




STANDARD ARTICLE

Time course and prognostic value of serum GFAP, pNFH, and S100 β concentrations in dogs with complete spinal cord injury because of intervertebral disc extrusion

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Background: A noninvasive biomarker is needed to predict recovery from severe spinal cord injury (SCI) because of thoracolumbar intervertebral disc extrusion (TL-IVDE). Proteins released from neural and glial cells can be detected in the blood and show promise as prognostic tools, but their concentration is influenced by time after injury.

Hypothesis/Objectives: Serum concentrations of glial fibrillary acidic protein (GFAP), phosphorylated neurofilament heavy chain (pNFH), and S100 β will follow different time courses; measurement of combinations of these proteins will predict outcome.

Animals: Thirty-one dogs with TL-IVDE causing paralysis with no pain perception.

Methods: Prospective study. Serum samples were taken at presentation and intervals over 56 days and banked at -80°C . Glial fibrillary acidic protein, pNFH, and S100 β concentrations were measured using ELISA tests and plotted against time from onset of nonambulatory status. Outcome was established at 6 months. The association between biomarker concentration and outcome was examined using logistic regression, receiver operator characteristics curve analysis, and model development.

Results: Thirty-one dogs participated, 3/31 (10%) developed progressive myelomalacia and 19/31 (62%) recovered ambulation. Glial fibrillary acidic protein and S100 β concentrations rose for the first 1 to 3 days, and were undetectable by 14 and 28 days, respectively. Phosphorylated neurofilament heavy chain concentrations peaked at 14 days and were detectable at 56 days. Glial fibrillary acidic protein concentrations in the first 72 hours after onset of nonambulatory status predicted recovery with an accuracy of 76.7%–89% depending on sample timing.

Conclusions and Clinical Importance: Serum GFAP concentrations can be used to predict outcome in clinically complete SCI. A rapid inexpensive bedside test is needed.

KEYWORDS

biomarker, intervertebral disc disease, myelomalacia, prognosis

Abbreviations: AUC, area under the curve; CNS, central nervous system; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; IVDE, intervertebral disc extrusion; LOCF, last observation carried forward; MRI, magnetic resonance imaging; PMM, progressive myelomalacia; pNFH, phosphorylated neurofilament heavy chain; ROC, receiver operator characteristics; SCI, spinal cord injury; TL-IVDE, thoracolumbar intervertebral disc extrusion.

1 | INTRODUCTION

Injury to the central nervous system (CNS) is associated with unique challenges because of the postmitotic status of neurons and their poor regenerative capacity. This makes quantification of injury severity at the time it occurs important for prognostication. Severe injury can cause neuronal death but injured neurons can go through a period during which they cannot generate and propagate action potentials but subsequently

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recover. Clinically these 2 scenarios appear identical at the time of injury and, as such, quantifying irreversible neuronal loss at the time of presentation in patients with CNS injuries, although extremely important, is extremely challenging. This scenario is illustrated well in dogs with spinal cord injury (SCI) because of acute thoracolumbar intervertebral disc extrusions (TL-IVDE). All dogs with incomplete injuries in which sensory function is preserved have a good prognosis for recovery of motor function and continence.¹⁻⁴ By contrast, dogs with complete injuries (paraplegic with no pain sensation in either hind limb or tail) have a 50%-60% chance of recovery that cannot be reliably predicted from the neurological examination.⁵⁻⁷ Many groups have evaluated different prognostic biomarkers in this group of dogs including quantification of magnetic resonance imaging (MRI) findings,⁸⁻¹⁰ and measurement of inflammatory mediators and neural proteins found in cerebrospinal fluid (CSF).¹¹⁻¹⁵ Although useful, these approaches involve considerable cost (MRI) and are clinically invasive (CSF sampling).

For a biomarker test to have impact clinically, it should be inexpensive, rapid, and noninvasive as well having high sensitivity and specificity.¹⁶ Central nervous system-specific proteins released from dying neurons and glial cells in sufficient quantities to achieve measureable blood concentrations have been studied in human brain injury in great detail.¹⁷ These include neuronal and glial (astrocyte and oligodendroglia) specific proteins for which canine-compatible ELISA tests are now available.¹⁶ Indeed, the presence of glial fibrillary acidic protein (GFAP) in serum might detect progressive myelomalacia (PMM).¹⁸ However, the ability to predict outcome in less extreme scenarios than PMM has proven more challenging, with sensitivity being sacrificed for specificity, perhaps in part because of dogs presenting at different times after injury, different release rates, and different half-lives of biomarkers.¹⁹

The purpose of this study was to describe the changes in serum concentrations of 3 biomarkers, GFAP, phosphorylated neurofilament heavy chain (pNFH), and S100 β over an 8-week period after severe (sensorimotor complete) SCI and to evaluate the predictive power of combinations of these biomarkers in the first 72 hours after onset of paralysis. We hypothesized that serum concentrations of these biomarkers would follow different time courses and that in dogs that recover motor function they would decrease rapidly to become undetectable, while those that fail to recover would have increasing concentrations because of ongoing neural damage. In addition, we hypothesized that combinations of these biomarkers would allow us to predict the final outcome (ambulatory at 6 months, yes or no) in dogs with complete thoracolumbar SCI with different thresholds for prediction at different times of sampling.

