Blood-based biomarkers for TBI Classification

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Bottom Line Upfront: Blood-based biomarkers (BBMs) have demonstrated utility as informative components of an improved classification system for TBI. The application of BBMs in a TBI classification system has potential to allow for a more adaptable and nuanced approach to triage, diagnosis, and treatment. Although these biomarkers may reflect underlying pathophysiology changes such as neuronal, astrocytic, or vascular injury, the extent to which these changes underlie the symptoms and signs used to diagnose TBI is not known. Current evidence supports the use of GFAP, UCH-L1 and S100B to assist in re-classification of TBI at acute time points (0-24 hours) primarily in ED settings, while NfL, GFAP and probably S100B have utility at subacute time points (1-30 days) in hospital and ICU settings. Blood levels of these biomarkers reflect the extent of structural brain injury in TBI and may be useful for describing the extent of structural brain injury in a classification system. While there is insufficient evidence to support a role for BBMs at chronic time points (>30 days), emerging evidence suggests that NfL and p-Tau may have a potential future role in this regard. Evidentiary details are contained in the Appendix.

A. Recommendations for biomarkers at ACUTE time points (0-24 hours)

GFAP

Context of Use (COU): Diagnostics, Prognostic biomarkers - Emergency Department, athletic training room

Indication: GFAP can aid in the identification of those who will, with high likelihood, have a normal head CT and thus may not require acute neuroimaging. Validated sensitivity of 0.98 and specificity of 0.36 at cutoff of 30pg/mL when combined with UCH-L1[1].

Indication: GFAP has potential to aid in the prediction of traumatic intracranial injuries not seen on head CT scan with AUC 0.78[2].

Indication: GFAP can aid in the diagnosis of TBI at acute time points based on its ability to discriminate between TBI and controls with AUCs ranging from 0.60 to 0.94[3-8]. Combining with UCH-L1 provides marginal improvement in AUC[3,4,8].

Indication: GFAP can aid in the prediction of mortality and global functional outcome 6-12 months after injury, with AUCs ranging from 0.63 to 0.81[8-11]. Combining with UCH-L1 provides marginal improvement in AUC[8].

Modifiers and Limitations:

- Current cut-offs are method and/or laboratory-specific, underscoring the need for certified reference materials and methods for proper assay standardization.
- Elevations in healthy young children and older adults suggests need for age-specific cutoffs[12].
- Elevations in other neurologic diseases (e.g., multiple sclerosis, stroke, Alzheimer's disease) have potential to reduce GFAP's specificity in certain patients[13,14,15,16].

UCH-L1

Context of Use (COU): Diagnostics, Prognostic biomarkers - Emergency Department, athletic training room

Indication: UCH-L1 can aid in the identification of those who will, with high likelihood, have a normal head CT and thus may not require acute neuroimaging. Validated sensitivity of 0.98 and specificity of 0.36 at cutoff of 360pg/mL when combined with GFAP[1].

Indication: UCH-L1 can aid in the diagnosis of TBI at acute time points based on its ability to discriminate between TBI and controls, with AUCs ranging from 0.66 to 0.94[3,5,8,17]. Combining with GFAP improves the AUC[3,8].

Indication: UCH-L1 can aid in the prediction of mortality and global functional outcome 6 months after injury, with AUCs ranging from 0.74 to 0.84[8,18]. Combining with GFAP improves the AUC[8,18].

Modifiers and *Limitations*:

- Short half-life among TBI biomarkers, 7–9 h[5,19].
- Lack of reference values by age.
- Lack of data in children.

- Current cut-offs are method and/or laboratory-specific, underscoring the need for certified reference materials and methods for proper assay standardization.

S100B
Context of Use (COU): Diagnostics biomarkers - Emergency Department, athletic training room.

Indication: S100B can aid in the identification of those who will, with high likelihood, have a normal head CT and thus may not require acute neuroimaging. Validated sensitivity 0.94 and specificity 0.19 in the mild low risk group within 6 hours of injury using a cutoff of 0.10 μg/L[20,21].

Indication: S100B can aid in the diagnosis of TBI at acute time points based on its ability to discriminate between TBI and controls, with AUCs ranging from 0.67 to 0.79[17,22,23].

Modifier and Limitations:
- Current cut-offs are method and/or laboratory-specific, underscoring the need for certified reference materials and methods for proper assay standardization.
- Increases with age suggest need for age-specific cutoffs[24]. However, population-based references ranges in younger age groups have been established[25,26].
- Has short half-life[27] and short sampling time window ≤6 h[21]. Three-hour cutoff has been proposed in pediatric cohorts[28]
- Elevations in other conditions (e.g., melanoma[29], extracranial trauma[30], intense physical exertion[31], epilepsy[32], stroke[33]) have potential to reduce S100B's specificity in certain patients.

