal injury acutely following TBI. (A) CT images showing indicative placement of cerebral microdialysis catheter in deep white matter (B)

Longitudinal trajectories NfL, tau and UCH-L1 and GFAP in brain extracellular fluid over the first 10 days post-TBI.

Imaging evidence of traumatic axonal injury

Diffusion MRI showed evidence of extensive TAI (Figure 5A). Individual z-score maps of fractional anisotropy (FA) were generated at each scanning site by comparing patients with local controls. Significant reductions in FA were present in a large number of white matter tracts at all three time points (Figure 5A,B). We examined the whole white matter skeleton and corpus callosum. Both showed significant reductions in FA at the subacute, six and twelve month time points (all P<0.001, Figure 5A, Supplementary Table 8). FA declined over time between the acute

time since injury (hours)

Biomarker concentration (pg/ml)

A

B

NfL

and six month assessments in a large number of white matter tracts including the whole white matter and corpus callosum regions (n=49, V=975, p<0.001)(Figure 5C,D). Further reductions were present from 6-12 months but were not significant (n=36, V=209, p=0.052). Focal lesions were excluded from the calculation of FA in all analyses (Supplementary methods) (27, 28).

Fig. 5. Axonal injury and neurodegeneration after moderate-severe TBI. (A) Fractional anisotropy (FA) in patients z-scored against local healthy controls in the whole white matter (WM, upper part) and the corpus callosum (CC, lower part), for subacute, 6 and 12 month visits in controls (blue) and patients (red). (B) Voxelwise comparison of zFA in patients vs controls with significant group differences in red, overlaid on the WM skeleton in green. Significant differences are seen at subacute (upper part), 6 month (middle) and 12 month (lower) visits. (C) Longitudinal change in FA measures between subacute and six month, and six to twelve month visits in WM and CC regions post-TBI. (D) Voxelwise map of regions with significant within-subject FA reductions between subacute and 6 months visits. (E) Jacobian determinant (JD) atrophy rates in grey matter (GM) and white matter (WM) in controls and patients. (F) Voxelwise map of significant longitudinal reduction in WM volume (in blue) between 6-12 months post-injury.

Post-traumatic neurodegeneration

Brain volume changes over time were investigated to assess neurodegeneration triggered by TBI (Figure 5E). The Jacobian determinant (JD) measure provides a sensitive index of atrophy rates (29). Grey matter JD was reduced, indicating progressive atrophy, in patients during the first six months post-injury (W=469, P<0.001) with accompanying CSF expansion (W=1214, P=0.002). In contrast, white matter JD was only reduced six to twelve months post-injury (W=309, P=0.001) with accompanying CSF expansion (W=769, P=0.005) but no abnormality of grey matter JD versus healthy controls. Linear mixed-effects modelling showed a significant interaction between tissue class and time (F(2)=8.4, P<0.001). This was the result of lower grey matter JD in the first six months (V=183, P=0.01), but no significant differences in white matter JD between subacute to six month, and six to twelve month intervals longitudinally, within patients. Whole-brain analyses showed significant longitudinal atrophy of right internal capsule and posterior thalamic radiation from six to twelve months post-injury (Figure 5F). No other significant group differences were present voxelwise between patients and controls at the first interscan interval, or for other tissue classes at the second interscan interval.

Peak concentrations of all blood biomarkers predicted grey matter atrophy rates in the first six months post-injury (all P<0.004, adj. R2 0.20 to 0.34, Supplementary Table 9, Figure 6A), which was strongest for tau (adj. R2 0.34, P<0.001). In contrast, only peak plasma NfL predicted late white matter atrophy in the six to twelve month interval (adj. R2 0.14, P=0.037). We have previously reported that axonal injury in the chronic phase after TBI measured by FA predicts later neurodegeneration measured by JD (18). Here, we found a similar voxelwise relationship between acute axonal injury and later white matter neurodegeneration (Figure 6B): subacute FA was correlated with longitudinal white matter atrophy between 6 and 12 months in a number of white matter tracts including the corpus callosum, right corona radiata, and left cerebral peduncle.