2 | METHODS

2.1 | Animals

Dogs with naturally occurring SCI because of TL-IVDE were used in this prospective descriptive study. Inclusion criteria were: neurologic status of paraplegic with loss of pain perception in both hind limbs and tail; onset of nonambulatory status within 72 hours of presentation to the hospital; diagnosis of TL-IVDE confirmed with MRI or computed tomography; and surgical decompression. Dogs showing signs of PMM were included in the study without surgical treatment if they

were euthanized for this complication at time of presentation and their diagnosis was confirmed at necropsy.

All dogs were managed as routine patients of the neurology service and were discharged to their owners when postoperative pain was managed adequately with oral analgesics and when they were either able to urinate voluntarily or when the owners were able to express their bladders manually. Data collected from the medical record included signalment (age, breed, and sex), duration of nonambulatory status before presentation as reported by the owner (categorized as <24 hours [1 day], 24-47 hours [2 days], or 48-72 hours [3 days]) and site of disc extrusion based on imaging findings. The first 10 dogs were reevaluated on days 14, 28, 56, and 180 at which time a complete physical and neurological examination was performed and they were assessed as ambulatory (able to take 10 consecutive unassisted weight bearing steps²⁰) or nonambulatory when walking on a nonslip surface. The remaining dogs were reevaluated at 14, 42, and 180 days to determine outcome. If owners could not bring their dogs in for any of the rechecks, ambulatory status was established over the telephone. All procedures were performed in accordance with the North Carolina State University Institutional Animal Care and Use Committee.

2.2 | Samples

Blood samples were obtained at the time of presentation to the hospital in all dogs. The first 10 dogs were used to generate pilot data to allow selection of optimal time points for sampling a larger group of dogs. In these first 10 dogs, sampling was repeated daily for 4 days and then on days 14, 28, and 56. Based on the results of these 10 dogs, in addition to the sample obtained at the time of presentation, blood samples were taken in the remaining dogs for the first 3 days of hospitalization and on day 14. Samples were taken into red top tubes, centrifuged, and serum was removed and banked at -80°C until analysis.

2.3 | ELISA tests

Serum samples were thawed on ice before ELISA analysis and all kits were used following the manufacturer's instructions. For GFAP measurement, a human GFAP ELISA kit (BioVendor, Asheville, North Carolina) was used. Serum was diluted 1:2 or 1:3 with provided diluent and standards ranged from 0.25 to 25 ng/mL. Absorbance was read at 450 and 630 nm on a VersaMax plate reader (Molecular Devices, Sunnyvale, California). For S100 β , an ELISA detection kit for Canine S100 β Protein from MyBiosource (San Diego, California) was used. Serum samples were diluted 1:2 with PBS. Standards ranged from 0.5 to 10 ng/mL. Absorbance was read at 450 nm on a VersaMax plate reader (Molecular Devices). Serum pNFH was measured with the version 1.2 ELISA kit from EnCor Biotechnology (Gainesville, Florida). Following the manufacturer's instructions, serum samples were diluted 1:2-1:12 in dilution buffer. Standards provided with the kit ranged from 0.012 to 12.5 ng/mL. Absorbance was read at 450 and 630 nm on VersaMax plate reader (Molecular Devices). For all 3 biomarkers, SoftMax Pro 5.0 Software (Molecular Devices) was used to subtract the 630 nm readings from the 450 nm readings (for GFAP and pNFH),

and values from blank wells were subtracted from values of samples and standards (all 3 biomarkers). To generate a curve from the standards, the mean absorbance of duplicate wells was plotted in a logarithmic scale, and a curve was generated by a 4-parameter algorithm, as described in the kit manuals.

3 | STATISTICAL ANALYSIS

Summary data were generated on the signalment, time from onset of nonambulatory status, and location of IVDE. The median and quantiles were calculated for each biomarker at each time point to document the change in their serum concentrations over time. Dogs were then grouped according to ambulatory status at 6 months, and scatter plots were generated for each biomarker to describe the changes in serum concentrations over time between dogs with and without successful recovery of ambulation.

