




# Serum Neurofilament Light as a Biomarker of Traumatic Brain Injury in the Presence of Concomitant Peripheral Injury

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Ker Rui Wong<sup>1</sup> , William T O'Brien<sup>1</sup>, Mujun Sun<sup>1</sup>, Glenn Yamakawa<sup>1</sup>, Terence J O'Brien<sup>1,2</sup> , Richelle Mychasiuk<sup>1</sup>, Sandy R Shultz<sup>1,2</sup> , Stuart J McDonald<sup>1,3\*</sup> and Rhys D Brady<sup>1,2\*</sup>

<sup>1</sup>Department of Neuroscience, Central Clinical School, Monash University, Melbourne, VIC, Australia. <sup>2</sup>Department of Medicine, Royal Melbourne Hospital, The University of Melbourne, Parkville, VIC, Australia. <sup>3</sup>Department of Physiology, Anatomy and Microbiology, School of Life Sciences, La Trobe University, Bundoora, VIC, Australia.

## ABSTRACT

**INTRODUCTION:** Serum neurofilament light (NfL) is an emerging biomarker of traumatic brain injury (TBI). However, the effect of peripheral injuries such as long bone fracture and skeletal muscle injury on serum NfL levels is unknown. Therefore, the aim of this study was to determine whether serum NfL levels can be used as a biomarker of TBI in the presence of concomitant peripheral injuries.

**METHODS:** Rats were randomly assigned to one of four injury groups: polytrauma (muscle crush + fracture + TBI; n = 11); peripheral injuries (muscle crush + fracture + sham-TBI; n = 12); TBI-only (sham-muscle crush + sham-fracture + TBI; n = 13); and triple-sham (n = 7). At 2-days post-injury, serum levels of NfL were quantified using a Simoa HD-X Analyzer.

**RESULTS:** Compared to triple-sham rats, serum NfL concentrations were higher in rats with peripheral injuries-only, TBI-only, and polytrauma. When compared to peripheral injury-only rats, serum NfL levels were higher in TBI-only and polytrauma rats. No differences were found between TBI-only and polytrauma rats.

**CONCLUSION:** Serum NfL levels did not differ between TBI-only and polytrauma rats, indicating that significant peripheral injuries did not affect the sensitivity and specificity of serum NfL as a biomarker of moderate TBI. However, the finding of elevated serum NfL levels in rats with peripheral injuries in the absence of a TBI suggests that the presence of such injuries may limit the utility of NfL as a biomarker of less severe TBI (eg, concussion).

**KEYWORDS:** Blood biomarker, extracranial injury, bone fracture, polytrauma, SIMOA

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**CORRESPONDING AUTHOR:** Rhys Brady, Department of Neuroscience, Central Clinical School, Monash University, 6th Floor, The Alfred Centre, 99 Commercial Road, Melbourne, VIC 3004, Australia. Email: rhys.brady@monash.edu

## Introduction

Individuals that experience high-energy impacts (eg, motor vehicle collisions) often suffer injuries to multiple body parts (ie, polytrauma).<sup>1,2</sup> For example, traumatic brain injury (TBI) is frequently combined with extremity fractures and muscle damage.<sup>3</sup> Due to the complex nature of polytrauma, it can be challenging to identify TBI and determine the appropriate treatment for these patients.<sup>1</sup> Hence, there is an urgent need for a biomarker that can accurately detect TBI in the context of polytrauma. For more than a decade, there has been increasing attention on several neuron-enriched proteins, such as, S100 calcium-binding protein B (S100B), neuron-specific enolase (NSE), and neurofilament light (NfL) as potential biomarkers of TBI.<sup>4–6</sup> S100B is one of the most studied biomarkers in TBI and was initially thought to be exclusively expressed by astrocytes.<sup>5</sup> Subsequent studies revealed it is also expressed by

extracerebral cell types such as, chondrocytes and adipocytes which, are involved in fracture healing.<sup>5</sup> NSE is a glycolytic enzyme that is predominantly expressed in neurons, although it is also released by neuroendocrine cells and erythrocytes.<sup>5</sup> Therefore, S100B and NSE may not be appropriate biomarkers for diagnosing TBI in the presence of significant concomitant peripheral trauma.<sup>7</sup>