Fig. 6. Relationship of TBI biomarkers brain atrophy rates and functional outcomes. Peak blood biomarker levels in the first six weeks post-TBI used for predictions, alongside the subacute z-scored FA in either corpus callosum (zFA CC) or white matter (zFA WM). (A) Peak biomarker concentrations and tissue-specific atrophy rates in grey matter from the subacute to six month visit (most strongly predicted by tau), and in white matter from six to twelve months post injury (predicted by NfL). (B) Areas of significant correlation between subacute white matter FA and atrophy rates from 6-12 months post injury. (C) Receiver operating characteristic curves predicting Glasgow Outcome Score Extended

(GOSE) >4 post-TBI at six (left) and twelve (right) months.

Plasma NfL correlates with diffusion MRI measures of axonal injury

Next we tested whether peak axonal injury blood biomarker levels correlated with diffusion measures of axonal injury. Plasma NfL showed a strong negative correlation with FA (ρ=-0.44, P<0.001) as did plasma tau (ρ=-0.34, P<0.001) (Figure 7A). Factor analysis of the peak blood biomarker levels and the corpus callosum FA was performed to assess the latent variable structure underlying these observations in a data-driven way (Figure 7B). Three factors explained 71% of the variance in the data. Peak NfL and subacute FA in the corpus callosum both loaded heavily on the same factor, suggesting a close association of these measures (loading strengths 0.56 and -0.99, respectively). The other two factors were loaded variably by the blood biomarkers, but with little contribution of corpus callosum FA and lower NfL loading.

Diffusion tensor MRI was also performed in 12 of 18 patients who had undergone microdialysis (mean 25 days after injury; SD 12). Across the group, FA was reduced relative to controls in the whole white matter (W= 339, P<0.001). More specifically, FA from a spherical region of interest around the catheter tip insertion site was also reduced (V=0, P=0.003). Individual variability in catheter site FA and microdialysate biomarker concentrations did not show a significant correlation using Spearman's rank test, across NfL, tau, UCH-L1 or GFAP.

Fig. 7. Correlations and factor structure of blood and neuroimaging biomarkers after TBI.
(A) Relationships of peak axonal injury plasma biomarker concentrations and subacute zscored fractional anisotropy (zFA) in the corpus callosum (CC) showing significant correlations. (B) Factor analysis showing three factors explaining latent variable structure of biomarkers, with axonal markers NfL and corpus callosum zFA both loading strongly on Factor two.

Biomarkers and functional outcomes

Long-term functional outcome was measured using the Glasgow Coma Scale Extended (GOSE). This was binarised using a score of six or higher to indicate favourable outcomes. Peak plasma NfL concentration in the first six weeks post-injury, during the subacute time period, had the highest pseudo R2 value in predicting the GOSE at six and twelve months post injury (McFadden's R2, 0.24, 0.48, respectively) (Figure 6C, Supplementary Table 9). Z-scored FA in the corpus callosum had the second highest pseudo R2, for both six and twelve month GOSE scores. Weaker but significant other predictors of outcome included peak plasma UCH-L1, plasma GFAP and

serum S100B, but not plasma tau.

Validation of axonal injury markers in an experimental injury model

A controlled cortical impact (CCI) model was used to validate plasma NfL against neuroimaging and histopathological evidence of axonal injury. Other fluid biomarkers were not assessed in the rodent work. Thirty-four rats underwent either sham-operation (n=4), 1mm CCI (n=15, 'mild injury') or 2mm CCI (n=15, 'moderate-severe injury'). Ten naïve animals served as histological controls. Plasma NfL increased over two weeks following 1mm or 2mm injury. Mixed effects modelling with time and injury as fixed effects showed a significant interaction between time and injury severity ($F(4,90)=14.9$, $P<0.001$). This was the result of similar plasma NfL concentrations at baseline, but higher plasma NfL at all post-injury timepoints in 1mm and 2mm CCI animals (all $P<0.001$, Figure 8A, Supplementary Table 10). Plasma NfL levels in sham/naive animals did not change over time (Supplementary Table 11). Cerebrospinal fluid was also sampled 15 days after CCI. CSF NfL was increased ($F(2,27)=24.2$, $P<0.001$) with higher levels in the 2mm group than the naïve/sham ($t=6.9$, $P<0.001$) and 1mm CCI animals ($t=5.0$, $P<0.001$, Supplementary Table 10). Peak plasma NfL correlated with CSF NfL ($P=0.003$, $\rho=0.52$) (Supplementary Figure 3).