In order to evaluate the ability to predict outcome when a dog first presents to the veterinarian, the relationship between serum concentration of each biomarker at the time of presentation to our hospital and outcome at 6 months was examined using multivariate logistic regression. *P* values <.05 were taken as significant. For biomarkers with a significant relationship with outcome, a receiver operator characteristic (ROC) curve analysis was performed and the sensitivity and specificity of serum biomarker concentrations for prediction of ambulatory status at 6 months was calculated. This allowed comparison with previously published data on these biomarkers in canine SCI.^{18,19} The accuracy of prediction of recovery using cutoffs established in the ROC curve analysis was calculated from the sum of true positive and true negative results divided by the total number of tests performed. This analysis was then repeated using the time from onset of nonambulatory status (categorized as 1, 2, and 3 days) in order to examine the influence of duration of nonambulatory status at time of sampling on the ability to predict outcome. These analyses were performed using JMP-14 (SAS Institute, Cary, North Carolina).

In order to determine whether an algorithm could be developed using combinations of the biomarker concentrations and duration of injury to enhance predictive power further, we performed exploratory modeling of probability of response using a neural network approach^{20,21} with combinations of the biomarker serum concentrations over the first 3 days after presentation to our hospital. We modeled the probability of response as a function of average biomarker concentrations for each biomarker combination using a neural network with a single hidden layer, a sigmoid activation function, and soft-max for predicting class probabilities^{21,22}; the hidden layer had 3 nodes, and was tuned using leave-1-out cross-validation. The R code for the model is provided in Supporting Information Data S1.

4 | RESULTS

Thirty-one dogs participated in the study. There were 15 dachshunds, 6 mixed breeds, 4 cocker spaniels, and 1 each of the following breeds: Corgi, Miniature Poodle, Pomeranian, French Bulldog, Beagle, and Labradoodle. There were 19 female dogs, 2 of which were intact, as

well as 10 castrated male and 2 intact male dogs. Eighteen dogs presented to the hospital within 1 day of onset of nonambulatory status, 8 presented within 1-2 days, and 5 presented within 2-3 days. Two dogs were euthanized on the day of presentation (without surgical decompression) and 1 dog on the following day (surgical decompression was performed) because they developed clinical signs consistent with PMM. These included loss of pelvic limb reflexes in combination with a caudal border of the cutaneous trunci reflex more than 2 vertebral levels above the level of their IVDE. These dogs had serum samples taken at presentation (all 3 dogs) and the following day (1 dog). The diagnosis was confirmed with histopathology in all 3 dogs and they were considered to have failed to recover ambulation for statistical analysis. Of the remaining 28 dogs, 15 recovered independent ambulation by 6 weeks and this number increased to 19 by 6 months. One dog was lost to follow-up after 6 weeks, and 2 were euthanized 3 months after injury. At the time of last evaluation, all 3 dogs were paraplegic with no pain perception and so they were considered to have failed to recover ambulation for statistical analysis, using the last observation carried forward (LOCF) convention. The clinical information of dogs that did and did not recover walking by 6 months is provided in Table 1.

4.1 | Time course of serum biomarker concentrations

Data from the initial 10 dogs demonstrated that serum concentrations of markers of astrocytic injury (GFAP and S100 β) followed a different time course to pNFH, the marker of axonal injury. Glial fibrillary acidic protein and S100 β were detected at extremely low concentrations, if at all, and were only measureable for the first 4 days after presentation, corresponding to up to 7 days after onset of nonambulatory status. By contrast, pNFH increased to a maximum at 14 days and was still measureable at 28 and 56 days. Sampling for the remaining dogs was therefore performed at time of presentation and on the first 3 days after presentation as well as on day 14. The median and quantiles of each serum biomarker for the whole case cohort is provided in Table 2.

4.2 | Relationship of biomarker concentrations to recovery

Scatter plots for each biomarker with dogs categorized as ambulatory at 6 months (yes or no) are provided in Figure 1. These data demonstrate that serum GFAP was undetectable in the majority of dogs that recovered, while concentrations peaked on day 3 after onset of nonambulatory status in dogs that did not recover, and were not detectable by 14 days. By contrast, S100 β was detectable at low concentrations (<4 ng/mL) in the majority of dogs over the first 14 days after onset of nonambulatory status, regardless of outcome. The pNFH serum concentration increased from the first day after onset of nonambulatory status to peak at 14 days, but it is notable that in dogs that did not recover, there was a dramatic increase in serum concentration between 3 and 14 days whereas in dogs that did recover, this increase was not as dramatic (Figure 1). The 3 dogs that had the worst outcome, developing PMM, had extremely high GFAP concentrations at time of

TABLE 1 Cohort characteristics of dogs with thoracolumbar intervertebral disc extrusion (TL-IVDE) categorized as walking or unable to walk at 6 months

