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Myelin degradation, axonal changes and expression trajectories of glial cells stimulated by rapid head insult in humans to estimate approximate time elapsed since trauma

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Abstract

Background Post severe traumatic brain injury (sTBI), axonal alterations lead to myelin loss and its degeneration. In the recovery phase, numerous intermingled biochemical pathways involving complex inflammatory reactions cloud the understanding of this yet undiscerned process that also varies with agonal period. In cases with dubious histories, approximating the survival time can be challenging, and expression levels of characteristic markers may aid forensic experts in the same.

Methods This exploratory study recruited 100 samples—68 sTBI, 22 non-TBI and 10 age- and sex-matched control samples. Male:female ratio was 87:13. Histochemical staining using H&E was used to characterize myelination pattern, and IHC of GFAP and CD-68 were performed to assess astroglial and microglial reactions with respect to survival time in specific sites.

Result Among sTBI, non-TBI and control recruits, sTBI patients depicted significant myelination abnormalities, astroglial proliferation and microglial reaction and varying with survival time. Non-TBI and control samples depicted nearly similar profiles.

Conclusion In order to untangle the complex mesh of biochemical responses, nuanced research on individual factors (both pre- and post mortem) with regard to specific site and survival time are warranted. Standardizing experimental data and converting it into empirical data shall aid forensic experts in suggesting approximate agonal period.

Keywords Forensic, Severe traumatic brain injury, Myelin degeneration, Axonal changes, Astroglia, Microglia

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Background

Traumatic brain injury (TBI) is an Armageddon for the medical community, be it critical care experts or forensic experts (Traumatic brain injury: epidemiology, classification, and pathophysiology - uptodate 2022). In forensic practice, it is imperative to obtain an estimation of the agonal period, even if it is deemed difficult due to scarce circumstantial and autopsy evidence (Ondruschka et al. 2019; Trautz et al. 2019). In this context, specific biomarkers may be used to distinguish severe traumatic brain injury from blunt trauma to other body parts and rule out possibilities of assault to head. An ideal biomarker is specific for a given brain compartment and reaches a threshold value with 100% sensitivity and specificity. However, due to peri- and post-mortem changes, TBI-related biomarkers are known to show variations. If there is an availability of sufficient empirical data on threshold values, several studies have suggested the possibility of using biomarkers for forensic purposes in the affirmative (Belsey and Flanagan 2016).

Rapid, high impact rotational/angular head trauma results in OL loss/death which leads to axonal breakdown and myelin degeneration. Myelin sheaths get damaged due to direct structural damage of the axon that is referred to as primary axotomy (Armstrong et al. 2016). Consecutively, myelin membrane may get degraded due to complex secondary reactions including calcium overload, mitochondrial dysfunction and oxidative stress resulting in release of glutamate and other excitotoxic enzymes (Armstrong et al. 2016; Benarroch 2009; Shi et al. 2015). During the remoulding phase (post brain injury), other neighbourring cells, including microglia, astrocytes and neurons, actively support oligodendrogenesis (Flygt et al. 2013; Caillava et al. 2011; Burda et al. 2016).

In the present study, we investigated the extent of myelin loss, progressive axonal changes in different sites and sTBI-induced changes in astrocytes and microglia in post-mortem brain tissues after sTBI that hold forensic as well as clinical significance. It thus provides a promising resource to establish the chain of biochemical reactions that may provide objective evidence for craniocerebral injuries as a cause of death with reference to survival time.

β-APP is a single transmembrane protein involved in various regulatory pathways, like cell adhesion, axonal transport and neurotrophic and neuroproliferative activity et al. Neurons synthesize β-APP in the perikaryon and transport through the axon by fast axonal transport (100 to 400 mm/day) (Turner et al. 2003; Kj et al. 1996). Any breakdown in the integrity of the axon leads to a time bound accumulation of this protein at the site of the break which can be detected by using antibodies to these proteins.

Neurofilament protein is intermediate filaments found in the cytoplasm of neurons, forming the neuronal cytoskeleton. These proteins are approximately 10 nm in diameter and many micrometres in length. They are believed to function primarily to provide structural support for axons and to regulate axon diameter, which influences nerve conduction velocity (Yaghmai and Povlishock 1992). Following TBI, neurofilaments go through proteolytic cleavage and collapse, and thus, NF proteins can be used as potential biomarkers for the identification of axonal injury (Okonkwo et al. 1998).

Robust and complex inflammatory reactions occur in the CNS post-TBI. This inflammatory response could be both detrimental and potentially beneficial (Woodcock and Morganti-Kossmann 2013; Bergold 2016; Hellewell et al. 2016; Corrigan et al. 2016). After TBI, macrophages/microglia cells migrate to the lesion site to mitigate the deleterious effects of inflammation process through (Progressive inflammation-mediated neurodegeneration after traumatic brain or spinal cord injury - PubMed 2022) phagocytosis of molecular and cellular debris post TBI (Donat et al. 2017).

In general network, there are two classes of microglia named M1 and M2 — the former produces interleukins and pro-inflammatory factors, and the latter are a source of anti-inflammatory factors and growth factors engaged in the adaptive immune response (Hu et al. 2015; Koshinaga et al. 2007). Cluster of differentiation-68 (CD-68) is a lysosomal protein highly expressed by circulating phagocyting macrophages and by tissue macrophages (microglia). Following axonal injury, cell membrane disruption, myelin debris and axonal degeneration stimulate microglia activation.

The key function of astrocytes is to re-establish normal brain microenvironment after brain trauma. Post sTBI, these cells migrate to the lesion site to form the glial scar, although this increase in astrocyte proliferation results in more severe form of injury, disrupting non-overlapping 'tiled' domains of astrocytes. Glial fibrillary acidic protein (GFAP) is the intermediate filament protein III and helps in providing support to resident neurons and maintaining mechanical strength.

Craniocerebral trauma is commonly encountered in forensic medical practice. Thus, to illustrate neuropathological changes with survival time in brain following trauma, utilization of biomarkers or proteins with specificity for structures of CNS shall be of great significance. In medicolegal cases presented with uncertain histories, use of immunopathological and histochemical markers to ascertain TBI as a cause of death could be crucial for legal purposes (Trautz et al. 2019; Finnie 2016).

Methods

This research work incorporated the autopsy cases conducted at designated post-mortem hall of the Forensic Medicine Department at Trauma Centre, AIIMS, New Delhi, India, between 2018 and 2020. Total of 456 decedents were selected; out of these, 100 cases: 68/22/10 from sTBI/non-TBI/control were included respectively after applying exclusion criteria. Survival time, also called agonal period, was defined as duration elapsed between time of incident and time of death. The precise survival time (ST) was known from the history of accident/trauma, the medical records or from the documents of police investigation.

Specimen collection and stratification

During autopsy, the brain tissue samples of gross precontusional area from grey-white matter interface, corpus callosum, thalamus and brain stem were collected only after taking consent from legally authorized representative (LAR) of deceased. The described work has been carried out in accordance with 'Declaration of Helsinki' (WMA- The World Medical Association-WMA Declaration of Helsinki – ethical principles for medical research involving human subjects 2022). Brain tissue samples were preserved into neutral-buffered 4% formaldehyde for fixation. Fixative was changed after every 24–72 h to ensure proper fixation of the samples. Samples were collected into three groups, which is sTBI/non-TBI and control as follows:

Study group A: Severe traumatic brain injury (sTBI) ($n=68$)

Cases with GCS score ≤ 8 (severe TBI cases) at the time of admission and age ranging between 18 and 60 years with positive CT findings (any abnormality in the brain like haemorrhage, contusions, abnormal basal cisterns, mid-line shift, mass effect, herniation and the presence of white cerebral signs) were included in the study. According to the survival time of the patients, the sTBI group A was divided into three major time phases, namely acute (T1: > 0–72 h), sub-acute (T2 > 72–240 h) and chronic (T3 > 240 h). T1 phase was further subcategorized into three time zones: T1a: > 0–6 h, T1b: > 6–24 h and T1c: > 24–72 h.