White matter integrity was assessed in the corpus callosum (CC) using DTI at 14 days post-injury. FA was reduced after injury (Fig. 8C,D) with a main effect of injury severity due to lower FA with 2mm impacts ($F(1,100)=28.3$, $P<0.001$) (Fig. 8D). Plasma NfL concentrations were not significantly correlated with zFA. Histopathological examination was performed at 15 days postinjury. WM tissue density in the corpus callosum was reduced (Fig. 8E,F), with a significant interaction between severity and segment of the corpus callosum ($F(8,80)=4.5$, $P<0.001$). Following 2mm impact, Luxol fast blue-positive tissue was markedly thinned proximally to the injury site (segments 2-4; all $P<0.02$, Supplementary Table 11), but unchanged following 1mm or sham injury (Fig. 8E,F). Mean CC thickness was positively correlated with zFA ($r=0.67$, $P=0.008$) and negatively correlated with peak plasma NfL ($\rho=-0.61$, $P=0.02$, Supplementary Figure 3).

Axonal integrity was assessed using fluorescent neurofilament staining. 2 mm, but not 1mm or sham injury was associated with disorganised swollen axons and axonal bundles, with axonal spheroid bulbs in CC segments proximal to injury (Fig. 8G). There was a significant main effect of injury severity ($F(2,80)=40.57$, $P<0.001$) and anatomical region ($F(4,80)= 4.2$, $P=0.004$), with a reduction in NF staining in segments 2-5 in 2mm-CCI animals (all $P<0.04$, Supplementary Table 11). Peak plasma NfL was negatively correlated with neurofilament stain intensity ($\rho=-0.48$,

P=0.042) but not significantly correlated with zFA (Supplementary Fig. 3).

Fig. 8. Axonal injury markers including NfL and DTI MRI in a controlled cortical impact model of TBI. (A) NfL concentration in plasma at baseline, 3, 7, 10, 14 days post-injury in naive, sham and after to 1mm and 2mm CCI. (B) CSF NfL 15 days post-injury (mean ± standard error of the mean). (C) DTI-MRI images from naïve/sham animals, 1 and 2mm CCI (D) zFA after 1mm / 2mm CCI vs. controls. (E) Representative half brain photomicrographs of sections stained with Luxol Fast Blue from naive/sham animals (left), 1mm (middle) and 2mm CCI (right). Magnifications (red rectangles) show ipsilateral corpus callosum (segments 2/3, dotted outline) of the respective groups. Black dotted scale bars correspond to 1 mm (half brain sections), black solid lines to 200 µm (magnifications). (F) Quantification of Luxol Fast Blue staining showing thickness of segments of ipsilateral CC (% of the respective contralateral segment) measured every 500µm. (G) Neurofilament Alexa 568 immunofluorescence (grayscale) in naïve/sham (left), 1mm (middle) and 2mm CCI (right block). Magnifications (blue rectangles) show the ipsilateral corpus callosum (segments 2/3, dotted outline) with a HALO overlay (threshold-based classification of intensity; red>orange>yellow). White dotted scale bars correspond to 1mm (half brain sections) and white solid lines to 100µm (inserts). (H) Quantification of neurofilament staining. Alexa 568 immunofluorescence intensity in the segments of the ipsilateral CC (% of the respective contralateral segment).