Characteristic		Walking at 6 m (n = 19)	Unable to walk at 6 m (n = 12)
Breed	Dachshund:	9	6
	Mix breed:	5	2
	Cocker Spaniel:	2	2 ^a
	Beagle:	1	1 ^a
	Labradoodle:	1	1
	Poodle:	1	
Age y: median (range)		4 (3-11)	3.75 (2-11)
Sex	F	1	1
	FS	9	8
	M	7	0
	MC	2	3
Onset	<24 h	12	6
	24-47 h	4	4
	48-72 h	3	2
Site	T11/12	2	1
	T12/13	4	2
	T13/L1	2	1
	L1/2	4	2
	L2/3	4	4
	L3/4	2	1
	L4/5	1	1

Abbreviations: h, hour; L, lumbar; m, month; T, thoracic; y, year.

^a Developed progressive myelomalacia (PMM).

presentation, at 7.63, 11.66, and 37.77 ng/mL. All 3 dogs also had measureable concentrations of pNFH (1.65, 4.89, and 60.4 ng/mL) and S100 β (0.036, 0.39, and 1.99 ng/mL).

The association between serum concentrations of each biomarker at the time of presentation to the hospital (which ranged from 1 to 3 days after onset of nonambulatory status) with the outcome (ambulatory yes or no at 6 months) was investigated first using multivariate logistic regression to examine the utility of a predictive test performed at presentation. Glial fibrillary acidic protein serum concentration, but not pNFH or S100 β , showed a significant association with outcome (Table 3). The importance of the timing of sampling was examined by repeating the analysis on each of the first 3 days after onset of nonambulatory status. There were significant differences in GFAP concentrations between those that did and did not recover on each of the first 3 days after onset of paralysis but not in pNFH or S100 β

(Table 3). Receiver operator characteristic curve analysis of GFAP serum concentrations had a high area under the curve, sensitivity, and specificity with an accuracy at predicting ambulatory status of >76% (Table 4), with the highest accuracy at day 3 after onset of nonambulatory status.

Preliminary exploratory analyses did not identify any benefit of combinations of the biomarkers over GFAP alone when evaluating a single time point. In order to take advantage of the diverging biomarker serum concentration profiles with time in dogs that do versus dogs that do not recover motor function, we extended the analysis to take the average of the biomarker concentrations over the first 3 days after presentation. Response status (walking at 6 months) was modeled as a function of log average of GFAP and pNFH, in which the averages were taken of the 3 samples obtained over the 72 hours from presentation. Dogs with low values of this combination of biomarkers are more likely to be responders relative to those with large values of 1 or more of these biomarkers. Figure 2 shows the estimated probability of response with the observed data overlaid; as anticipated, dogs with lower values of biomarkers have a higher estimated probability of response, with the combination of average GFAP and average pNFH giving the best prediction. The leave-1-out cross-validation error for this model is 0.133 (an accuracy of 87%) and the in-sample error is 0 (outcome was correctly predicted in all cases); therefore, a ROC curve analysis provides no further information.

5 | DISCUSSION

This study examined the change in serum concentrations of GFAP, pNFH, and S100 β over an 8-week period after clinically complete SCI because of TL-IVDE. We found that the glial biomarker, GFAP, increased over the first 3 days after onset of nonambulatory status and then decreased and was largely undetectable by the 4th day. By contrast, pNFH, a marker of axonal injury, peaked at 14 days and could still be detected at 56 days and S100 β was present at low concentrations for 14 days. When comparing the biomarkers, serum concentrations of GFAP had the highest accuracy for predicting long-term recovery at a single time point in this group of severely injured dogs, whereas S100 β was not useful, and pNFH had limited utility. Moreover, detecting GFAP in the serum at the time of presentation predicted outcome better than assessment of pain perception alone.

Dogs with clinically complete SCI (paraplegic with no pain perception) were chosen for this study because of their extremely variable recovery and the difficulty in predicting that recovery at the time of presentation.

TABLE 2 Median values and range for serum concentrations of each biomarker over 56 days after onset of nonambulatory status

Biomarker Conc (ng/mL) median (range)	Day 1 N = 18	Day 2 N = 18	Day 3 N = 28	Day 4 N = 19	Day 14 N = 27	Day 28 N = 9	Day 56 N = 9
GFAP	0 (0-13)	0 (0-4.6)	0 (0-37.8)	0 (0-39.5)	0 (0-0.3)	0 (0-0)	0 (0-0)
S100b	1.05 (0-3.7)	0.03 (0-3)	1.3 (0-4.4)	0 (0-3.6)	1.6 (0-3.3)	0 (0-0)	0 (0-0)
pNFH	0.6 (0-65)	0.45 (0-5.6)	1.5 (0-90.1)	3.2 (0-150.9)	14.8 (0-119.4)	0.4 (0-10.9)	0.1 (0-0.6)

Abbreviations: Conc, concentration; GFAP, glial fibrillary acidic protein; N, number; pNFH, phosphorylated neurofilament heavy chain.