Study group B: non-traumatic brain injury (non-TBI) ($n=22$)

This group included those who died after a clinical course due to injuries other than TBI (such as blunt trauma abdomen, amputation, assault/gunshot in abdomen) with GCS between 13 and 15. NCCT Head reported 'no abnormalities detected' (NAD).

Control ($n=10$)

Age- and gender-matched individuals without any injury, who had died due to systemic causes and not suffering from any neurodegenerative disorders/CNS diseases, were recruited in this group. GCS score of all control samples was 3 (E1, V1, M1) as they were brought dead. NCCT Head reported 'no abnormalities detected' (NAD).

Exclusion criteria

Patients who had post-trauma craniectomy or any neurosurgical intervention, any neuropsychological illness/neurodegenerative disease, age > 60 years, penetrating injury to head and history of alcoholism were excluded. Moreover, cases where autopsy was performed more than 24 h after death (due to progressive autolytic changes) were excluded.

Routine Hemotaxylin and Eosin (H&E) Staining

After ensuring proper fixation of the tissue, gross examination was done. Transverse sections measuring 1–2 cm from region of interest (ROI) from every site in each case were taken. After tissue processing, 5 µm (using Microm HM 355 S)-thick paraffin-embedded serial sections from brain tissues were obtained on superfrost slides (Thermo Scientific). The slides were stained with haematoxylin and eosin (H&E) and graded semiquantitatively to assess the severity of white matter damage according to Brun and Englund (1986) (grading enumerated in Table 1). The analysis was standardized to equal area of tissue for each section.

Table 1 Grading for Severity of White Matter Damage (H&E Staining)

Grade	Severity	White matter damage
Grade 1	Normal	• Normal white matter
Grade 2	Mild	• No appreciable reduction in axonal meshwork density • Easily recognized long axons • Occasional axonal debris • A slightly increased number of reactive astrocytes
Grade 3	Moderate	• A slight reduction of axonal meshwork density • Reduction of oligodendroglial cell nuclei • Increased no. of reactive astrocytes
Grade 4	Severe	• A marked reduction of myelin, axons and oligodendroglial cell nuclei • Marked astrocytic reaction • Loosely scattered macrophages but no complete cerebral infarct

When several grades were observed in one section, the dominant grade represented the section. *Brun and England (1986)

Immunohistochemistry (IHC)

Sections from different sites were immunostained using fully automated autostainer Ventana benchmark XT (Roche Tissue Diagnostics) using XT ultraview DAB V3 Detection Kit (as per manufacturer's recommendations). Paraffin sections of 5 µm thickness in each case were obtained on poly-L-lysine-coated slides. Sections from ROI were immunostained for β-APP (β-amyloid precursor protein)/NFP (neurofilament protein) to investigate the axotomy and other neuronal defects post-traumatic head injury with time, GFAP (glial fibrillary acidic protein) and CD-68 (cluster of differentiation-68) to examine astrogliosis and microglial changes with survival time (details mentioned in Table 2) For positive controls, histological sections of normal brains were used, and for negative controls, phosphate buffer solution was used instead of primary antibody. Consensus of two (or more) independent observers unbeknownst to demographic and clinical information was considered for all histological and immunohistochemical examination.

Immunohistochemical Examination

The sections were examined using high magnification 400 × to assess the distribution and pattern of β-APP/NFP immunoreactivity at the same time. The amount and pattern of APP staining were recorded and scored for each section (Jensen et al. 2014). For analysis of GFAP, positive astrocytes were evaluated in 25 optical fields by two (or more) independent observers blind to the demographic and clinical information in sTBI and non-TBI and for control cases using 400 × magnification (Nikon 80i, five headed teaching microscope). Median (25–75%) percentiles were calculated for each case. For analysis, CD-68-positive cells were evaluated in region of interest (ROI) by two independent observers blind to the demographic and clinical information in sTBI and non-TBI and for control cases using 400 × magnification (Nikon 80i).

Statistical analysis

Categorical variables were summarized by frequency (%), and χ^2/Fisher exact test as appropriate was used to compare frequencies between sTBI, non-TBI and controls. Quantitative variables were assessed for approximate normality. Variables following normal distribution were summarized by mean ± SD, and Student's *t*-test was used to compare mean. One-way ANOVA/Kruskal–Wallis H test and Bonferroni/Dunn test were used for multiple comparisons. Variables following nonnormal distribution were summarized by median and range/interquartile range. Wilcoxon's rank-sum test was used to compare distribution of nonnormal variables. STATA 14.0 statistical software was used for data analysis. p-value ≤ 0.05 was considered statistically significant (Table 2).

Results

This exploratory study was conducted to evaluate myelin and axonal alterations as well as secondary changes in astroglial and microglial cells following severe traumatic brain injury (sTBI).

Demographic details

Demographic details of the cohort including age, sex, GCS, causative instrument of head trauma and circumstances of head trauma have been enumerated in Table 3. Younger population (21–30 years) were more vulnerable to traumatic events in both sTBI and non-TBI groups (31.3% and 41.7%, respectively). Males were more susceptible in all groups of recruits. All sTBI patients were severely comatose with GCS ≤ 8 in sTBI group; all non-TBI recruits had ante-mortem GCS score 9–15, whereas all control recruits had GCS score 3 as these were brought dead cases that had died due to hanging, myocardial infarction, poisoning, etc.

In the sTBI group, motor vehicular accidents (MVA) accounted for the majority of deaths (75%), followed by fall from height (20.5%), whereas only 3% cases exhibited human-animal encounters. Evaluation of circumstance of head trauma revealed that majority of MVA involved rear impact crash (27.5%) and side impact crash (23.5%) with only 11.7% front impact crash and skids (Table 3). CT head examination depicted cerebral contusion (50.8%), subdural haematoma (SHD) (51.1%), intraventricular haemorrhage (10.3%) and subarachnoid haemorrhage (SAH) (34.7%). A total of 16.3% of the patients with severe CNS injury exhibited midline shift (MLS) of ≥ 5 mm.