B. Recommendations for biomarkers at SUBACUTE time points (1-30 days)
Neurofilament light chain (NfL)
Context of Use (COU): Diagnostics, prognostic biomarker - Emergency Department, ICU, clinic

Indication: NfL can aid in the diagnosis of TBI at subacute time points based on its ability to discriminate between TBI and controls with AUCs ranging from 0.78 to 0.92[7,34].

Indication: Subacute levels of NfL can aid in the prediction of white matter atrophy and reduced white matter integrity weeks to months after injury injury[35,36,37].

Indication: NfL can aid in the prediction of global functional outcome and persistent symptoms 6-12 months after injury with AUCs ranging from 0.71 to 0.97[11,34,35,38].

Modifiers and Limitations:
- NfL levels peak 7-14 days post injury, and can remain elevated for months. [36,39,40].
- Elevations among healthy older adults suggests need for age-specific cutoffs[12,41].
- Reference limits may be assay- and even laboratory-specific, , underscoring the need for certified reference materials and methods for proper assay standardization.
- Elevations in other conditions (e.g. stroke[16], amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer's disease[42]) have potential to reduce NfL's specificity for TBI in certain patients.

GFAP
Context of Use (COU): Prognostic biomarker - ICU, clinic

Indication: GFAP can aid in the diagnosis of TBI at subacute time points based on its ability to discriminate between TBI and controls, with AUCs ranging from 0.74 to 0.94[5,7,34].

Indication: GFAP can aid in the prediction of mortality and global functional outcome 6 to 12-month post-injury with AUCs ranging from 0.69 to 0.88[9,43].

Indication: GFAP can aid in the prediction of traumatic abnormalities on neuroimaging with AUCs ranging from 0.82 to 0.97[44,36].

Modifier and Limitations: Same as for ACUTE

S100B
Context of Use (COU): Prognostic biomarker - ICU

Indication: S100B can aid in the prediction of mortality with AUCs ranging from 0.73 to 0.83[45].

Indication: S100B can aid in the prediction of delayed radiologic pathology with AUC of 0.86[46,36].
Modifier and Limitations: Same as for ACUTE

C. Recommendations for biomarkers at CHRONIC time points (> 30 days)

No biomarkers recommended for use at time points 1 month and beyond, due to limited data.
Markers with future potential:
NfL
Context of Use (COU): Prognostic biomarker
Indication: NfL levels have potential to aid in the prediction of post-TBI cerebral atrophy and reduced microstructural integrity[34,35,47].

pTau (phosphorylated Tau)
Context of Use (COU): Prognostic biomarker
Indication: p-Tau has potential to aid in the prediction of global functional outcome and persistent symptoms 6-12 months after injury respectively[48,49].

D. Recommendations for biomarker ANALYSIS AND INTERPRETATION

1. Establish appropriate reference ranges for clinical decision-making [50,51].
2. Harmonize / standardize assays across platforms, taking into account
   - Robustness of biomarker assays to variations in pre-analytical factors such as sample collection, processing, and storage[52].
   - Analytical precision: such as accuracy, precision, linearity, limit of detection, limit of quantification, selectivity, recovery, reproducibility, and stability[53,54-56].
3. Improve transparency in assay development through large-scale data sharing of methods and results[57-59].

E. Knowledge Gaps and Future Research Directions

Gaps
- Lack of certified reference methods and materials for assay standardization.
- Lack of data relating blood-based biomarkers to specific TBI pathoanatomic endophenotypes.
- Lack of data on biomarker panels (e.g. ≥3 biomarkers) in different settings
- Very few studies measuring blood-based biomarkers in TBI patients at a month or more post injury.
- Lack of data in special populations (e.g., paediatrics, geriatrics).

Future Directions
- Develop certified reference methods and materials for standardization of the most promising biomarkers.
- Refine reference ranges for S100B, GFAP, UCH-L1 and NFL in diverse populations, as determined by age, sex, comorbidities, polypharmacy, and potentially other lifestyle factors.
- Develop practical, cost-effective and rapid point of care analyzers to enable the integration of biomarkers in TBI classification in out-of-hospital environments.
- Use a reverse translational approach to clarify the connection between blood based biomarkers and specific pathoanatomic endophenotypes (e.g., axonal injury, microglial activation, oxidative stress, etc.) similar to the A/T/N system in Alzheimer's disease[60].
- Promising candidate biomarkers should be studied in well-characterized in clinically representative cohorts using predetermined sampling times based on the current knowledge.
- Encourage investigative efforts to discover novel biomarkers that can fill current knowledge gaps such as the lack of strong biomarkers for prognostication in the less severely injured patients and limited biomarkers available for the chronic phase of injury.

