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Longitudinal plasma biomarker profiles predict neurological outcome in traumatic spinal cord injury

Running title: Plasma NfL, GFAP, and CNTN-1 in SCI

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Summary for Social Media if Published

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What is the current knowledge on the topic?
Traumatic spinal cord injury (SCI) is diagnosed by imaging and clinical scoring using the American Spinal Injury Association Impairment Scale (AIS). These methods have limited value for prognosis.

What question did this study address?
In this study, the prognostic value of plasma neurofilament-light (NfL), glial fibrillary acidic protein (GFAP) and contactin-1 (CNTN-1) was analysed longitudinally up to 18 weeks post-injury.

What does this study add to our knowledge?
Plasma NfL, GFAP and CNTN-1 levels were shown to correlate with and predict neurological outcome over a long time period up to 18 weeks post-SCI, with the highest predictive ability for clinical outcome when analyzed together. This highlights the usefulness of NfL, GFAP and CNTN-1 as prognostic biomarkers for SCI and that their measurement does not have to be performed in strict time-windows.

How might this potentially impact on the practice of neurology?
These plasma biomarkers are a promising tool supporting informed decision-making during initial treatment and rehabilitation as well as patient stratification in clinical trials, reducing sample size and maximizing therapeutic efficacy.

Abstract

Objective: Traumatic spinal cord injury (SCI) is diagnosed by imaging and clinical scoring using the American Spinal Injury Association Impairment Scale (AIS). These methods have limited value for prognosis. Here, the prognostic value of plasma neurofilament-light (NfL), glial fibrillary acidic protein (GFAP) and contactin-1 (CNTN-1) was analysed.

Methods: Biomarker levels were determined in the plasma of traumatic SCI patients (*n*=37) and healthy controls (*n*=22 ~~each~~). SCI samples (*n*=~~70~~112) were collected at different time points from 0-4 days to 18 weeks post-injury. NfL and GFAP were measured by Simoa technology, CNTN-1 by Luminex. Baseline and outcome AIS and motor scores were collected as a measure of injury severity.

Results: NfL, GFAP and CNTN-1 showed different kinetics in SCI patients over time. Baseline biomarker levels could identify AIS-A SCI patients (NfL+GFAP) and discriminate between patients with a motor score change <5 and those with a change ≥5 (NfL+GFAP+CNTN-1). Longitudinally, NfL could identify AIS-A patients up to 12 weeks post-SCI and discriminate between patients with a motor score change <5 and those with a change ≥5 up to 18 weeks post-SCI. Further, baseline biomarker levels positively (NfL+GFAP) or negatively (CNTN-1) correlated with outcome injury severity and together could accurately predict AIS conversion (AUC 0.863) and motor score change (AUC 0.857). This predictive ability was maintained in subacute/chronic SCI stages.~~NfL and GFAP levels at baseline and NfL levels at 3 weeks post-SCI were significantly higher in outcome motor-complete versus motor-incomplete SCI patients. NfL levels were even significantly higher up to 12 weeks post-injury in SCI patients with a motor score change < 5 compared to SCI patients with a motor score change ≥ 5. Baseline biomarker levels positively (NfL+GFAP) or negatively (CNTN-1) correlated with outcome injury severity and could accurately predict AIS conversion (AUC: NfL 0.788, GFAP 0.800, CNTN-1 0.800, NfL+GFAP+CNTN-1 0.813) and motor score change (AUC: NfL 0.844, GFAP 0.867, CNTN-1 0.822, NfL+GFAP+CNTN-1 0.800).~~

Interpretation: In conclusion, plasma NfL, GFAP and CNTN-1 are potential prognostic biomarkers in SCI. This is important for patient stratification in clinical trials, prediction of neurological outcome and informed decision-making in SCI treatment and rehabilitation.

Keywords: spinal cord injury; neurofilament-light; glial fibrillary acidic protein; contactin-1; clinical outcome

Introduction

Traumatic spinal cord injury (SCI) results from damage to the spinal cord, leading to temporary or permanent motor and sensory loss.[1] Diagnosis involves the evaluation of neurological symptoms and imaging of the spinal cord. MRI is used in the acute phase to evaluate spinal cord compression, transection, oedema and haemorrhage.[2] The International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) including the American Spinal Injury Association (ASIA) Impairment Scale (AIS) determine injury severity using myotomal-based motor examination, dermatomal-based sensory examination and anorectal examination.[3] The AIS includes complete injuries (A), sensory incomplete injuries (B), motor incomplete injuries with less than half (C) or at least half (D) of key muscles below the neurological level having a muscle grade greater than or equal to 3, and normal functioning (E).[3,4] However, MRI and AIS have limited value as prognostic tools and are not always accessible, feasible or reliable during the acute phase, especially in unstable patients.[5] Additionally, AIS artificially reduces the variability of the heterogeneous SCI patient population.[5,6] Protein biomarkers have been studied for their potential to determine neuronal injury but these have limited clinical applicability due to their restriction to the CSF.[7-10]