Table 2 Details of immunohistochemical markers used

S. no	Antibody	Dilution	Host species	Target	Product no./supplier
1	β-APP	1:100	Polyclonal rabbit	Axons	Abcam AB15272
2	NFP	1:100	Monoclonal mouse	Axons	Thermo Fisher Scientific BSD5818
3	GFAP	1:1000	Polyclonal rabbit	Astrocytes	Dako (Z0334)
4	CD-68	1:100	Monoclonal mouse	Microglial cells/macrophages	Dako(M0814)

β-APP beta-amyloid precursor protein, *NFP* neurofilament protein, *GFAP* glial fibrillary acidic protein, *CD-68* cluster of differentiation-68

Table 3 Demographic details of recruited cases among sTBI, non-TBI and control groups

Variables	sTBI ($n=68$)	Non-TBI ($n=22$)	Control ($n=10$)	*p*-value
Age category [*n* (%)]				0.06
< 21 years	0 (0.00)	1 (4.2)	1 (10)	
21–30 years	21 (31.3)	10 (41.7)	1 (10)	
31–40 years	14 (20.3)	5 (22.7)	6 (60)	
41–50 years	16 (23.4)	4 (16.7)	1 (10)	
51–60 years	17 (25.1)	2 (9.1)	1 (10)	
Sex (male:female)	55:13	10:1	9:1	
Initial GCS: 3–8	68 (100)	–	10(100)	< 0.001
9–15	0 (0.0)	22 (100)	–	
Survival time (hours) [median (min.-max.)]	40.5 (0–1121.5)	1.34 (0–550.1)	0 (0.0–0.0)	< 0.001
Causative instrument of head trauma [*n* (%)]				< 0.001
Motor vehicular accident	51 (75.0)	12 (54.5)	–	
Fall	14 (20.5)	3 (13.6)	–	
Assault/homicide	1 (1.5)	6 (27.4)	–	
Suicide	0 (0.0)	0 (0.0)	10 (100)	
Human-animal encounter	2 (3.0)	1 (4.5)	–	
Circumstances of head trauma [*n* (%)]				
Front impact crash	6 (11.7)	1 (8.3)	0	0.67
Rear impact crash	14 (27.5)	4 (33.4)	0	
Side impact crash	12 (23.5)	5 (41.7)	0	
Skid	8 (15.7)	1 (8.3)	0	
Hit fixed object	4 (7.9)	0 (0.0)	0	
Unknown	7 (13.7)	1 (8.3)	0	

sTBI severe traumatic brain injury, *non-TBI* nontraumatic brain injury, *GCS* Glasgow Coma Score, *PM* post mortem, *n* number, *%* percentage, *SD* standard deviation, *min.* minimum, *max.* maximum; *p*-value < 0.05

Myelination abnormalities

CORPUS CALLOSUM (CC): On analysis of H&E-stained sections, a statistically significant difference was observed in severity of myelin degeneration between sTBI group compared to non-TBI and control cases (*p*-value: < 0.001). Majority of patients (73.5%) in sTBI group showed myelin degradation changes varying from mild to severe. However, a small fraction of cases (8.3%) in non-TBI group showed mild demyelination (grade 2) which could be attributed to oblique fall after bullet shot, and none of the control cases exhibited any myelin degeneration changes (Table 4; Fig. 1).

Grade 3 and grade 4 (in other words, moderate to marked myelin degeneration) changes were noted in 37.8% cases of acute phase (T1). There was significant increase in severity of myelin degeneration with increasing time period compared to acute phase (37.8%): 80% of cases in subacute phase (T2) and 66.7% of cases in chronic phase (T3) showed grade 3 and grade 4 myelin loss (*p*-value: 0.04) (Table 5; Fig. 1). Within different time zones in the acute phase, there was an increase in myelin degeneration with 33.3%, 45.5% and 92.8% of cases in T1a, T1b and T1c time zones, respectively (*p*-value: 0.03) (Table 5; Fig. 1).

Grey White Matter Interface (GWMI): Myelin degeneration was markedly higher in sTBI patients as compared to non-TBI and control groups (*p*-value: < 0.02). In comparison with the corpus callosum, myelin deterioration was less marked in grey-white matter interface as only 38.4% cases showed mild to moderate myelin degeneration changes. None of the cases in non-TBI and control group showed evidence of myelin degeneration (Table 4; Fig. 2). *Myelin degeneration is thus observed better in corpus callosum which is predominantly white matter.*

A substantial proportion of cases in subacute and chronic phase showed varied degree (mild to moderate to severe) of myelin degeneration changes — 58.3% and 70% cases respectively, with only 20% cases in acute phase (*p*-value: < 0.006) (Table 5; Fig. 2). With successive hours within acute phase, myelin degradation rose significantly — 0% in first 6 h, 10% cases in the first 24 h and 50% cases in > 24–72 h (Table 5; Fig. 2) (*p*-value < 0.03). This refers to the following: *rapid myelin degradation follows the initial hours after injury*.

Table 4 Sitewise comparison of various histo/immunohistochemical and molecular markers among sTBI, non-TBI and control groups

Feature	Site	Grade	sTBI	non-TBI	Control	*p*-value
Myelin degradation via H&E staining	CC	Grade 1 (normal)	26.5%	91.7%	100%	< 0.001
		Grades 2, 3, 4 (mild, moderate, severe)	73.5%	8.3%	-	
	GWMI	Grade 1 (normal)	61.5%	100%	100%	0.02
		Grades 2, 3, 4 (mild, moderate, severe)	38.4%	-	-	
IHC β-APP and NFP [median (min–max]	CC	-	8 (1–10)	1 (1–2)	0 (0–2)	< 0.001
	Thalamus		4 (0–10)	1 (0–3)	0 (0–2)	
	MB		4 (0–10)	1 (0–3)	0 (0–4)	
	Pons		6 (0–10)	1 (0–2)	0 (0–3)	
	MO		4 (0–10)	1 (0–4)	0.5 (0–4)	
	GWMI		3 (0–9)	1 (0–3)	0 (0–2)	
Astrogliosis IHC-GFAP [median (min–max]	CC	-	131 (13–461)	87.5 (3–215)	0 (0–185)	< 0.001
	GWMI	-	191 (41–563)	108.5 (7–271)	96.5 (30–175)	
Microglial reaction	-	-	67.2%	4.2%	0.0	< 0.001

sTBI severe traumatic brain injury, *non-TBI* nontraumatic brain injury, *GWMI* grey-white matter interface, *CC* corpus callosum, *MB* midbrain, *MO* medulla oblongata, *β-APP* beta-amyloid precursor protein, *NFP* neurofilament protein, *GFAP* glial fibrillary acidic protein, *CD-68* cluster of differentiation-68, *H&E* haematoxylin and eosin, *%* percentage, *SD* standard deviation; *p*-value < 0.05

Axonal changes: IHC β-APP and NFP

Higher extent of axonal damage was seen in corpus callosum (median score: 8), followed by pons (median score: 6). The changes were relatively less evident in other regions (median score: 3–4). However, the extent of damage was significantly higher in all sites in sTBI cases as compared to non-TBI and control cases (*p*-value < 0.001) (Table 4; Fig. 3).

Maximum axonal changes were seen in corpus callosum region, and there was significant difference in extent of axonal damage in acute vs. subacute phase and acute vs. chronic phase (*p*-value: < 0.001). The extent of damage was relatively less in pons and medulla oblongata, whereas minimal axonal alterations were seen in other sites (Table 5; Fig. 3).

Overall severity of damage was appreciably better on immunostaining of β-APP and NFP as compared to H&E examination alone. Even in patients who died within 0–6 h of head injury, axonal changes were seen in the region of corpus callosum and medulla oblongata with median score 6 for each. However, minimal changes were seen for other sites evaluated in first 6 h (median range: 1.5–3). Corpus callosum showed higher extent of axonal damage in T1c (24–72 h), and Pons was the second most affected area with apparent and significant changes in T1b (> 6–24 h) post injury (*p*-value: < 0.05) (Table 5; Fig. 3).

Astrogliosis

Extensive gliosis was noted in cases with sTBI [median (min.-max.) — 131 (13–461)] which is more pronounced compared to non-TBI [87.5 (3–215)] and control [0 (0–185)] groups (*p*-value: < 0.001). Interestingly, extent of gliosis also revealed a significant difference in non-TBI cases when compared with control cases (*p*-value < 0.01) (Table 4; Fig. 4).