- The pros and cons of using biomarkers as qualitative, categorical or continuous values in a TBI classification system have not been well studied.

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A.Recommendations for biomarkers at ACUTE time points (0-24 hours)

GFAP

Context of Use (COU): Diagnostics, Prognostic biomarkers - Emergency Department, athletic training room

Indication: GFAP can aid in the identification of those who will, with high likelihood, have a normal head CT and thus may not require acute neuroimaging.
- GFAP is detectable within 1 h of injury and peaks within 20–24 h with proposed half-life of 24–48 h[61]. At a cutoff of 30 pg/mL GFAP has validated **sensitivity of 0.98** and **specificity of 0.36** for traumatic intracranial injury on head CT scan among those with GCS 9-15 when measured within 12 hours of injury and combined with UCH-L1[1]. In isolation, clinically adequate sampling window is probably 24 h from injury[62,63]. Cutoffs for use in a point-of-care test device have been derived and validated [64,65]. In combination with UCH-L1, GFAP is FDA- and EMA-cleared for prediction of normal CT (Abbott i-STAT and Alinity; bioMerieux's Vidas platform). GFAP is recommended in French Mild TBI Practice Guidelines[66].

Indication: GFAP has potential to aid in the prediction of traumatic intracranial injuries not seen on head CT scan.
- GFAP levels within 24 hours of injury among ED patients with GCS 13-15 and a normal head CT distinguished those with and without traumatic abnormalities on MRI 7-18 days post-injury with an **AUC 0.78** (95%CI 0.73-0.83)[2].

Indication: GFAP can aid in the diagnosis of TBI at acute time points based on its ability to discriminate between TBI and controls with AUCs ranging from 0.60 to 0.94[3-8]. Combining with UCH-L1 provides marginal improvement in AUC[3,4,8].

Post-injury time of GFAP measurement	TBI Patient Group	Comparison Control Group	AUC (95% CI)	Reference
0-6 hrs	Concussed collegiate athletes	Contact sport athletes	**0.68** (0.61-0.75) GFAP **0.72** (0.65-0.79) GFAP+UCH-L1	McCrea (2020)[3]
0-6 hrs	Concussed military cadets during combat training	Military cadets	**0.75** (0.64-0.87) GFAP **0.80** (0.68-0.93) GFAP+UCH-L1	Giza (2021)[4]
4-24 hrs	ED patients with GCS 9-15	ED trauma controls	Ranged from **0.73** (0.68-0.77) – **0.83** (0.74-0.91)	Papa (2016)[5]
0-24 hrs	Hospitalized patients with GCS 13-15 and +head CT	Healthy controls	**0.94** (p<0.0001) (95% CI not provided)	Bogoslovsky (2017)[6]
0-24 hrs	ED patients with GCS 13-15	ED trauma controls	**0.92** (0.88-0.97)	Clarke (2021)[7]
0-24 hrs	ED patients with GCS 3-15 (83% GCS 13-15)	Uninjured controls	**0.91** (0.88-0.94) GFAP **0.94** (0.92-0.96) GFAP+UCH-L1	Diaz-Arrastia (2014)[8]

Indication: GFAP can aid in the prediction of mortality and global functional outcome 6-12 months after injury, with AUCs ranging from 0.63 to 0.81[8-11]. Combining with UCH-L1 provides marginal improvement in AUC[8].

Post-injury time of GFAP measurement	TBI Patient Group	Outcome predicted	AUC (95% CI)	Reference
Hospital admission	GCS ≤8	Mortality at 6mo	**0.76** (0.61-0.92)	Lei (2015)[9]
		Unfavorable GOS (1-3) at 6mo	**0.82** (0.70-0.94)	

Hospital admission	GCS 3-15 (57% GCS 13-15)	Unfavorable GOS (1-3) at 6-12mo	**0.72** (0.60-0.81)	Takala (2016)[10]
		Incomplete recovery (GOS-E 1-7) at 6-12mo	**0.63** (0.52-0.71)	
0-24 hrs	ED patients with GCS 3-15 (83% GCS 13-15)	Unfavorable GOS-E ($\leq$4) at 6mo	**0.74** (0.61-0.87) GFAP **0.81** (0.70-0.91) GFAP+UCH-L1	Diaz-Arrastia (2014)[8]
0-24 hrs	ED patients with GCS 13-15	Favorable GOS-E (5-8) at 6-12mo	**0.76** (0.63–0.88)	Hossain (2019)[11]

Modifiers and Limitations:

- Current cut-offs are method and/or laboratory-specific, underscoring the need for certified reference materials and methods.
- Elevations in healthy young children and older adults suggests need for age-specific cutoffs. Among 900 Canadians, the upper 97.5th percentile for GFAP was 226 pg/ml in those 3-9 years old, 92 pg/ml for those 10-59 years old, and 228 pg/ml for those 60-79[12].
- Elevations in other neurologic diseases (e.g., multiple sclerosis, stroke, Alzheimer's disease) have potential to reduce GFAP's specificity in certain patients. GFAP levels were higher among 33 elderly adults at risk for AD (cognitively normal but with abnormal amyloid-beta load on positron emission tomography) compared to 63 elderly adults with normal PET scans (240 pg/mL vs 151 pg/mL)[13]. GFAP levels were higher among 13 adults with secondary progressive multiple sclerosis (146 pg/ml) and 25 adults with primary progressive multiple sclerosis (131 pg/ml) compared to 20 healthy controls (92.3 pg/ml)[14]. Mean serum GFAP level was significantly higher among 79 adult MS patients compared to 13 healthy controls (120 vs 79.3 pg/ml, p=0.031)[15]. Among 30 adults mean age 75.1 years, GFAP levels were 6,300 pg/ml within 1 day of acute ischemic stroke[16].

UCH-L1

Context of Use (COU): Diagnostics, Prognostic biomarkers - Emergency Department, athletic training room

Indication: UCH-L1 can aid in the identification of those who will, with high likelihood, have a normal head CT and thus may not require acute neuroimaging.

- Detectable within 1 h from TBI, peaks 8 h after TBI[5,19]. Validated **sensitivity 0.98**, **specificity 0.36** among TBI patients with GCS 9-15 needing head CT within 12 hours of injury at a cut off of 360 pg/mL, when combined with GFAP[1]. In combination with GFAP, UCH-L1 is FDA- and EMA cleared for prediction of normal CT. Recommended for use in the French Mild TBI Practice Guidelines[66].

Indication: UCH-L1 can aid in the diagnosis of TBI at acute time points based on its ability to discriminate between TBI and controls, with AUCs ranging from 0.66 to 0.94[3,5,8,17]. Combining with GFAP improves the AUC[3,8].

Post-injury time of UCH-L1 measurement	TBI Patient Group	Comparison Control Group	AUC (95% CI)	Reference
0-6 hrs	Concussed collegiate athletes	Contact sport athletes	**0.66** (0.58-0.74) UCH-L1 **0.72** (0.65-0.79) UCH-L1+GFAP	McCrea (2020)[3]
0-6 hrs	Concussed high school athletes	Non-contact athletes Contact sport athletes	**0.79** (0.70-0.88) **0.74** (0.65-0.83)	Meier (2020)[17]
0-24 hrs	ED patients with GCS 3-15 (83% GCS 13-15)	Uninjured controls	**0.87** (0.83-0.90) GFAP **0.94** (0.92-0.96) GFAP+UCH-L1	Diaz-Arrastia (2014)[8]
4-24 hrs	ED patients with GCS 9-15	ED trauma controls	Ranged from **0.63** (0.58-0.68) – **0.67** (0.53-0.81)	Papa (2016)[5]

Indication: UCH-L1 can aid in the prediction of mortality and global functional outcome 6 months after injury, with AUCs ranging from 0.74 to 0.84[8,18]. Combining with GFAP improves the AUC[8,18].

Post-injury time of UCH-L1 measurement	TBI Patient Group	Outcome predicted	AUC (95% CI)	Reference
0-24 hrs	ED patients GCS 3-12	Mortality at 6mo	**0.76** (95% CI: 0.71–0.81) UCH-L1 **0.84** (0.80–0.89) UCH-L1+GFAP	Korley (2022)[18]
0-24 hrs	ED patients GCS 13-15	Unfavorable GOS-E ($\leq$4) at 6mo	**0.70** (0.62–0.79)	Korley (2022)[18]
0-24 hrs	ED patients GCS 3-15 (83% GCS 13-15)	Unfavorable GOS-E ($\leq$4) at 6mo	**0.74** (0.60-0.91) UCH-L1 **0.81** (0.70-0.91) UCH-L1+GFAP	Diaz-Arrastia (2014)[8]

Modifiers and *Limitations*:
- Short half-life among TBI biomarkers, 7–9 h[5,19].
- Lack of reference values by age.
- Lack of data in children.
- Lack of certified reference methods and materials for assay standardization.

S100B

Context of Use (COU): Diagnostics biomarkers - Emergency Department, athletic training room.