Neurofilament-light chain (NfL) and glial fibrillary acidic protein (GFAP) are structural proteins in neurons and astrocytes, respectively, that are released into the blood following neuronal damage.[11-13] They have already been described as biomarkers of disease activity in several neurological disorders.[11,12] In SCI, GFAP and/or NfL serum levels have been examined in the acute phase (1-4 days post-injury) up to 7 days post-injury.[14-16] Baseline levels correlated with AIS grade and could predict neurological outcome. However, to accurately assess the prognostic value of NfL and GFAP, determining their long-term temporal profile is crucial. Understanding the time frame in which these markers provide prognostic information is highly necessary in case of delays in diagnosis[17,18] or difficulties in collecting or storing blood samples during the acute SCI stage. Contactin-1 (CNTN-1) is an axonal protein involved in CNS myelin formation.[19] Reduced CSF and serum CNTN-1 levels correlated with disease and disability progression in multiple sclerosis patients[20,21] and were also described in patients with chronic inflammatory demyelinating polyneuropathy.[22] To comprehensively capture processes of neuronal, astrocytic and myelin damage following traumatic SCI, we determined longitudinal plasma levels of NfL, GFAP and CNTN-1 up to 18 weeks (w) post-injury and evaluated the potential of these biomarkers to predict neurological outcome.

Materials and methods

Sample collection

Traumatic SCI patients were recruited in Belgium at Ziekenhuis Oost-Limburg (Genk), University Hospitals Leuven (Leuven), Algemeen Ziekenhuis (AZ) Turnhout (Turnhout), Jessa Hospital (Hasselt), Antwerp University Hospital (Antwerp) and in The Netherlands at Adelante Centre of Expertise in Rehabilitation (Hoensbroek) and Maastricht UMC+ (Maastricht). Healthy controls (HC) were recruited at Hasselt University (Hasselt, Belgium). The study was approved by the institutional Medical Ethics Committees. Written informed consent was obtained from all participants according to the Declaration of Helsinki.

In total, 112~~70~~ plasma samples were collected from traumatic SCI patients (*n*=372~~2~~, Table 1, Supplementary Table 1) at different time points post-injury: 0w (0-4 days, *n*=372~~2~~), 3w (*n*=231~~6~~), 6w (*n*=191~~2~~), 12w (*n*=181~~1~~) and 18w (*n*=159). Missing time points were due to organizational issues or patient withdrawal. Injury severity was determined by the AIS at baseline (0w, available for 37/372~~2/22~~ patients) and outcome (latest available measurement before September 2023~~2~~, available for 34/371~~8/22~~ patients). Individual motor scores were also collected at baseline (0w, available for 27/371~~4/22~~ patients) and outcome (available for 29/371~~5/22~~ patients). Cross-sectional HC samples (*n*=22) were matched to SCI patients as closely as possible regarding age and sex (Table 1). Peripheral blood was collected in lithium heparin tubes and centrifuged at 400g for 10 minutes. Plasma was collected, centrifuged at 1500g for 10 minutes, aliquoted, and stored at -80°C in the University Biobank Limburg.

NfL, GFAP and CNTN-1 measurements

NfL and GFAP were measured using single molecule array (Simoa, Quanterix, MA, USA) with the commercial NF-Light advantage Kit and GFAP Discovery Kit on an HD-X platform according to the manufacturer's instructions. CNTN-1 analysis was done using the Human contactin-1 Magnetic Luminex Assay (R&D Systems, Minneapolis, USA) on a Bio-Plex 200 system (Bio-Rad Laboratories, Veenendaal, The Netherlands), following the manufacturer's instructions. Use of plasma samples was in-house validated according to Andreasson et al.[23] Bridging samples (*n*=20) were included to control for batch effects. Mean intra-assay and inter-assay coefficients of variation were 3.7% and 8.5% for NfL, 4.4% and 8.4% for GFAP and 2.5% and 2.0% for CNTN-1, respectively. All measurements were performed at the

Neurochemistry laboratory of the Amsterdam UMC location VUmc using standard operating procedures.[24] Samples were randomized and analyzed in duplicate, blinded to disease status or clinical information.

Statistical analysis

Data analysis was done using Prism software version 9.4 (GraphPad Software, San Diego, CA, USA) and JMP Pro 17.0.0 software (SAS Institute, Cary, NC, USA). Baseline differences in biomarker levels between HC and the total SCI cohort or between subgroups of SCI patients were tested with Mann-Whitney U test or ANOVA followed by Tukey's post-hoc multiple comparisons test, respectively. Associations between NfL, GFAP or CNTN-1 levels and AIS scores, motor scores or age were explored by means of Spearman correlation tests.

For the total SCI cohort, changes in biomarker levels were examined utilizing a linear mixed model. This model considers the correlation between measurements within the same patient and yields valid inferences even in the presence of missing data, assuming they are missing at random (MAR). Age at baseline and time point were incorporated as fixed effects, and patient as a random effect. Based on this model, all pairwise differences between time points were estimated.