Extensive astrogliosis was observed in all three time phases (T1, T2, T3). The astrocytic-positive cell count in acute, sub-acute and chronic phase was [median (min.-max.): 127 (18–461), 137 (23–362) and 183 (13–449) respectively. Difference in extent of gliosis between the three phases was statistically nonsignificant (*p*-value: 0.4) (Table 4; Fig. 4). The median cell count in T1a, T1b and T1c time zones within acute phase was 162 (24–278), 91 (24–461) and 102 (18–322), respectively (*p*-value: 0.3) (Table 5; Fig. 4).

The observations in grey-white matter interface were similar to corpus callosum. Reactive gliosis was observed in all three groups with the median (min.-max.) — 191 (41–563), 108.5 (7–271) and 96.5 (30–175) in sTBI, non-TBI and control groups, respectively (*p*-value: < 0.001). *Interestingly, control cases revealed astrogliosis which may be attributed to some curious event during lifetime of the diseased individual* (Table 4; Fig. 4).

The extent of reactive gliosis in acute, subacute and chronic phases respectively was [median (min–max): 179.5 (41–401), 191 (93–563) and 325.5 (47–482)] with *p*-value: 0.3 (Table 5; Fig. 4). When different time zones were assessed, higher number of reactive glial cells was observed in patients who died in T1c time zone [median (min–max): 214.5(41–401)] as compared to T1b [198 (146–394)] and T1a [160 (48–246)] time zone; however, the difference in positive astrocytic cells was statistically non-significant (*p*-value: 0.2) *(*Table 5*;*

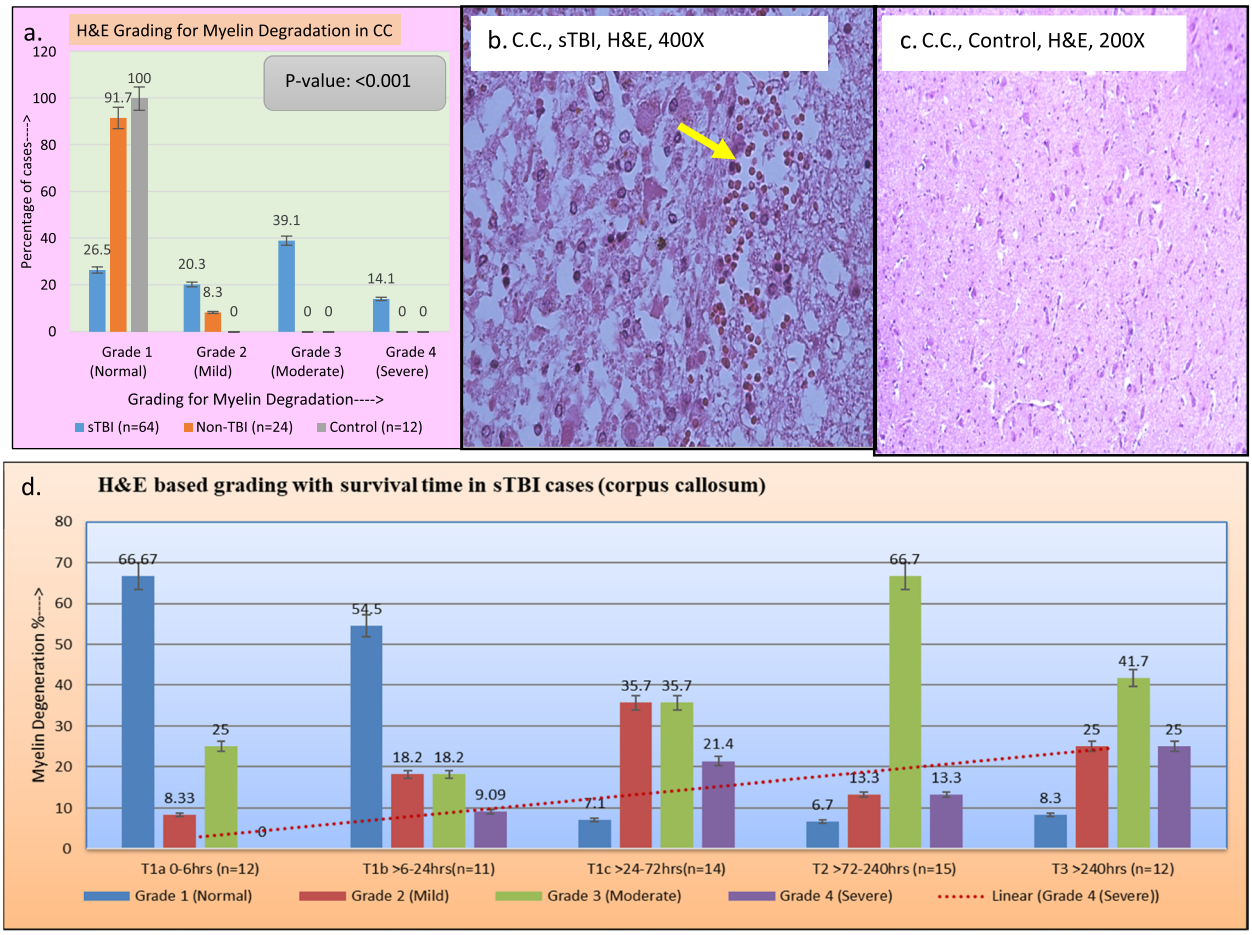

Fig. 1 H&E staining of corpus callosum (CC). **a** Percentage of cases depicting various grades for myelin degeneration in corpus callosum among sTBI and non-TBI control groups. **b** Section from corpus callosum in sTBI case, arrow showing collection of microglial cells with reduction in myelin, axons and oligodendroglial cell nuclei (grade 4 myelin degeneration) survival time: 8 days (H&E staining 400 ×). **c** Section from corpus callosum in control case, showing normal brain pathology. **d** Correlation of H&E-based grading for myelin degeneration with survival time among sTBI cases in corpus callosum

Fig. 4*). Thus. reactive gliosis is appreciable in the corpus callosum and grey-white matter interface of patients in the first 6 h post injury.*

Microglia reaction

Microglial proliferation, assessed using IHC-CD-68, revealed that majority of cases in sTBI group [43 cases (67.2%)] showed macrophage proliferation which was more prominent in grey-white junction. Only 1 (4.2%) case in the non-TBI group exhibited this feature, while none of the cases in control group showed microglial activation. The difference in microglial reaction among sTBI, non-TBI and control group was statistically significant (p-value: < 0.001) (Table 4; Fig. 5).

There was an appreciable increase in CD-68 positivity with survival time albeit non-significant. Majority of cases in the chronic phase 'T3' (> 10 days) showed microglial reaction with 83.3% CD-68 positivity as against 56.7% in the acute phase 'T1' (0–3 days). Among the different time zones of acute phase, microglial/macrophage activation increases from T1a to T1c (41.6 to 71.4%) (Table 5; Fig. 5).