Indication: S100B can aid in the identification of those who will, with high likelihood, have a normal head CT and thus may not require acute neuroimaging. Recommended for use in French[66] and Scandinavian[21] TBI clinical decision guidelines.
- Detectable within 1 h after TBI with a peak in <6 h and has a half-life of 0.5–2 hr[67]. Validated as part of the Scandinavian TBI clinical decision-making guidelines in mild-low risk patients within 6 hours of injury, using a cutoff of 0.10 µg/L[20,21]. **Sensitivity 0.94**, **specificity 0.19** in the mild low risk group within 6 hours of injury using a cutoff of 0.10 µg/L [20]. Demonstrated safety and cost-effectiveness[68,69].
- Similar performance among paediatric cohorts, with **sensitivities** ranging from **97-100%** and **specificities** ranging from **34-37.5%**[70].

Indication: S100B can aid in the diagnosis of TBI at acute time points based on its ability to discriminate between TBI and controls, with AUCs ranging from 0.67 to 0.79[17,22,23].

Post-injury time of S100B measurement	TBI Patient Group	Comparison Control Group	AUC (95% CI)	Reference
0-6 hrs	Concussed high school athletes	Non-contact athletes Contact athletes	**0.79** (0.70-0.88) **0.68** (0.60-0.77)	Meier (2020)[17]
0-1 hr	Concussed elite athletes	Contact athletes	**0.67** (0.52-0.83)	Shahim (2014)[22]
0-6 hrs	ED patients with GCS 13-15	Healthy controls	**0.71** (0.68-0.74)	Bazarian (2013)[23]

Modifier and Limitations:
- Current cut-offs are method and/or laboratory-specific, underscoring the need for certified reference materials and methods.
- Increases with age suggest need for age-specific cutoffs[24]. However, population-based references ranges in younger age groups have been established[25,26].
- Has short half-life[27] and short sampling time window ≤6 h[21]. Three-hour cutoff has been proposed in pediatric cohorts[28]
- Elevations in other conditions (e.g., melanoma[29], extracranial trauma[30], intense physical exertion[31], epilepsy[32], stroke[33]) have potential to reduce S100B's specificity in certain patients.
- Lack of certified reference methods and materials for assay standardization.

B. Recommendations for biomarkers at SUBACUTE time points (1-30 days)

Neurofilament light chain (NfL)

Context of Use (COU): Diagnostics, prognostic biomarker - Emergency Department, ICU, clinic

Indication: NfL can aid in the diagnosis of TBI at subacute time points based on its ability to discriminate between TBI and controls.
- NfL levels at 30 days discriminated healthy controls from TBI patients with a GCS of 13-15, 9-12, and 3-8 with **AUCs** of **0.84**, **0.92** and **0.92**, respectively (95% CI not provided)[34].
- NfL levels at 2 weeks and 3 months discriminated patients with GCS 13-15 from trauma controls with **AUC 0.83** (95% CI 0.75 – 0.91) and **AUC 0.78** (0.70-0.87), respectively [7].

Indication: Subacute levels of NfL can aid in the prediction of white matter atrophy and reduced white matter integrity weeks to months after injury.
- Increased NfL levels at 6 days associated with a decrease volume in total gray matter and white matter (ß = −6,958, p = 0.004 and ß = −6,389, p = 0.019, respectively), and in central and posterior portions of the corpus callosum (ß = −8.8, p = 0.0016 and ß = −9.2 p = 0.023, respectively) in a clinic based cohort of mixed severity TBI patients (GCS 13-15 in 55%, <12 in 45%) 1-6 weeks post injury[35].
- Peak NfL levels during the first 6 weeks post-injury among those with GCS 3-12 correlates with lesion volume on MRI acquired at 1-6 weeks (ρ=0.50, p<0.001)[36].
- NfL levels 1-24 hours after injury among those with GCS 13-15 correlated with reductions white matter integrity (reductions in whole brain FA [ρ=-0.323, p=0.002] and increases in MD [ρ=-0.343, p<0.001]) on DTI 4-15 months after injury[37].

Indication: NfL can aid in the prediction global functional outcome and persistent symptoms 6-12 months after injury with AUCs ranging from 0.71 to 0.97[11,34,35,38].

Post-injury time of NfL measurement	TBI Patient Group	Outcome predicted	AUC (95% CI)	Reference
1-24hrs	GCS 13-15	Unfavorable GOS-E (<4) at 6-12mo	**0.83** (0.69-0.96)	Hossain (2019)[11]
24 hrs	GCS <8	Unfavorable GOS (1-3) at 12mo	**0.71** (95% CI not provided)	Shahim (2016)[38]
6 days	Concussed elite athletes	Post-concussive symptoms at 12mo	**0.97** (0.94-1.0)	Shahim (2020)[35]

- NfL measured at 30 days after injury in a clinic based cohort of TBI patients GCS 3-15 (GCS 13-15 in 55%) correlated with an improvement in GOS-E at 90 days (ρ=0.85, p=0.0004)[34].