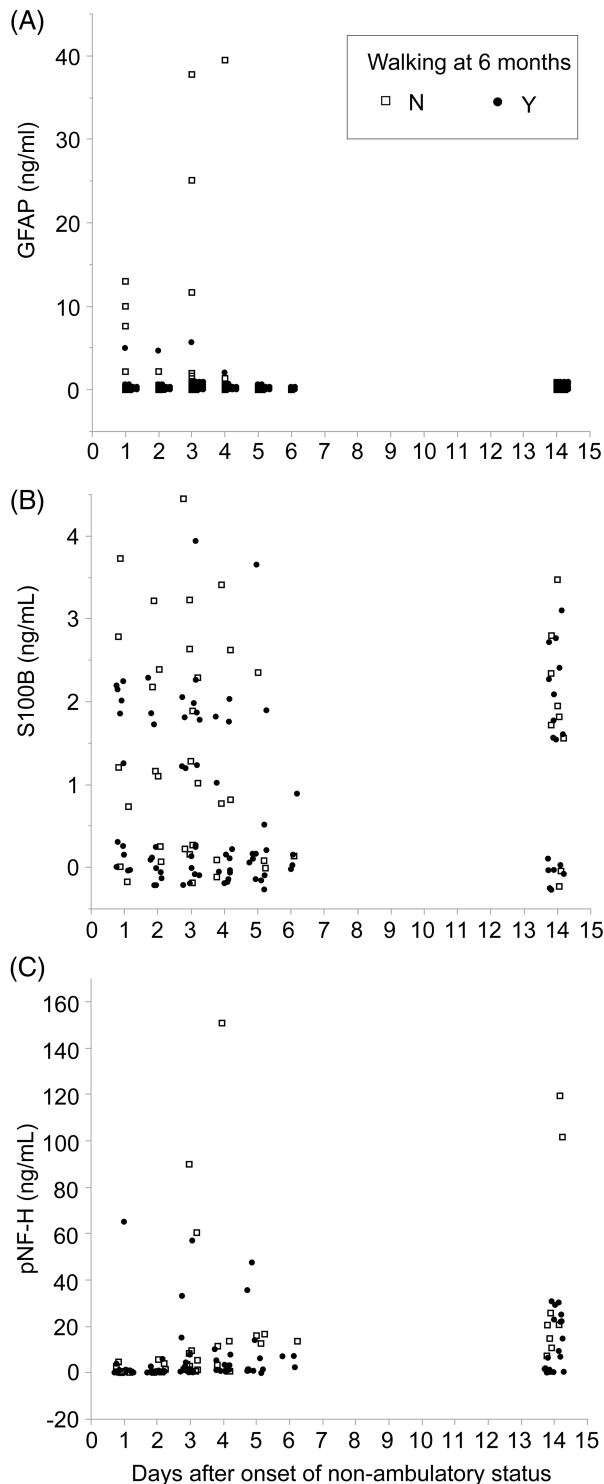


FIGURE 1 Scatter plots of A, GFAP; B, s100 β ; and C, pNFH serum concentrations in 31 dogs over 14 days after onset of nonambulatory status. Dogs are identified according to whether or not they are walking at 6 months, with white squares representing dogs that did not recover and solid black circles representing dogs that did recover. GFAP, glial fibrillary acidic protein; pNFH, phosphorylated neurofilament heavy chain

Among the many different biomarkers of CNS injury, neuron- and glial-derived biomarkers are of particular interest because of their specificity to the nervous system. Neuron-specific enolase, microtubule-associated protein tau, myelin basic protein, GFAP, neurofilaments, and S100 β have all

been investigated in both brain and SCI in people.^{17,23–26} We selected biomarkers reflecting both glial and axonal integrity, for which canine compatible ELISA tests were commercially available, in order to capture a more complete picture of the cellular processes occurring.