Discussion

Biomarkers are known to show significant differences following TBI compared to atraumatic controls (Goyal et al. 2013; Kumar et al. 2015; Zwirner et al. 2022). Severe traumatic insult to the brain results in axonal damage and glial injuries followed by neuroinflammation involving astrogliosis and microglial reaction/proliferation (Lier et al. 2020; Eng 1985). Microscopic H&E examination revealed changes in the nervous tissue in the form of haemorrhage, hypoxic changes and retraction of neuronal cell body at around 0–6 h. Blood-derived monocytes and reactive microglia were visible from 12 to 24 h. Small number of hemosiderin-containing macrophages

Table 5 Comparison of various histo/immunohistochemical and molecular markers with survival time in sTBI

Feature	Site	Grade	0–6 h	> 6–24 h	> 24–72 h	> 72–240 h	> 240 h	*p*-value
Myelin degradation via H&E staining [*n* (%)]	CC	1 (normal)	66.7%	54.5%	7.1%	6.7%	8.3%	0.03
		2/3/4 (mild, moderate, severe)	33.3%	45.5%	92.8%	93.3%	91.7%	
	GWMI	1 (normal)	100%	90%	50%	41.7%	30%	0.003
		2/3/4 (mild, moderate, severe)		10%	50%	58.3%	70%	
IHC β-APP and NFP [median (min–max]	CC	-	6 (1–8)	7 (1–10)	8 (1–10)	9 (4–10)	7 (1–10)	< 0.001
	Thalamus		1.5 (0–9)	4 (1–8)	3 (1–10)	5 (1–10)	5 (5–10)	0.1
	MB		2.5 (0–7)	3 (0–7)	5 (1–9)	4 (0–10)	4 (2–8)	0.2
	Pons		2.5 (0–8)	6 (2–9)	6.5 (1–10)	7 (2–10)	7 (1–9)	< 0.001
	MO		6 (0–9)	4 (2–8)	4 (1–10)	3 (1–9)	3 (2–9)	0.2
	GWMI		3 (0–5)	3 (1–8)	4 (1–9)	5 (1–8)	3 (2–7)	0.3
Astrogliosis IHC-GFAP [median (min–max]	CC	-	162 (24–278)	91 (24–461)	10 2 (18–322)	137 (23–362)	183 (13–449)	0.3
	GWMI	- -	160 (48–246)	198 (146–394)	214.5 (41–401)	191 (93–563)	325.5 (47–482)	0.2
Microglial reaction [*n* (%)]	-	-	41.6	54.5	71.4	80	83.3	0.3

sTBI severe traumatic brain injury, *non-TBI* nontraumatic brain injury, *GWMI* grey-white matter interface, *CC* corpus callosum, *MB* midbrain, *MO* medulla oblongata, *β-APP* beta-amyloid precursor protein, *NFP* neurofilament protein, *GFAP* glial fibrillary acidic protein, *CD-68* cluster of differentiation-68, *H&E* haematoxylin and eosin, *%* percentage, *SD* standard deviation; *p*-value < 0.05

were noted in cases with long survival time. After 24 h, astrocytic reaction with large cytoplasm, referred to as gemistocytic astrocytes, were identified which eventually led to formation of glial scar.

Histopathological analysis revealed evidence of axonal injury in the form of axonal swelling/myelin breakdown and transection changes similar to a study by Bisht et al. (2013). With increasing survival time post trauma, these features become more evident; as compared to the subacute phase, the chronic phase exhibited significant histopathological changes, whereas least changes were revealed in the first 3 days after TBI (acute phase). Repair processes involved neovascularization along with hyalinized blood vessels, oedema, etc. in concordance with other studies (Zwirner et al. 2022; Gusmão and Pittella 2002; Romero et al. 2018; Adams et al. 1982; Finnie 2016).

Microscopic features such as loss of oligodendroglial cell nuclei, reduction of axonal meshwork density, increased number of reactive astrocytes and the presence of loosely scattered macrophages indicate more severe forms of myelin degeneration observed in sTBI cases.

A total of 33.3% cases at T1a (0–6 h) zone exhibited features similar to observations by Maxwell William L. et al. where myelin dislocations occur within internodal myelin of larger axons within 1–2 h of TBI. In the acute phase (0–3 days), only 56.7% cases showed myelination degenerative changes. Adjacent to the impact site, long axonal networks that traverse corpus callosum and grey-white matter interface are stimulated by forces of strain, tension, torsion and compression and subsequently lead to the degradation of axolemma and cytoskeletal breakdown as the cell injury triggers biomechanical pathways (Montanino and Kleiven 2018).

Assessing the level of myelin degradation may also enable in prediction of survival time, given the sequential and significant difference in the same. Corpus callosum revealed significantly higher myelin abnormalities with varying degrees of myelin degradation in subacute (100%) and chronic (91.7%) phases following sTBI. These may be explained with reference to secondary changes resulting in further demyelination of viable axons. Maxwell William L. et al. also demonstrated that waves of Ca^{2+} depolarization extend from the initial locus injury for perhaps hundreds of microns after TBI (Maxwell 2013).

Significantly higher extent of axonal damage was revealed by β-APP and NFP (IHC) scoring (Table 4; Fig. 3) in patients who died due to sTBI as shown by other studies (Blumbergs et al. 1995). Corpus callosum happens to be the most vulnerable part of the brain as it is located in the midline and thus highly susceptible to secondary injury due to the elevated intracranial pressure (Ljungqvist et al. 2017; Rutgers et al. 2008; Gallyas et al. 2006). Moreover, being the largest commissural white matter bundle in the brain with high myelin content (Fitsiori et al. 2011), it was found to be more sensitive and vulnerable to severe demyelination compared to grey-white matter interface. The shearing stress that develops in and around the brain stem and tentorial notch makes pons the second most affected region in sTBI (higher β-APP and NFP score), thus