Neurofilament light (NfL) is primarily expressed in sub-cortical myelinated axons.<sup>8</sup> When neurons are damaged, which occurs in TBI, NfL is released into the extracellular space, cerebral spinal fluid, and blood.<sup>9</sup> As such, serum NfL has been postulated to reflect neuroaxonal damage and is a candidate biomarker of TBI.<sup>7,10–13</sup> In humans, cerebrospinal fluid and serum NfL levels have been shown to be elevated following mild-, moderate-, and severe TBI, peaking in the weeks following injury and potentially remaining elevated for several months or years.<sup>11,14,15</sup> There is some clinical evidence that serum NfL levels may predict TBI outcome.<sup>11,15</sup> In rodents,

\*These authors contributed equally to this work.



serum NfL levels peak in the days following mild TBI and correlate with sensorimotor deficits.<sup>16,17</sup> However, NfL is also found in peripheral nerve fibers,<sup>18</sup> and the effect of peripheral injuries on serum NfL levels in the presence and absence of TBI remains unknown. It is therefore important to establish how extracranial injury may impact the utility of NfL as a TBI biomarker. Using our rat model of polytrauma, this study examined how peripheral injuries modified serum NfL levels in the presence and absence of a moderate TBI.

## Methods

### Animals

Forty-three, 7-week old male Sprague Dawley rats were obtained from the Monash Research Platform (Clayton, Australia), and individually housed on a 12-hour light/dark cycle with access to food and water ad libitum. All procedures were approved by the Alfred Animal Ethics Committee.

### Experimental groups

Rats were randomly assigned to one of four injury groups: polytrauma (muscle crush + fracture + TBI;  $n=11$ ); peripheral injuries (muscle crush + fracture + sham-TBI;  $n=12$ ); TBI-only (sham-muscle crush + sham-fracture + TBI;  $n=13$ ); and triple-sham ( $n=7$ ). 3 polytrauma, 4 peripheral injury, and 5 TBI-only rats were excluded due to death immediately post-injury, euthanasia during recovery, or a comminuted fracture. Therefore, there were 8 rats in each experimental group and 7 triple-sham rats.

### Injury models

Injuries were administered as previously described.<sup>19</sup> Briefly, under isoflurane induced-anesthesia, analgesic (0.05 mg/kg buprenorphine in sterile saline) was given subcutaneously and the muscle crush was performed. A 1.2 kg impactor (diameter: 1 cm, width: 1.5 cm), was released from a height of 55 cm, guided by 2 metal rods to impact the hamstring.

Immediately after the muscle injury, femoral fracture was performed. An incision was made medial to the patella and Kirschner-wire (diameter = 1.1 mm) was inserted into the marrow cavity to stabilize the fracture for the duration of the experiment. A 500 g weighted impactor (diameter = 3 mm) was released from 55 cm and guided to the femoral midshaft to induce a transverse non-comminuted femoral fracture. The fracture was confirmed via X-ray.

A moderate TBI was administered following the femoral fracture using the lateral fluid percussion model.<sup>20</sup> As previously described,<sup>19</sup> after craniotomy (5 mm in diameter, 4.5 mm posterior, 2.5 mm left of bregma) a hollow plastic cap was attached over the craniotomy using dental cement. The rat was then connected to the fluid percussion device via the hollow cap, and a fluid pulse (2.6-3.0 atmosphere; atm) was delivered.

Apnea and atm were recorded immediately after the injury as indicators of TBI severity.<sup>21,22</sup> For all sham injuries, all incisions were made and sutured, however, the injury was not given.

### Serum collection and NfL quantification

At 2-days post-injury, blood was collected into 600  $\mu$ l SST Microtainers via terminal cardiac puncture. Blood was allowed to clot at room temperature for 30 minutes prior to centrifugation at 6000g for 90 seconds at 4°C. Serum NfL levels were quantified using a single-plex assay, "Simoa® NF-light Advantage Kit" on the Simoa HD-X Analyzer (Quanterix, USA).<sup>16</sup> A single assay was performed on 8 randomly selected samples per group, and was run in a temperature-controlled laboratory by an experimenter blinded to the experimental conditions. Samples were tested in duplicate, with a total serum volume for each sample of 106  $\mu$ l. All samples measured above the lower limit of quantification for NfL (0.174 pg/ml).

### Statistical analysis

A Mann-Whitney test was used to analyze apnea duration. Two-way ANOVA was used to analyze weight. Serum NfL levels were natural log transformed to decrease skewness of distributions and analyzed using two-way ANOVA. Tukey's post-hoc comparisons were used as appropriate. Significance was set at  $P < .05$ . Area under the receiver operating characteristics (AUROCs) were estimated for NfL.

## Results

At the time of injury acute injury severity measures were taken. At 2 days post-injury serum was collected from rats and was analyzed using the Simoa HD-X Analyzer.