DISCUSSION

We present the results of BIO-AX-TBI, a multi-centre prospective study of moderate-severe TBI. Our goal was to investigate neuroimaging and fluid biomarkers of axonal injury, a key type of brain injury that remains difficult to diagnose using standard clinical tools. We focus on the assessment of neurofilament light (NfL), a promising marker of axonal damage. Our results show that plasma NfL levels are significantly elevated in plasma after TBI. Variability in NfL plasma levels is not explained by CT findings, as high concentrations are seen in patients with no visible pathology on CT. However, NfL correlates with the extent of axonal damage seen on diffusion MRI and predicts subsequent white matter neurodegeneration and late functional outcomes. For the first time, we directly measure NfL from damaged white matter using invasive cerebral microdialysis. This shows that plasma NfL measurement reflects levels in damaged white matter within the brain. Microdialysate levels were around 100 times higher than plasma, with brain

extracellular fluid and plasma concentrations strongly correlated. NfL also correlated with histopathologically-defined axonal injury within the white matter produced by an experimental injury model. Tau, GFAP, UCH-L1 and S100B were also elevated after TBI in plasma and brain extracellular fluid post-injury, and plasma tau concentrations predicted cortical atrophy in the first six months after injury. Notably, levels of NfL peaked ten days to six weeks after TBI, in contrast to the acute peak seen for all other blood biomarkers assessed. The results show that plasma NfL is a sensitive and clinically-relevant measure of axonal injury, which provides important prognostic information when measured subacutely post-TBI.

Neurofilament light has previously been studied in a range of neurological conditions characterised by axonal degeneration (5). NfL constitutes a core part of the axonal backbone in conjunction with higher molecular weight neurofilaments (medium and heavy), and cytoplasmic proteins including α-internexin and peripherin (30, 31). High expression is seen within the long myelinated axons of the cerebral deep white matter (14), which are susceptible to TAI that arises from shearing forces of rotational acceleration in head injury (13). Plasma and cerebrospinal fluid NfL increase after various types of TBI and previous studies have indicated the potential of NfL for predicting outcomes after single moderate/severe injury (6, 8, 11, 12). NfL levels are known to increase even after mild TBI (32, 33). Concentrations have previously been shown to be highest early after injury but to remain elevated in some individuals in the long term. NfL is elevated in a range of proteinopathies (5). Hence, NfL levels potentially provide information about the extent of posttraumatic

neurodegeneration long after injury (6, 9, 34, 35).

Our work provides direct evidence that plasma NfL is an informative biomarker of TAI. Most importantly, we show that microdialysate taken directly from damaged white matter contains very high levels of NfL. We used an innovative approach to microdialysis, with commercially available 3% Dextran 500 perfusion fluid used for the first time, facilitating multi-centre microdialysis sampling. For the first time, ultrasensitive Simoa analysis of microdialysate and plasma were carried out on simultaneously acquired samples. Acute elevations of tau, UCH-L1 and GFAP were also seen in microdialysate Brain extracellular fluid concentrations of NfL, as well as tau and UCH-L1, were around 100 times higher than plasma. Blood and brain concentrations of NfL, tau and UCH-L1 were each highly correlated, indicating that plasma samples provide an accurate reflection post-traumatic changes in damaged white matter.

The time-course of increased NfL in the acute and subacute period after TBI was distinct from other biomarkers. Plasma NfL peaked subacutely between ten days and six weeks post-injury. This contrasted with tau, UCH-L1 and glial markers GFAP and S100B, which all peaked in the first 24 hours. This could be due to the longer half-life of NfL, which is estimated to be around 3 weeks from mouse modelling (36). Alternatively, NfL might also continue to be released from degenerating axons, a process that is known to progress for years after injury (37, 38). The delayed peak of NfL has important implications for optimally timing clinical measurement and measuring NfL 2-4 weeks after brain injury might be optimal for TAI diagnosis and prognostication.

Plasma NfL was closely related to diffusion MRI, which was used to quantify the extent of TAI. DTI provides a sensitive measure of axonal damage (13, 39), which can often be missed on CT or standard MRI (28). In keeping with previous work (3, 40), we observed widespread FA reductions after moderate/severe TBI. These abnormalities were seen in a large number of white matter tracts, including the corpus callosum where similar changes in FA were also seen in the experimental injury model. One previous study has shown that serum NfL at 30 days post-injury correlates with corpus callosum FA at this time (6). We extend this work by showing that NfL measured subacutely correlates with diffusion MRI measures of TAI. This relationship was stronger for NfL than tau, and a factor analysis provided further evidence that plasma NfL and DTI are closely related in quantifying underlying axonal injury. High levels of NfL were seen in patients irrespective of CT or MRI evidence of focal brain injury, showing that plasma NfL can be an informative biomarker of traumatic axonal injury in patients without abnormalities on CT imaging.