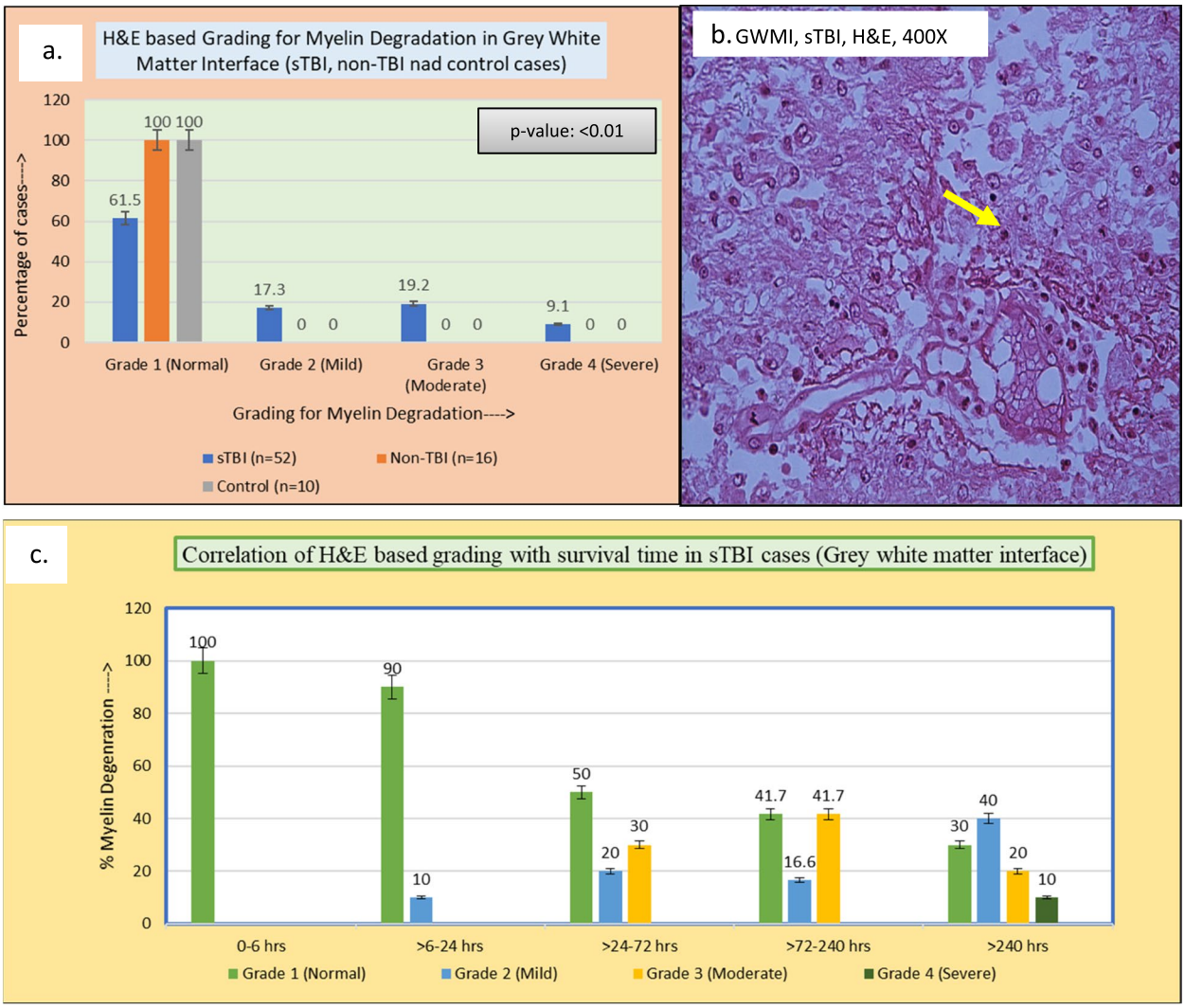

Fig. 2 H&E staining of grey-white matter interface (GWMI). **a** Percentage of cases depicting various grades for myelin degeneration in grey-white matter interface among sTBI (blue), non-TBI (orange) and control (grey) groups. **b** Section from grey-white matter interface, arrow showing microglial cell collection (grade 4 myelin degeneration) with survival time > 10 days. **c** Correlation of H&E-based grading for myelin degeneration with survival time among sTBI cases in grey-white matter interface

being a reliable indicator of axonal injury. Neiss et al. and (Niess et al. 2002) Oehmichen et al. both reported simultaneous proof of DAI in the pons and corpus callosum (Oehmichen et al. 1998).

Axonal breakdown changes were significantly well appreciated in the first 6 h with higher β-APP and NFP scores in the regions of corpus callosum and brainstem as reported by Bisht et al. Another study by Johnson et al. indicates that the extent of axonal pathology diminished over time from the acute-injury phase (> 2 weeks) (Johnson et al. 2013a), as was observed in the present study, which may be due to increased concentrations of APP-degrading enzymes such as caspase-3 within the injured axons. Thus, in head injury MLCs with incongruous history, prediction of approximate survival time might be possible and enable in distinguishing acute survivors versus subacute and chronic survivors using difference in β-APP/NFP expression. These investigations shall also help in ruling out cases of high impact CNS injury when features of blunt trauma to abdomen are present too.

Various animal as well as human studies revealed higher levels of GFAP after traumatic CNS insult (Flygt et al. 2013; Sullivan et al. 2013; Namjoshi et al. 2017; Neri et al. 2018). In the present study, we recorded significant GFAP immunoreactivity detected in terms of

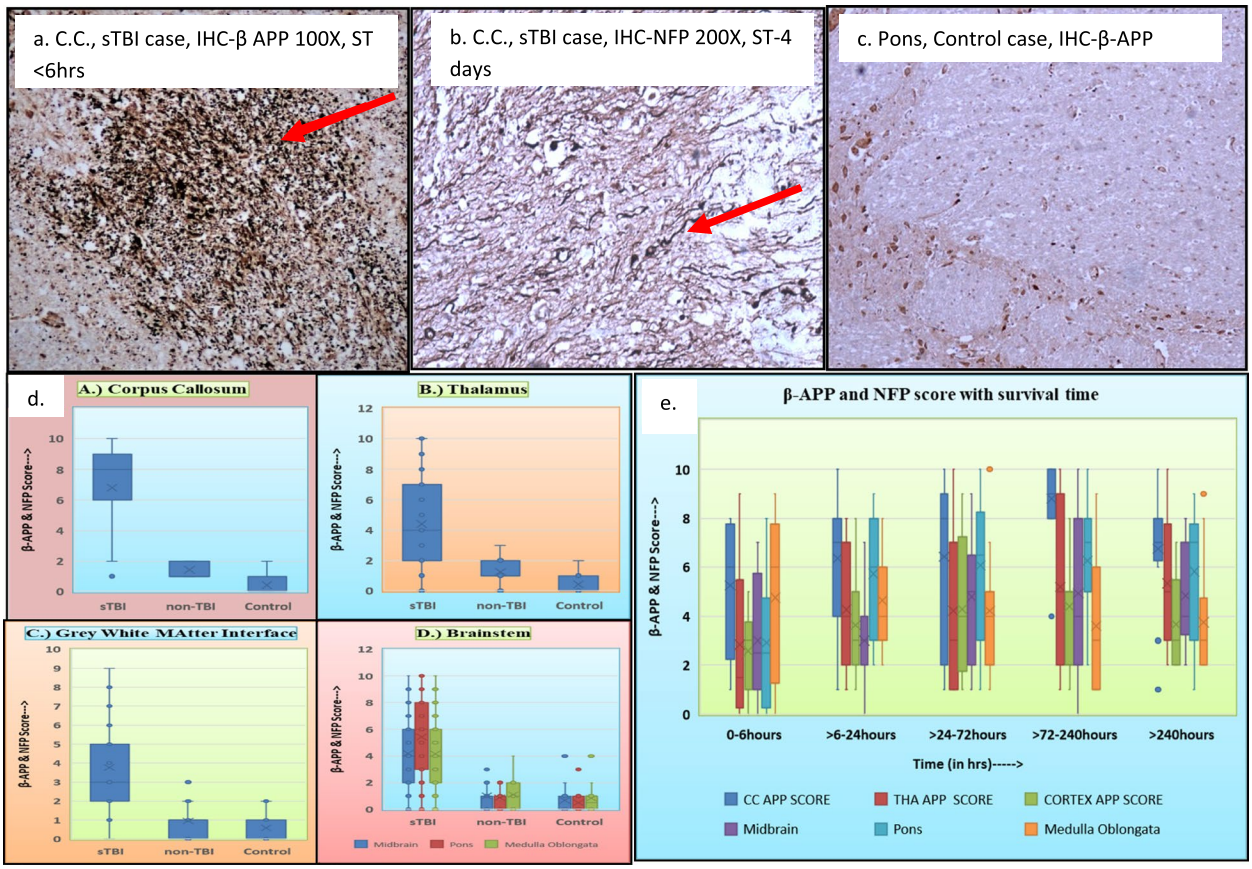

Fig. 3 IHC β-APP/NFP. **a** Section from corpus callosum (CC) in sTBI case (survival time < 6 h) arrow showing immunoreactive axons (IHC-β APP, 100 ×). **b** Section from corpus callosum in sTBI case (survival time — 4 days) arrow showing axonal varicosities (immunoreactive axons) (IHC-NFP 200 ×). **c** Section from pons in control case showing normal brain pathology (IHC-β APP 100 ×). **d** IHC-β APP/NFP score in different sites (clockwise: corpus callosum, thalamus grey-white matter interface (GWMI), brain stem (midbrain pons, medulla oblongata in series), and among different cohort groups, viz. sTBI, non-TBI and control. **e** Variation of IHC-β APP/NFP score with survival time in different sites (in series: corpus callosum, thalamus, grey-white matter interface, midbrain, pons, medulla

increase in the number of GFAP-positive astrocytes both in the corpus callosum and grey-white matter, with higher concentration in the latter.