Modifiers and Limitations:
- NfL levels peak 7-14 days post injury, and can remain elevated for months. [36,39,40].
- Elevations among healthy older adults suggests need for age-specific cutoffs. Among 900 Canadians, the upper 97.5th percentile for NfL was 11.3 pg/ml in those 3-9 years old, 17.1 pg/ml for 10-59 year olds, and 47.1 pg/ml for 60-79 year olds[12]. Among 1724 Swedes the upper 97.5th percentile for NfL was 7 pg/mL for 5-17 year olds, 10 pg/mL for 18-50 year olds, 15 pg/mL for 51-60 year olds, =; 20 pg/mL for 61-70 year olds, and 35 pg/mL for those 70 + years[41]. Reference limits and cut-offs may be assay- and even laboratory-specific.
- Elevations in other conditions (e.g. stroke[16], amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer's disease[42]) have potential to reduce NfL's specificity for TBI in certain patients.
- Lack of certified reference methods and materials for assay standardization.

GFAP

Context of Use (COU): Prognostic biomarker - ICU, clinic

Indication: GFAP can aid in the diagnosis of TBI at subacute time points based on its ability to discriminate between TBI and controls, with AUCs ranging from 0.74 to 0.94[5,7,34].

Post-injury time of GFAP measurement	TBI Patient Group	Comparison Control Group	AUC (95% CI)	Reference
1-7 days	hospitalized patients with GCS 9-15	trauma controls	Ranging from **0.81** (0.70-0.93) to **0.94** (0.78-1.00)	Papa (2016)[5]
30 days	hospitalized patients with GCS 9-12	healthy controls	**0.89** (95% CI not provided)	Shahim (2020)[34]
	hospitalized patients with GCS 3-8		**0.89** (95% CI not provided)	
0-3 days	ED patients with GCS 13-15	trauma controls	**0.74** (0.69 – 0.80	Clarke (2021)[7]

Indication: GFAP can aid in the prediction of mortality and global functional outcome 6 to 12-month post-injury with AUCs ranging from 0.69 to 0.88[9,43].

Post-injury time of NfL measurement	TBI Patient Group	Outcome predicted	AUC (95% CI)	Reference
1-5 days	GCS≤8	Mortality at 6mo	Ranging from **0.79** to **0.77** (95% CI not provided)	Lei (2015)[9]
		Unfavorable GOS (1-3) at 6mo	Ranging from **0.69** to **0.85** (95% CI not provided)	
7 days	GCS 4-8	Unfavorable GOS (1-3) at 6mo	**0.76** (0.61-0.90)	Raheja (2016)[43]
		Unfavorable GOS (1-3) at 12mo	**0.82** (0.65-0.98)	
		Mortality at 6mo	**0.88** (0.71-1.00)	
		Mortality at 12 mo	**0.81** (0.61-1.00)	

- GFAP measured at 30 days after injury in a clinic based cohort of mixed severity TBI patients (GCS 13-15 in 55%, GCS <12 in 45%) correlated with an improvement in GOS-E at 90 days (ρ=0.64, p=0.019)[34].

Indication: GFAP can aid in the prediction of traumatic abnormalities on neuroimaging
- Increased GFAP levels at time points between 1 and 7 days post-injury distinguished those with and without traumatic intracranial injury on head CT scan with AUCs ranging from **0.82** to **0.97** (lower 95% CI range:0.70-0.93)[44]. AUCs were not appreciably different with the addition of UCH-L1.
- Peak GFAP levels during the first 6 weeks post-injury among those with GCS 3-12 correlated with lesion volume on MRI 1-6 weeks post-injury (ρ=0.53, p<0.001)[36].

Modifier and Limitations: Same as for ACUTE

S100B

Context of Use (COU): Prognostic biomarker - ICU

Indication: S100B can aid in the prediction of mortality
- In a meta-analysis of 9 studies, S100B levels within the first 24 hours in hospitalized patients predicted in-hospital mortality and morality within 1 year of injury with average **AUCs** of **0.832**$\pm$0.134 and **0.732**$\pm$0.09, respectively[45].