Traumatic axonal injury is a trigger for progressive neurodegeneration. We have previously shown that patterns of axonal injury measured by FA predict neurodegeneration in the chronic phase postinjury

(18). NfL is used widely as a biomarker in neurodegenerative disease (5) and levels correlate with atrophy rates in Alzheimer's (41), and other diseases (42-44). Hence, we hypothesised that peak NfL early after TBI would predict late axonal degeneration because it would quantify the extent of early neurodegenerative insult. Peak plasma NfL predicted late white matter neurodegeneration six to twelve months post-injury. Furthermore, diffusion abnormalities subacutely predicted spatial patterns of late white matter atrophy. Peak tau plasma levels also predicted grey matter atrophy in the first six months after injury. These observations suggest that plasma NfL and diffusion measures of TAI provide information about the extent of the whitematter

predominant post-traumatic neurodegeneration after TBI, and may be useful biomarkers for clinical trials aimed at limiting the late effects of TBI (45).

We went on to use animal modelling to confirm that isolated brain injury caused elevated plasma NfL in a dose-response manner. CCI modelling produced plasma and CSF NfL levels that increased with more severe injuries. These increases were accompanied by histopathological evidence of axonal injury and accompanying diffusion abnormalities within the corpus callosum seen in our human work (reduced FA). The animal work was conducted over two weeks postinjury, with imaging and CSF sampling at around 14 days. This timing paralleled our acute/subacute human studies, facilitating direct comparison. Two weeks after CCI there was evidence of axonal injury within the corpus callosum, with disorganised swollen axons, axonal bundles and axonal spheroid bulbs. We have previously reported related results in detail and shown how computational modelling of biomechanical strain produced at the time of injury predicts these histopathological changes and the accompanying diffusion abnormalities (13). In this study, NfL levels in CSF and plasma correlated with extent of axonal damage quantitatively, indicated by reduced white matter thickness and reduced neurofilament immunofluorescence staining in the callosum.

Increased plasma NfL levels were seen in trauma patients without TBI, although levels were 5 -15 times higher in the TBI group, with a larger difference developing with increasing time since injury over the first two weeks. This increase in non-TBI patients might indicate that TBI had occurred but was not apparent on our clinical assessment. However, we used stringent criteria to exclude even mild injuries. Vertebral fractures were present in some non-TBI patients, and though there was no evidence of spinal cord injury in any case, we cannot exclude this as a possible NfL source (46). While previous work showed that additional extracranial injuries alongside TBI did not change the incremental value of NfL in predicting CT changes in TBI, biomarker changes in non-TBI patients had not specifically been assessed (47).

There were several potential limitations of the study. One concern is the potential influence of focal post-traumatic lesions on the calculation of atrophy rates and diffusion MRI measures. To mitigate this, each scan was assessed for focal lesions, with custom ‘masks’ created to exclude these areas, with FA and JD measures only sampled from non-lesioned voxels. A further possible concern is inter-site variability, though we carefully aligned data collection and analysis, with scanner differences specifically considered. We imaged healthy age and sex-matched controls and

used these to generate a normalised FA map for each patient per-site voxelwise. Volumetric longitudinal healthy control data were acquired at a single centre, compared with patient data which was collected in three different sites. As the atrophy rate calculation uses an individual's scan pair, each patient effectively acts as their own control, so we do not expect scanner differences to influence atrophy rates. All blood and microdialysate samples were centrally analysed using the same test kits and reagent batches to further minimise cross-site variability and facilitate comparisons.

In summary, we report the prospective BIO-AX-TBI study of advanced fluid and imaging markers of axonal injury after moderate-severe TBI. Combining human data with experimental modelling, we show that NfL is released from white matter proportionately to injury severity, and that it can be accurately assessed in blood. Plasma NfL is highly correlated with FA on DTI MRI, an established neuroimaging biomarker of axonal injury, and when assessed subacutely, predicts chronic white matter atrophy and functional outcomes at six and twelve months post injury. Total tau predicted grey matter atrophy rates after injury, providing complementary information to NfL. We conclude that plasma NfL measured subacutely after TBI reflects axonal injury within the brain. These longitudinal dynamics lend themselves to clinical assessment of individuals with persistent symptoms after injury who consult clinicians in the outpatient setting, and where there may be uncertainty about whether any significant axonal damage was produced at the time of injury.