### Acute injury severity measures

There were no differences between the TBI-only and polytrauma groups on the measures of apnea (Table 1). None of the groups differed in weight.

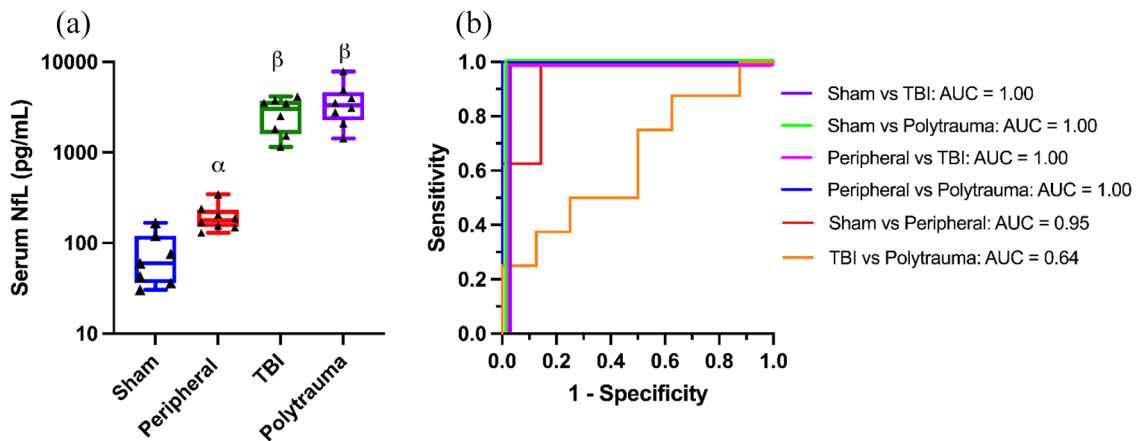
### Serum NfL levels

There was a main effect of TBI ( $F_{(1, 27)} = 338.5$ ;  $P < .0001$ ) and peripheral injury ( $F_{(1, 27)} = 14.7$ ;  $P = .0007$ ), and a TBI  $\times$  peripheral injury interaction ( $F_{(1, 27)} = 5.3$ ;  $P = .0294$ ) on serum NfL levels at 2-days post-injury (Figure 1). Post-hoc analysis revealed that when compared to triple-sham rats, serum NfL concentrations were higher in rats with peripheral injuries-only ( $P = .001$ ), TBI-only ( $P < .0001$ ), and polytrauma (TBI + peripheral injuries;  $P < .0001$ ). When compared to peripheral injury-only rats, serum NfL levels were higher in TBI-only ( $P < .0001$ ) and polytrauma rats ( $P < .0001$ ). No differences were found between TBI-only and polytrauma rats ( $P = .692$ ).

**Table 1.** Body weight and apnea.

	TRIPLE-SHAM	PERIPHERAL INJURIES	TBI-ONLY	POLYTRAUMA	P-VALUE
Weight (g)	252.9 ± 14.1	247.1 ± 4.3	258.8 ± 7.8	249.3 ± 5.1	TBI: $P = .63$ ; peripheral injury: $P = .37$ ; interaction: $P = .82$
Apnea (s)	0 ± 0	0 ± 0	56.3 ± 3.3	76.5 ± 27.8	TBI-only vs polytrauma: $P = .32$

Mean ± SEM.



**Figure 1.** Serum NfL levels at 2-days after combinations of trauma. (a) NfL concentrations were increased by the presence of peripheral injury alone ( $\alpha$ =different to sham injuries-only,  $P = .001$ ). Rats with TBI-only and polytrauma had higher serum NfL levels than rats with sham or peripheral injuries-only ( $\beta$ =different to both sham and peripheral injuries,  $P < .0001$ ). No differences were observed between rats with TBI-only and polytrauma. Individual data points are presented, with lines indicating median group concentrations. Note that serum NfL levels were natural log transformed for statistical analysis. (b) Sensitivity and specificity of serum NfL. NfL was able to correctly identify sham from peripheral injury with (AUC=0.95,  $P < .01$ ), sham from TBI (AUC=1.00,  $P < .01$ ), sham from polytrauma (AUC=1.00,  $P < .01$ ), peripheral from TBI (AUC=1.00,  $P < .001$ ), peripheral from polytrauma (AUC=1.00,  $P < .001$ ), but not TBI from polytrauma (AUC=0.64,  $P = .35$ ).