Indication: S100B can aid in the prediction of delayed radiologic pathology.
- Individual-specific increases in S100B 48 hours or more after injury in hospitalized patients with GCS 3-15 (GCS ≤8 in 70%) heralded by a mean of 2 days the development of new pathology visible on CT or MRI (infarction, hematoma, edema, herniation, or sinus thrombosis) with an **AUC 0.855** (95% CI not provided). An S100B increase of > 0.05 µg/L was **80% sensitive** and **89% specific** for prediction of new radiographic pathology[46].
- Peak S100B levels during the first 6 weeks post-injury among those with GCS 3-12 correlates with lesion volume on MRI acquired at 1-6 weeks (ρ=0.45, p<0.001)[36]

Modifier and Limitations: Same as for ACUTE

C. Recommendations for biomarkers at CHRONIC time points (> 30 days)

- **No biomarkers recommended** for use at time points 1 month and beyond, due to limited data.
- *Future potential*

NfL

Context of Use (COU): Prognostic biomarker

Indication: NfL levels have potential to aid in the prediction of post-TBI cerebral atrophy and reduced microstructural integrity.

- NfL levels at 180 days predicted WM volume loss from 180 days to 1 year (ß = −3,881, p = 0.001)[34,35]
- NfL levels at 1 year was associated with the rate of volume loss in the mid anterior corpus callosum from 1 to 2 years (ß = −34.5, p = 0.0001)[34,35]
- NfL levels at 8 months post injury predicts increased MD (DTI metrics of injury) from 8 months to >5 years with adjusted R^2 of 0.48 (p<0.001)[47].

pTau (phospho-Tau)

Context of Use (COU): Prognostic biomarker

Indication: p-Tau has potential to aid in the prediction of global functional outcome and persistent symptoms 6-12 months after injury

- In TBI patients with GCS 3-12, 1-6month mean pTau levels predicted unfavorable 12-month GOS-E (1-4 vs 7-8) and Disability Rating Scale (15-30 vs 0-4) with AUCs of **0.67** (p=0.03) and **0.71** (p=0.01), respectively[48].
- Elevations of serum exosomal p-tau levels in Veterans with combat-related mild TBI (mean 7-11 years post-injury) were significantly correlated with post-traumatic and post-concussive symptoms with correlation coefficients ranging from 0.29 to 0.40[49].

D. Recommendations for biomarker ANALYSIS AND INTERPRETATION

Establish appropriate reference ranges for clinical decision-making.

Blood-based biomarkers show potential for multiple uses in TBI: (1) as a way to discern between injured vs. non-injured; (2) as an indicator for severity of injury; (3) as an indicator of specific mechanisms of injury within the TBI milieu (e.g., excitotoxicity, inflammation, etc.); and (4) as a prognostic predictor of recovery trajectory. In each of these contexts, potential biomarkers require a reference point or reference range in order to provide meaningful, actionable information. As brain injury biomarkers become incorporated into clinical practice, ongoing work to refine reference ranges in diverse populations - as determined by age, sex, comorbidities, polypharmacy, and potentially other lifestyle factors - will be needed to aid the accuracy of biomarker measures. **The successful incorporation of blood based biomarkers into a classification schema requires an improved understanding of the aspect of injury pathobiology they capture and appropriate comparison values.**

Individual baseline comparison vs. normalized reference ranges

Informative biomarker measures can make use of two different reference points for comparison: either (1) the patient's individual "baseline" measure of that biomarker, or (2) comparison to a normalized, appropriate reference range. In the ideal world, every patient would have their own baseline, non-pathological biomarker reference points available for comparison; an individualized biomarker "reference library" would serve as the most informative indicator of change following injury. However, this is not a realistic expectation for current clinical contexts. At present, the best option available for biomarker "set points" is to compare a patient's biomarker measures to a normalized reference range. Establishing normalized reference ranges for specific blood-based brain injury biomarkers in healthy populations is critical for interpreting results. This work is already underway - for example, one study validating a GFAP assay examined reference values in 181 healthy individuals, finding a range of 0 - 0.08 ng/mL[50]. Reference ranges provided to clinicians

should be based on normative data from a random selection of individuals in the general population. Key considerations for developing reference ranges include recruiting adequate sample sizes, often at least 120 subjects. Importantly, these ranges should include an adequate representation of persons from all age, sex, and racial groups. Stratified sampling by key demographics like age and sex allows determining appropriate reference values for different groups.

Specialized population considerations

Clinically informative blood-based biomarkers for brain injury will require reference ranges selected appropriately for a patient's individual characteristics. For example, while high GFAP levels could indicate TBI they could also be elevated due to age or neurodegenerative diseases In contrast, GFAP may appear deceptively low even in a post-injury state due to low-level baseline GFAP ranges seen in patients with higher body mass index, diabetes, or tobacco use[51]. Historically, "complicating" factors like comorbidities and age have resulted in the exclusion of subjects from clinical research. Ironically, rather than resulting in "cleaner" biomarker data, this has hobbled our ability to establish the informative reference ranges necessary for widespread biomarker applicability. The biological variability of different analytes should be investigated across contexts, and reference change values should be estimated accordingly. This will inform the interpretation of serial measurements. As brain injury biomarkers become incorporated into clinical practice, ongoing work to refine reference ranges in diverse populations - as determined by age, sex comorbidities, polypharmacy, and potentially other lifestyle factors - will be needed to aid the accuracy of biomarker measures.