Glial fibrillary acidic protein is an insoluble intermediate filament present in abundance in astrocytes. It is released after injury as soluble fragments that can be detected in CSF and serum.^{18,25} There has been extensive work on CSF and serum GFAP (and its breakdown products) concentrations in human traumatic brain injury, and there is a clear association between serum concentrations and death.^{17,27,28} In human SCI, GFAP CSF concentrations show promise as a prognostic tool but there are limited data on serum concentrations.^{25,29–31} A study in spinal cord injured rats reported changes in a variety of biomarkers including GFAP in CSF and serum over a period of 7 days.³² They reported peak serum concentrations between 4 and 24 hours (depending on injury severity) and a return to normal levels within 7 days along with a direct relationship with injury severity. Importantly, a previous study in dogs reported that presence of GFAP in the serum at time of presentation had a high sensitivity and specificity at identifying PMM.¹⁸ In the current study, although GFAP serum concentrations in dogs with PMM were high, there were only 4 observations from 3 dogs with this condition, and so a statistical analysis was not attempted. Importantly, presence of GFAP in the serum occurred in many dogs that did not develop PMM and so quantifying the GFAP concentration will likely be important. Glial fibrillary acidic protein was only detectable for 3–4 days after injury, likely reflecting the time course of recovery of membrane integrity or death of astrocytes. Moreover, there was clear differentiation between dogs that did and did not recover motor function with the vast majority of dogs that recovered having undetectable GFAP concentrations at all time points. Not surprisingly, GFAP proved to be a good predictor of long-term motor recovery with an accuracy of >76% when samples were taken at any point in the first 3 days after onset of nonambulatory status and we propose that measurement of GFAP in dogs with severe SCI would be a useful addition to the clinical evaluation. The ELISA tests used required batching and were time and resource intensive. Development of a rapid bedside test that measures GFAP concentrations would be valuable to the veterinary community.

Neurofilaments are structural proteins found specifically in neurons. There are 3 different subunits, light, medium, and heavy chain, and of these, the light chain (NFL) and pNFH have been studied as biomarkers in human SCI.^{30,33–35} A meticulous study in rats established pNFH as a useful biomarker of axonal injury³⁶ and evaluated the time course of release after spinal cord transection and contusion. They showed serum concentrations peaked rapidly around 16 hours after injury, and then a second broader peak occurred on the 3rd day with a decrease to normal levels by 2 weeks. By contrast, in humans with SCI, serum pNFH concentrations remained elevated for 21 days, and here we show that they are still elevated (albeit at low concentrations) 8 weeks after injury in dogs with the most severe injuries. A previous study examined serum pNFH concentrations in dogs with IVDE causing paraplegia with or without pain perception and found a significant association of pNFH concentrations at the time of presentation with a failure to recover ambulation.¹⁹ A sensitivity and specificity of 34.8 and 100%, respectively, was reported when using a cutoff of 1590 pg/mL during the first 3 days of onset of paralysis. By

TABLE 3 Serum concentrations of GFAP, pNFH, and S100β expressed as median and range in 31 dogs categorized as walking at 6 months, yes or no and grouped as time of presentation as well as time of sampling after onset of nonambulatory status (1, 2, or 3 days). The number of samples analyzed at each time point is listed beneath the range

Time	GFAP (ng/mL)			pNFH (ng/mL)			S100β (ng/mL)			
	Walking at 6 months Yes (n = 19) or No (n = 12)									
	Yes	No	P value	Yes	No	P value	Yes	No	P value	
At presentation	0 (0-5) n = 19	2.2 (0-37.8) n = 12	.0005	0.5 (0-65) n = 19	0.4 (0.04-60.4) n = 12	.3	1.2 (0-3.9) n = 19	1.3 (0-3.7) n = 12	.73	
Time from onset of nonambulatory status (d)	1	0 (0-5) n = 12	4.9 (0-13) n = 6	.005	0.7 (0-65) n = 12	0.6 (0-4.9) n = 6	.4	1.3 (0-2.2) n = 12	1.1 (0.03-3.7) n = 6	.28
		2	0 (0-4.6) n = 13	0.2 (0-2.2) n = 5	.011	0.5 (0-5.6) n = 13	0.5 (0.2-5.5) n = 5	.57	0 (0-2.5) n = 13	1.3 (0-3) n = 5
	3	0 (0-5.6) n = 18	1.3 (0-37.8) n = 10	.004	0.8 (0-56.7) n = 18	3.7 (0.6-90.1) n = 10	.34	1.4 (0-3.9) n = 18	1.2 (0-4.4) n = 10	.21

Abbreviations: d, days after onset of nonambulatory status; GFAP, glial fibrillary acidic protein; n, number; pNFH, phosphorylated neurofilament heavy chain.

contrast, in our study, pNFH alone was a poor predictor of recovery in the first 3 days potentially highlighting the importance of sample timing and suggesting that additional work is needed to refine the most appropriate discriminating serum concentration. It was notable that combining pNFH with GFAP over the 3 days after surgery did provide additional predictive information, likely reflecting the divergence of serum concentrations over time in dogs that do and do not recover ambulation.