MATERIALS AND METHODS

Study design

BIO-AX-TBI is a multi-centre cohort study of patients after TBI (26). The study was designed to identify the most informative biomarkers of axonal injury in adult moderate-severe TBI patients. The sample size was based on the number (140 patients, 12 controls per study site) of participants needed to reliably test the contribution of DTI to prognostic modelling (type 1 error=0.05, power=0.95). Patient data from the BIO-AX-TBI study was analysed once the required number of patients with DTI and acute/subacute blood biomarkers had been followed-up at 6 months (Figure 1A, N=146 in the final analysis group) (26) (see Supplementary Methods).

Prospective cohort study overview

Patients with moderate-severe TBI, defined by the Mayo classification (48), were recruited from

eight trauma centres. Patients unable to provide consent underwent an assent process and were reconsented

if they were subsequently regained capacity. Inclusion criteria were age between 18 to 80 at time of injury and a diagnosis of moderate-severe TBI. Exclusion criteria were pre-existing neurological disease, previous TBI requiring hospitalisation, significant drug or alcohol abuse, pregnancy and for the MRI component, the presence of typical contraindications such as ferrous implants.

Clinical and demographic information were collected electronically using TBI common data elements. Blood biomarkers were assessed twice in the first ten days following injury. Patients were followed-up at a subacute timepoint ten days to six weeks post-injury. Follow-up visits included blood, MRI and clinical assessment (Figure 1B). Additional visits were performed at six and twelve months post-injury with a functional assessment including the GOSE (49). In a subset of sites (Figure 1B), six and twelve month assessments included blood and neuroimaging. Cerebral microdialysis was performed acutely at two sites, and in patients undergoing cerebral microdialysis, plasma biomarker assessment was performed up to twice per 24 hours acutely.

Healthy controls were recruited at each study site according to the same exclusion criteria. Controls underwent a single MRI and blood biomarker assessment. A separate group of longitudinallyscanned controls were included, comprising healthy volunteers who had participated in more than one study on the same scanner system with an aligned acquisition protocol (50).

Non-TBI patients were recruited at one site and underwent blood biomarker assessment on two occasions in the first ten days following injury. Inclusion criteria were the extracranial injuries requiring hospitalisation. As with the main patient group, exclusions included premorbid significant neurological disease in addition to any suspicion of TBI (i.e. symptomatic possible injuries), or a diagnosis of spinal cord injury, specific to this group.

Fluid Biomarker Analysis

Plasma samples were analysed for NfL, tau, GFAP and UCH-L1 using a Simoa-HD1 platform. Serum S100B was measured using a Millipore ELISA kit (Supplementary Materials). Samples were measured in duplicate by a technician using the same batch of reagents blinded to clinical information. Mean results were used for analyses. Peak biomarker concentrations within an individual were defined as the highest concentration over 6 weeks post-injury. Microdialysis samples were analysed in a similar manner on a Simoa-HD1 platform (see Supplementary

Materials).

Neuroimaging Acquisition

Participants in all sites had a high resolution structural T1 magnetisation prepared rapid gradient echo (MPRAGE), T2 fluid-attenuated inversion recovery (FLAIR), susceptibility-weighted imaging (SWI) and diffusion tensor imaging (DTI). Acquisition was harmonised at each site (see Supplementary Analysis). Structural MRIs were reported by neuroradiologists. Focal lesions were manually segmented, volumes calculated, and lesioned voxels individually excluded from analyses. A summary of the image analysis pipeline is provided (Supplementary Figure 4).