Following TBI, astrocytes become reactive and rapidly produce GFAP, possibly to maintain the integrity of the CNS cells (Li et al. 2009; Mellergård et al. 2011). GFAP immunoreactivity has been detected at 3 h post injury in areas surrounding the lesion site (Dressler et al. 2007), in concordance with the present study where GFAP-positive cells were well appreciated as early as 6 h after injury in both corpus callosum and grey-white matter junction. In the present study, we observed a gradual increase in astrocytic changes with survival time, and maximum changes were detected in the chronic phase (> 10 days) (Neri et al. 2018; Trautz et al. 2019). Reactive astrocytes initiate an edema while healing the surrounding damaged tissue from secondary lesions. Glial swellings that are one of the key mediators in diffuse brain edema get induced after TBI (Kimelberg 1995; Astrocytic edema in CNS trauma - PubMed 2022). It can be hypothesized that this could be one of the reasons for the increasing numbers in GFAP-positive astrocytes with increasing survival time. Interestingly, control cases revealed astrogliosis that may be attributed to some unrelated event during lifetime of the deceased individual.

Myelin damage leads to an accumulation of myelin debris that can stimulate microglial activation (Clarner et al. 2012). Majority of cases in sTBI group (67.2%) showed macrophage proliferation which was more prominent in grey-white junction. Consistent with astrocytic activity, microglial reaction with CD-68 positivity was observed to be highest in the chronic phase depicting secondary pathological changes in long-term survivors.

Multifaceted models of white matter injury showed that loss of reactive astrocytes leads to inflammation and neuronal loss, although astrocytes are also known to inhibit remyelination (Brambilla et al. 2009; Voskuhl

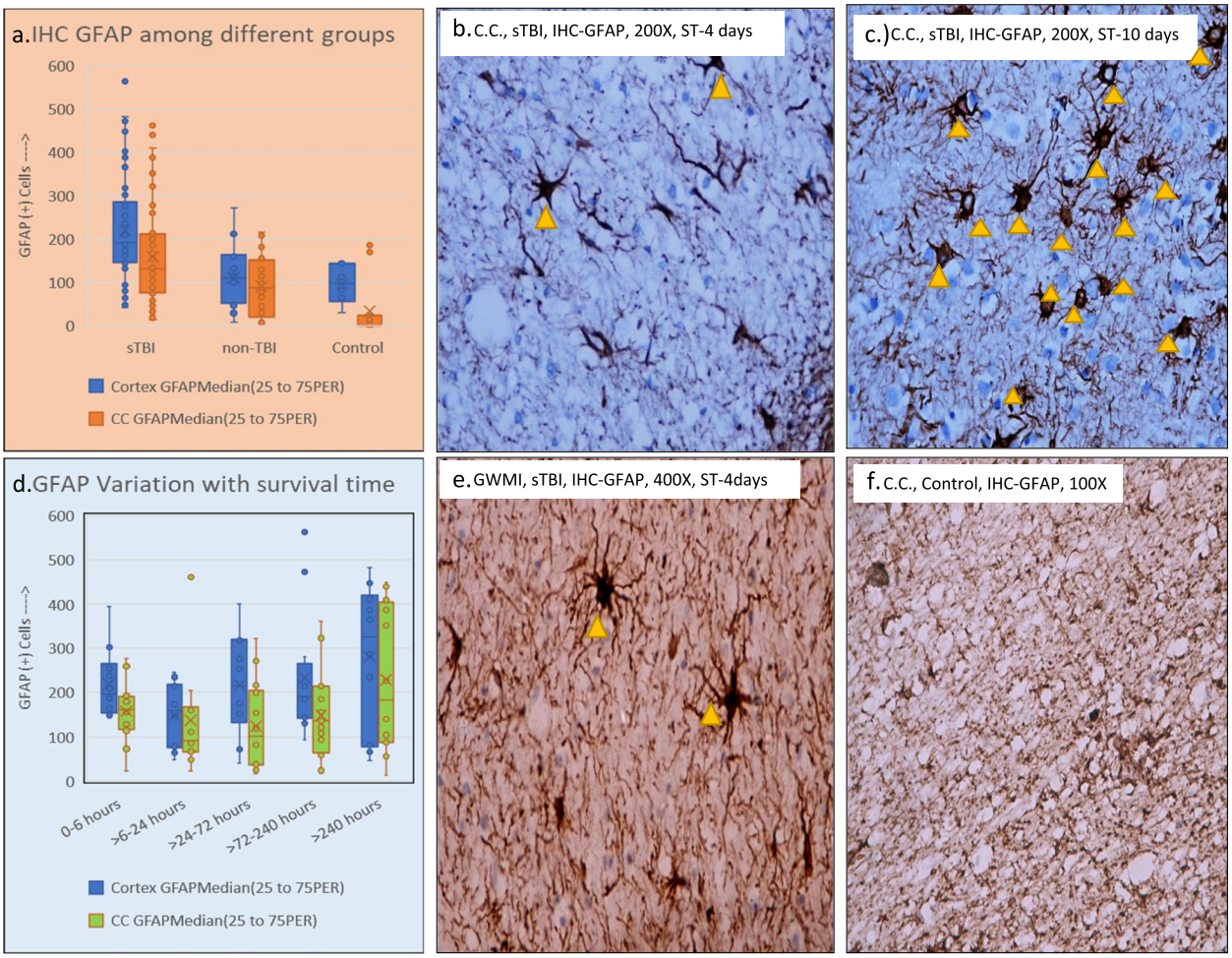

Fig. 4 HC GFAP. **a** Percentage of GFAP-positive cells in cortex (GWMI, grey-white matter interface: blue) and corpus callosum (orange), among different groups of cohort (IHC-GFAP). **b** Section from corpus callosum in sTBI case yellow triangles showing reactive astrocytes (IHC-GFAP 200 ×). Survival time — 4 days. **c** Section from corpus callosum in sTBI case yellow triangles showing reactive (IHC-GFAP 200 ×). Survival time — 10 days. **d** Variation of IHC-GFAP score with survival time in different sites (grey-white matter interface, blue and corpus callosum, green) among sTBI patients. **e** Section from corpus callosum in sTBI case yellow triangles showing single astrocyte with larger cell size, darker cytoplasmic staining with long processes (IHC-GFAP 400 ×). Survival time — 4 days. **f** Section from corpus callosum in control case, showing normal brain pathology (IHC-GFAP 100 ×)

et al. 2009). One of the key areas for further research would be to comprehend the nonhomogenous nature of astrocytes and their response curves (Glial development: the crossroads of regeneration and repair in the CNS - PMC 2022).

Through rigorous evaluation of the various markers, it was observed that β-APP serves as the best marker of severe TBI and resulting axonal damage showcasing marked differences in the regions of corpus callosum and pons, thus reiterating β-APP to be the gold standard in this avenue (Johnson et al. 2013b). However, TBI biomarkers are affected by various factors such as age, sex, time elapsed since trauma, pre-mortem pathology, autolysis and storage conditions. As such, each biomarker reveals unique details, and thus, it is suggested that using various biomarkers at different sites simultaneously provides a more robust and holistic idea for estimation of survival time. In this context, it is important to arrive at empirical conclusions utilizing diverse biomarkers as per feasibility.