S100β is a small calcium binding protein that is particularly abundant in, although not specific to, astrocytes. It is released by injured and dying cells and can be detected in the peripheral blood within 3 hours of a brain injury. Its half-life in humans has been estimated at 1-2 hours.³⁷ Although its utility as an indicator of mild traumatic brain injury is contentious because of its release from other tissues, such as fat and chondrocytes,^{17,28,38,39} its predictive power in moderate and severe brain injury has been established.^{37,40} Most human SCI studies have evaluated CSF concentrations of this biomarker,^{29,30} but there are experimental and clinical studies demonstrating an association between S100β serum concentrations and SCI severity.^{41,42} The experimental

study in rats demonstrated peak serum concentrations 3 days after injury with undetectable concentrations by day 6, while the study in humans looked within 24 hours only and could differentiate controls from those with vertebral fracture and presenting severity on the ASIA scale. In our study, we were able to detect S100β at concentrations in the blood comparable to those reported in other species for 6 days after onset of paralysis. Although the lack of differentiation between dogs that did and did not recover motor function suggests it is not a useful prognostic biomarker in this population, measurement of this biomarker at the time of presentation may be worth additional investigation, given the relatively small size of our study.

We looked to enhance the accuracy of our testing by considering combinations of biomarkers but did not find any benefit of biomarker combinations over GFAP alone when looking at a single time point. Given the release of these proteins increases over the first 2 to 3 days (or 14 days, for pNFH) in dogs that do not recover while dogs that recover show no elevation or rapid decrease to normal, taking the average values from the first 3 days after presentation allowed a highly predictive model to be generated. These data suggest that

TABLE 4 Results of ROC curve analysis of GFAP serum concentrations at time of presentation and subdivided based on duration of nonambulatory status

Time period		AUC (95% CI)	GFAP cutoff (ng/mL)	Sensitivity % (95% CI)	Specificity % (95% CI)	Accuracy %
At presentation		0.80 (0.60, 0.93)	0	79 (56.7, 91.5)	73 (43.4, 90.2)	76.7
Time from onset of nonambulatory status	1 day	0.79 (0.55, 0.95)	<2.18	91.7 (66.7, 98.6)	66.7 (30, 91.3)	83.3
	2 days	0.83 (0.67-0.99)	0	83.3 (55.2, 95.3)	83.3 (43.7, 97)	81.3
	3 days	0.86 (0.67-0.96)	<0.63	94.4 (67.2, 97)	80 (49-94.3)	89

Abbreviations: . AUC, area under curve; GFAP, glial fibrillary acidic protein; ROC, receiver operator characteristic.