Neuroimaging Analysis - diffusion

Diffusion-weighted images were processed following a standard tract based spatial statistics (TBSS) approach and underwent quality control checks pre-and post-processing (Supplementary Methods). Images were normalised across centres to account for the inter-site scanner differences using z-scoring. In each site, raw FA maps in patients were normalised in a voxelwise manner to the local site-specific healthy control FA maps, who were age and sex matched to TBI patients across the group. Mean z-scored FA was calculated for the whole white matter skeleton. To account for any potential issues with scan quality diminishing at the boundaries, we also sampled zFA within the corpus callosum (28). Voxelwise analysis of zFA was performed using thresholdfree cluster enhancement (TFCE) multiple comparisons correction and a reported at a threshold significance level of $P<0.05$. Longitudinal and cross sectional analysis of diffusion metrics was conducted using the general linear model with non-parametric permutation testing (10,000) in FSL Randomise (51), with age and gender included as a nuisance covariates in cross sectional analyses, and individualised lesion masking. In patients with microdialysis, the catheter tip was identified on CT imaging. CT scans were registered to DTITK space and a 10mm radius spherical region of interest was generated. The spherical region of interest was overlaid on the white matter skeleton and FA sampled within the overlapping voxels.

Neuroimaging Analysis - Structural

Volumetric data were analysed using standard approaches to produce JD atrophy rate maps (see Supplementary Materials). Voxelwise analyses of longitudinal volumetric imaging data were conducted using non-parametric permutation testing (10,000) with age and sex as nuisance covariates, with individualised lesion masking. Given the strong prior hypothesis of the direction of change (atrophy post-TBI), we report a one-sided t test for this analysis. Voxelwise correlation

between JD and DTI metrics were conducted after registration of DTI images to MNI152 space, using non-parametric permutation testing (n=10,000).

Experimental injury model overview

Experiments were conducted under a Home Office license and were in keeping with the Animal [Scientific Procedures] Act 1986 and EU legislation. Thirty-four Male Sprague Dawley rats (~8-9 weeks old) were housed in standard conditions (13). MRI was performed in 18 animals prior to group assignment. Animals were then randomised to sham-operation (n=4), mild/1 mm CCI (n=15) and moderate/2 mm CCI (n=15). Surgery and CCI were performed as previously (22) (Supplementary Methods). Sham-operated animals were subjected to all drugs and surgical procedures except craniotomy and impact. Blood was taken at baseline (two to four days prior to surgery), 3, 7, 10 and 14 days post-injury (Supplementary Figure 5). Fourteen days post-impact, animals underwent a second MRI. The next day, the rats underwent terminal anaesthesia followed by transcardial perfusion and tissue harvesting. Ten additional naïve animals served as histology controls and were only subjected to anaesthesia prior to transcardial perfusion.

A one-way ANOVA was used to test differences in plasma NfL levels at baseline and CSF NfL concentrations. A mixed-effects model, with time and injury as fixed effects, were used to test differences in plasma NfL levels across different animal injuries and time since injury. CSF NfL concentrations was not significantly different between naïve animals and shams, and hence these groups were combined as controls. DTI changes were analysed using a mixed effects model with injury and segment as fixed effects. White matter integrity, as shown by LFB staining, and neurofilament expression pattern and density, as indicated by fluorescence intensity, was assessed using a two-way ANOVA with segment and impact severity as factors.

Statistical analysis

Standard t-tests were used for comparisons of normally distributed continuous data, and the nonparametric

Wilcoxon test for non-normally distributed continuous variables. Paired t-tests, repeated measures ANOVA designs or linear mixed effects models, were used for longitudinal within-subject data, as appropriate. Owing to the data distribution, linear regressions on fluid biomarker concentrations were performed on log10 transformed data. Bonferroni multiple comparison corrections were performed to correct for the number of timepoints and biomarkers/groups assessed, other than for NfL where a clear a-priori hypothesis existed. Hence.

NfL comparisons were corrected for the groups/timepoints only. In exploratory analysis of other biomarker levels and within the experimental injury model, a correction for the number of biomarkers was conducted. Factor analysis was performed using the R core function 'factanal'. To assess the relationships between biomarker concentrations in blood and cerebral microdialysate, a linear mixed effects model was used. Missing data was imputed independently for the model to facilitate analysis of plasma/brain at aligned timepoints (for further detail, see Supplementary Methods).

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