### Sensitivity and specificity of serum NfL

AUROC analysis was used to compare the performance of serum NfL for distinguishing between sham, peripheral injury, TBI and polytrauma rats at 2-days post-injury. As shown in Figure 1, NfL was able to accurately distinguish sham from peripheral injury with (AUC=0.95,  $P < .01$ ), sham from TBI (AUC=1.00,  $P < .01$ ), sham from polytrauma (AUC=1.00,  $P < .01$ ), peripheral from TBI (AUC=1.00,  $P < .001$ ), peripheral from polytrauma (AUC=1.00,  $P < .001$ ), but not TBI from polytrauma (AUC=0.64,  $P = .35$ ). For injury group comparisons featuring overlap between serum NfL levels (ie, AUC < 1.00), an un-transformed cut-off of 125.4 pg/ml for sham versus polytrauma had a sensitivity of 1.00 and specificity of 0.86, and a cut-off of 2657 pg/ml for TBI versus polytrauma gave a sensitivity of 0.75 and specificity of 0.50. For sham versus peripheral injury the positive predictive value was 1.0 and negative predictive value 0.86, and TBI versus polytrauma the positive predictive value was 0.58 and negative predictive value 0.75.

### Discussion

Fluid-based biomarkers have emerged as candidates to assist in the clinical care of patients with suspected TBI. As specificity for TBI is important to biomarker utility, it is essential that the contributions of extracranial factors such as musculoskeletal

trauma are understood. This study found no difference in serum NfL levels between TBI-only and polytrauma rats, suggesting that the presence of significant peripheral injuries does not affect the utility of serum NfL as a biomarker of moderate TBI. This finding is congruent with what has been observed in humans during the acute period following TBI and concomitant polytrauma.<sup>23</sup>

We also observed elevated serum NfL levels in the peripheral injury-only group when compared to sham-only rats, suggesting that increased serum NfL levels can arise from extracranial sources. Given that NfL is only expressed in the soma and axon of neurons,<sup>18</sup> this finding is likely attributed to peripheral nerve damage. This notion is supported by studies that have reported increased serum NfL levels in patients with peripheral neuropathies<sup>24</sup> and proximal femur fracture.<sup>25</sup> Our finding that peripheral injuries increase serum NfL levels suggests that it may be difficult to delineate a patient with extracranial injury in the absence of TBI from a patient with a milder form of TBI (eg, concussion) that does not result in such a substantial increase of serum NfL. Concerningly, studies in a rat model of mild TBI found that serum NfL levels in the first week post-injury were similar to that observed in the peripheral injury group in the current study.<sup>16,17</sup> As such, our findings indicate that significant extracranial injury may limit the utility of serum NfL as a biomarker of mild TBI in the context of polytrauma.

Aside from acquired TBI, evidence of NfL as a potential prognostic biomarker in other CNS conditions such as multiple sclerosis and Alzheimer's disease has been increasing, where studies have found strong positive correlation between increased levels of serum NfL and progression of neuronal damage in these patients.<sup>26</sup>

One limitation of the current study is the lack of female rats which may provide a more comprehensive overview of how serum NfL may reflect axonal damage following TBI in both sexes. This study used male rats due to the high mortality rate of this injury model and the fact that young adult males are more likely to experience TBI and polytrauma than females.<sup>27</sup> Nonetheless, the lack of female rats in this study prevents understanding of how serum NfL may reflect axonal damage following TBI in both sexes. In addition, future studies are required to determine whether peripheral injuries alter serum levels of other potential TBI biomarkers such as tau, ubiquitin C-terminal hydrolase L1, and glial fibrillary acidic protein in the presence and absence of a moderate TBI.

## Conclusion


Serum NfL levels did not differ between TBI-only rats and TBI rats with polytrauma, indicating that significant peripheral injuries did not affect the sensitivity and specificity of serum NfL as a biomarker of moderate to severe TBI. However, our finding of elevated serum NfL levels in rats with musculoskeletal trauma in the absence of a TBI indicates that the presence of such injuries may limit the utility of NfL as a biomarker of less severe TBI.

## Author Contributions

All authors contributed to the writing of the manuscript. R.D.B., S.R.S., R.M., T.J.O., S.J.M., and K.W., conceptualized and designed the experiment. R.D.B. and K.W. administered the injury methods. M.S., W.T.O., S.J.M., and K.W. completed the NfL quantification.

## ORCID iDs

Ker Rui Wong  <https://orcid.org/0000-0001-7874-0672>

Terence J O'Brien  <https://orcid.org/0000-0002-7198-8621>

Sandy R Shultz  <https://orcid.org/0000-0002-2525-8775>

## Data Availability

The data that support the findings of this study are available from the corresponding author, (R.D.B.), upon reasonable request.

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