Harmonize / standardize assays across platforms, taking into account
For blood-based protein biomarkers to usefully inform injury status, standardized thresholds should be established for specific analytes. Cross-assay reliability must be ensured to maintain consistency across assay platforms: in order to apply standardized thresholds, the output of an analyte assay for one group needs to be reliably comparable to another group's assay for the same analyte. This requires assay harmonization and standardization, a challenge that is also being tackled by biomarker investigators in other pathologies, including AD, DM and EBV. Several considerations must be addressed to resolve this challenge, the most important of which are to develop certified reference methods and materials for assay standardization.

1) **Robustness of biomarker assays to variations in pre-analytical factors.** Additional analytical validation on existing platforms to establish robustness for routine clinical use. Pre-analytical factors typically account for 50-70% of test error, the effect of variables such as time from collection to processing and freezing, freeze-thaw stability, diurnal or post-prandial effects, repeatability and reproducibility (by assessing the effect of different reagent lots, instruments, and laboratory technicians) must be established.
   Pre-analytical considerations for assay harmonization include sample collection, processing, and storage. Assays comparing blood-based biomarkers in samples that are collected and stored under different circumstances may yield dramatically different measures of the targeted analyte. In proteomic biomarker discovery and clinical research, variations in preanalytical processing (including sample preparation, storage and processing, fraction of the blood used for testing, and sample additives such as EDTA) present a substantial source of variability in laboratory testing[52].

2) **Analytical precision:**
   Assays of biomarkers utilized in the classification of TBI should undergo rigorous analytical validation. Analytical validation is a critical step in the development of brain injury biomarker assays. It establishes that the assay is accurate, precise, selective, sensitive, reproducible, and stable[53]. Without rigorous analytical validation, brain injury biomarker results cannot be trusted or meaningfully interpreted. Analytical validation typically assesses accuracy, precision, linearity, limit of detection, limit of quantification, selectivity, recovery, reproducibility, and stability. For example, accuracy and precision determine how close measured values are to the true value, and how much measurements vary on repeated testing. Linearity helps ensure the assay responds appropriately over the range of expected biomarker concentrations. Selectivity indicates the assay can distinguish the biomarker from interfering substances. Robust analytical validation gives confidence that the assay performs to the standards required for clinical and research use. Numerous peer-reviewed publications provide recommendations and examples of analytical validation

of biomarker assays[54-56]. Adherence to established analytical validation principles and guidance is essential for developing reliable brain injury biomarker assays.

Improve transparency in assay development through large-scale data sharing of methods and results.

Furthermore, there currently exists a lack of standardization or transparency in assay design and methodological procedures. This variability makes it difficult to compare the results of one analyte assay to another. For example, consider a scenario where two research groups attempt to quantify GFAP in blood (five hours post mild TBI) on an enzyme-linked immunosorbant assay (ELISA), and results show entirely different values. In this example, understanding assay design specifics is essential to understanding differing signal outputs. Transparency is needed from each research team regarding what protein subunit of GFAP is being plated; what antibodies and antibody epitope targets are being utilized; what washing techniques and blocking techniques are being implemented; and how the plate reader parameters are being set. All of these assay design factors will affect the resultant output signal, which in turn determine if a clinical biomarker threshold is being met or not. In the current environment of non-standardized assay design and lack of transparency, it is nearly impossible to compare signal output from one setting to the other, even for the same targeted protein epitope, because of the other variables (often unknown) being juggled at the same time. The challenging lack of consistency, quality control, and transparency in TBI blood-based biomarker methods has been discussed at length by numerous researchers[57-59].

1) A centralized hub for "recipe sharing" of methodologies and results via common data elements will allow for enhanced cross-methods comparison of biomarker detection techniques.
2) Develop and distribute reference reagents for candidate proteins a "universal calibrator" library for use in comparing diverse assays tied by a common analyte. Cross-calibration and standardization across assay platforms and laboratories will require both recombinant qualified protein standards, as well as authenticated matrix-specific pooled control and TBI samples in different biofluids (serum, plasma, CSF), to ensure the accuracy and reliability of results
3) Incentivize participation from both academia and industry / commercial: Assay manufacturers should inform clinicians of what the assay is measuring - for example intact protein versus protein degradation products, free versus combined, post-translational modifications. Ideally the target epitopes of capture antibodies should be disclosed.

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