Limitation

Reactive gliosis can also be a feature of infection or hypoxic insult to the brain parenchyma, and thus, GFAP is not a very specific biomarker for documentation of sTBI cases. Hence, in addition to GFAP, other site-wise IHC parameters must be examined for better categorization of sTBI cases and predicting time of traumatic event

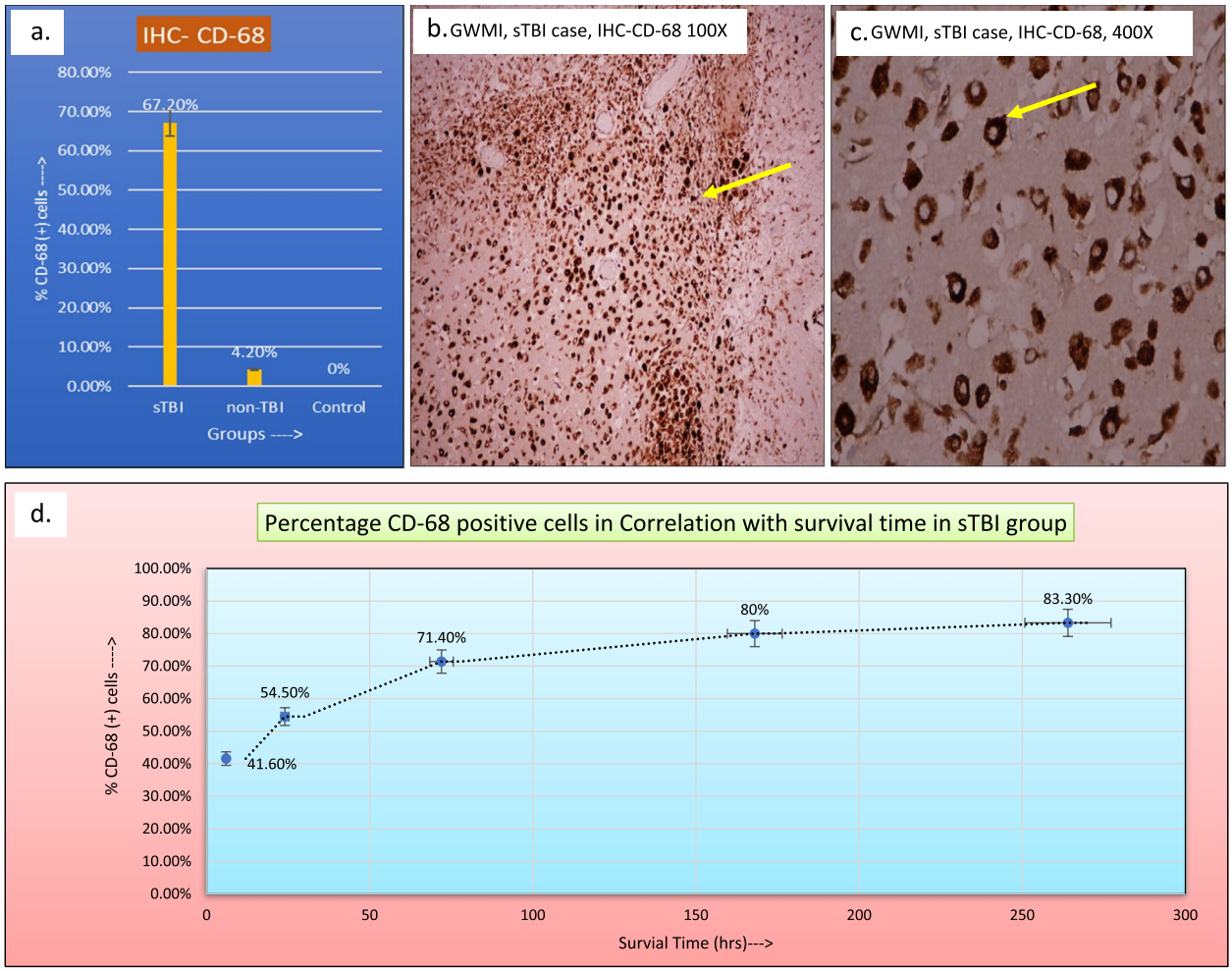

Fig. 5 IHC CD-68 in grey-white matter interface (GWMI). **a** Percentage of decedents with IHC CD-68-positive cells among sTBI, non-TBI and control groups. **b** sTBI case: collection of macrophages (yellow arrow) in GWMI (IHC-CD-68-positive 100 ×). Survival time: 20 days. **c** sTBI case: collection of macrophages (yellow arrow depicting single macrophage) in GWMI (IHC CD-68-positive 400 ×). Survival time: 20 days. **d** Variation of percentage of CD-68-positive cells with survival time in sTBI group

prior to death from a forensic point of view. Due to ante- and post-mortem changes, TBI-related biomarkers are known to show variations. Markers that did not reveal sufficient tangible conclusions must not be disregarded yet as insufficient sample size or procedural lapses may act as limiting factors in ascertaining objective conclusions. We also reiterate that individual cases of pre-mortem pathology and post-mortem interval may have had an effect on the expression of factors examined in this study.

Conclusions

Reconstructing TBI scenario can be challenging in medicolegal cases where gross features of TBI are absent and circumstances leading to death are dubious. A holistic approach using biomarkers has the potential to indicate approximate survival time if threshold levels of the same are determined through empirical data. Insights into the functional interplay of a panel of markers post sTBI hold significant value in justice delivery and ought to be better standardized for reproducibility and reconnaissance. Post traumatic brain injury, there are myriad interlinked changes in the brain environment like axonal damage, myelin degradation, secondary inflammatory changes like astrogliosis and microglial reaction, among numerous others. Thus, in this context, it is important to arrive at empirical conclusions utilizing diverse biomarkers as per feasibility. It is also reiterated that further studies are warranted to support robust evidences in routine forensic practice.

Abbreviations

sTBI	Severe traumatic brain injury
GCS	Glasgow Coma Score
H&E	Haematoxylin and eosin
IHC	Immunohistochemistry
β-APP	Beta-amyloid precursor protein
NFP	Neurofilament protein
GFAP	Glial fibrillary acidic protein
CD-68	Cluster of differentiation-68
CNS	Central nervous system
MLC	Medicolegal case
DAI	Diffuse axonal injury

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Authors' contributions
MS, AS and VS helped in concepts, design, literature search, data acquisition, manuscript preparation and gaining of ethical approval. MS and NC designed the article layout and wrote and edited the document. PM, SP, DA, RMP and AR helped in review and editing. RM and SL helped in concept, screening of intellectual content and manuscript editing and review. The manuscript has been reviewed and approved by all authors.

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Availability of data and materials
The corresponding author hereby declares that all datasets are available.

Declarations

Ethics approval and consent to participate
This study has been approved by the ethics committee of All India Institute of Medical Sciences (AIIMS), New Delhi, India (Ref no: IECPG-469/27.009.2018). Following the ethical clearance, written and informed consent were obtained from legally authorized relative (LAR) of every recruited case in the study. The entire work was carried out in accordance to Declaration of Helsinki and other protocols.

Consent for publication
The corresponding author, on behalf of all co-authors, hereby grants the consent for publication.

Competing interests
The authors declare that they have no competing interests.

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