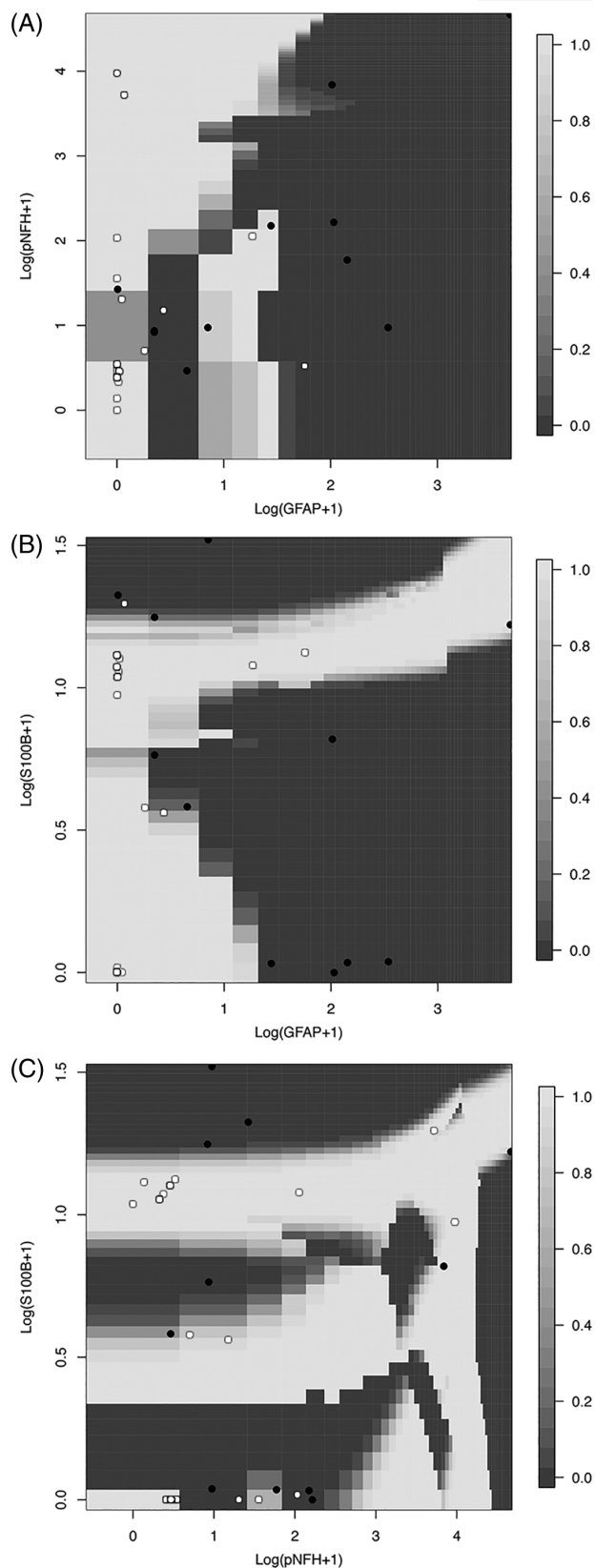


FIGURE 2 The estimated probability of response (ambulatory yes or no) at 6 months with the observed data for A, log average of GFAP and pNFH; B, log average of GFAP and S100 β ; and C, log average of pNFH and S100 β overlaid. The heat map scale of probability of response ranges from 0, indicating recovery of walking, to 1, indicating no recovery of walking. White squares are dogs that did not recover; black circles are dogs that did recover. GFAP, glial fibrillary acidic protein; pNFH, phosphorylated neurofilament heavy chain

combining sequential measurements over a 3-day period, while not helpful for decision making at time of presentation, could help with early decisions on postoperative care, and as new subacute phase therapies such as cellular transplants⁴³ are developed, could allow early initiation of these therapies. However, it is important to note that our findings including the findings of the neural network analysis need to be replicated in a separate population of dogs, distinct from those used to build the model. The data generated in this study could be used to determine the appropriate number of samples at each time point to perform a well powered future study.

It was noticeable that the biomarkers chosen for their glial specificity showed a short-term increase and then rapidly decreased again, suggesting that there was early release by cells around the time of injury, but that this phase ended within a few days as astrocytes either recovered cellular membrane integrity or died and were removed. By contrast, the serum concentrations of pNFH peaked at 14 days and were still detectable 8 weeks after injury in some cases, likely reflecting ongoing Wallerian degeneration in white matter.³⁴ It was also noticeable that some dogs that recovered had rapid resolution of their serum pNFH concentrations, suggesting that conduction block made a large contribution to the severity of their presenting clinical signs. These serial measurements have been made in dogs that underwent surgical decompression and it would be interesting to make the same measurements in dogs managed conservatively. However, very few paraplegic pain perception negative dogs referred to a specialty practice are managed conservatively. The fluctuations of these biomarkers have lent insight into the pathophysiological events occurring within the spinal cord of dogs with severe SCI because of TL-IVDE that are treated by surgical decompression.

This study was limited by a small sample size and needs to be replicated in a larger cohort. One of the aims was to determine the influence of timing of sampling on the predictive accuracy of the biomarkers, but the limited number of cases meant that time of sampling had to be categorized by days rather than smaller fractions; examining the effect of timing on the utility of these biomarkers at 12 hour intervals rather than 24 hour intervals would be important in a larger case population. Allocating duration to the injury is also always challenged by relying on client observations, and the difficulty in determining when the timing of injury should start. For example, it could be argued that injury onset occurs at the first sign of back pain or hind limb abnormalities. However, given the potential for these dogs to have long and poorly definable onsets, we chose to define onset as the time that dogs were recognized as nonambulatory by their owners at which time it is clear that there has been a significant SCI. The timing of collection of samples on subsequent days is also a weakness because of challenges in collecting samples reliably from clinical cases out of hours and because some owners elected to take their dogs home soon after surgery. This resulted in uneven numbers of samples at each time point. We also considered a successful outcome as motor recovery, without considering recovery of pain perception. This decision was made based on published data that suggest dogs that do not recover pain perception but do recover ambulation have less severe injuries than those who do not.^{44,45} In addition, owners of these pain perception negative walking dogs define their outcome as successful. Finally, 3 dogs were euthanized before the 6-month end point. Given their lack of motor or sensory recovery, they were

treated using the LOCF convention as having failed to recover but it is possible that they could have recovered independent walking.

In conclusion, serial measurement of serum GFAP, pNFH, and S100 β supports rapid resolution of glial membrane integrity but prolonged axonal degeneration. The presence of serum GFAP in the first 3 days after onset of paralysis can predict motor recovery in dogs with complete paralysis because of acute IVDE with good, although not perfect, accuracy. In addition, taking the average value of serum concentrations of a combination of GFAP and pNFH over the first 3 days after hospitalization might provide additional information to predict outcome and could be used to target postsurgical treatment most appropriately. Development of a bedside test that measures serum concentrations of both GFAP and possibly pNFH would be clinically useful.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All procedures were approved by the North Carolina State University Animal Care and use Committee.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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