Differences in biomarker levels among subgroups of SCI patients (subgroup based on injury severities: separate AIS classes or motor-complete versus motor-incomplete~~AIS-A/B versus AIS-C/D/E~~, subgroup based on disease outcome: AIS conversion yes versus no, subgroup based on motor score change: < 5 versus ≥ 5) were analyzed using linear mixed models. Time point, an indicator for the subgroup, and their interaction were included as fixed effects, and the patient as a random effect. Based on this model, a comparison between subgroups was conducted at each time point.

To render a normal distribution of residuals in the described mixed effects models, biomarker levels were log-transformed. Given the exploratory nature of the study and to minimize the type II error, the reported *P*-values for the pairwise comparisons have not undergone correction for multiple testing.

Logistic regression was used to assess the predictive value, represented as odds ratio (OR) and 95% confidence interval (CI), of individual and combined biomarker levels for AIS conversion and motor score change using likelihood ratio tests. Receiver operating characteristics (ROC) curves were created, and estimates of the area under these curves (AUC) were obtained. Cross-

validation was not performed due to the limited number of observations. A $P<0.05$ was considered significant.

Data Sharing Statement

Study data are available from the corresponding author, upon reasonable request, subject to institutional agreements and ethical approvals.

Results

Plasma NfL, GFAP and CNTN-1 show different kinetics in SCI patients over time

NfL, GFAP and CNTN-1 were measured in the plasma of traumatic SCI patients (n=3722) at five time points (0w, 3w, 6w, 12w, 18w post-SCI) and HC (n=22). At baseline (0w), SCI patients had higher NfL (11.215x) and GFAP (95.5118x) levels compared to HC ($P<0.0001$), while CNTN-1 levels were decreased in SCI patients (1.67x, $P<0.0001$) (Fig. 1A). Over time, NfL levels in SCI patients peaked at 3w and gradually declined thereafter (0w-3w/6w, 3w-12w/18w, 6w-18w: $P<0.0001$; 0w-6w: P=0.044; 3w-12w: P=0.0002; 3w-18w: P=0.00026w-12w: P=0.0002, 12w-18w: P=0.0053) (Fig. 1A). In contrast, GFAP levels peaked at baseline, decreased at 3w ($P<0.0001$) and then gradually returned to HC levels ($P<0.0001$, all time points vs baseline). CNTN-1 levels gradually increased after baseline to HC levels (0w-6w/12w/18w, 3w-12w: $P<0.0001$; 3w-12w6w: P=0.00081; 30w-18w: P=0.00312). Similar findings were observed in terms of days post-injury (Supplementary Fig. 1). Biomarker levels were similar in SCI patients with cervical or thoracic lesions (data not shown). NfL and GFAP levels significantly correlated with age in HC but not in SCI patients, while CNTN-1 levels did not correlate with age (Fig. 1B).

Plasma NfL, GFAP and CNTN-1 levels correlate with neurological outcome

Next, NfL, GFAP and CNTN-1 plasma levels were correlated with disease outcome (AIS) at the end of the clinical follow-up. Compared with HC, baseline plasma NfL and GFAP levels

were increased in AIS-A (44x and 296x, respectively, both $P<0.0001$), AIS-B (6x, $P<0.01$ and 128x, $P<0.0001$, respectively), AIS-C (8x and 17x, respectively, both $P<0.01$), AIS-D (4x, $P<0.05$ and 21x, $P<0.001$, respectively) and AIS-E (GFAP only, 30x, $P<0.01$) SCI patients (Fig. 2A). Within the SCI patient group, baseline NfL and GFAP levels were higher in AIS-A SCI patients compared with AIS-B (NfL only, 7x, $P<0.01$), AIS-C (5x and 17x, respectively, both $P<0.01$), AIS-D (12x, $P<0.01$ and 14x, $P<0.001$, respectively) and AIS-E (19x, $P<0.0001$ and 10x, $P<0.001$, respectively) SCI patients at outcome. For GFAP alone, baseline levels were also increased in AIS-B patients versus AIS-E patients (4x, $P<0.05$). Baseline CNTN-1 levels were decreased in AIS-A (2x, $P<0.0001$), AIS-B (2x, $P<0.001$), AIS-C (1.5x, $P<0.5$) and AIS-E (1.4x, $P<0.05$) SCI patients compared with HC, with only a difference between AIS-A and AIS-D SCI patients (1.7x, $P<0.01$).

When analyzing the different time points, higher NfL levels were demonstrated in AIS-A SCI patients only at baseline compared with AIS-B and AIS-C SCI patients but from baseline up to 12w post-SCI compared with AIS-D and AIS-E SCI patients (Fig. 2B, Supplementary Tables 2-4). Differences in GFAP levels between the AIS classes (A vs C/D/E and B vs E) were found only at baseline, while CNTN-1 levels were lower in AIS-A patients compared with AIS-D/E patients at 3w (D/E) and 6w (D) post-SCI. Importantly, biomarker levels at baseline (NfL, GFAP, CNTN-1), but also at 3w and 6w post-SCI (NfL, CNTN-1) could discriminate between motor-complete and motor-incomplete SCI patients at clinical endpoint (Supplementary Fig. 2). ~~Furthermore~~In addition, NfL and GFAP levels positively correlated with injury severity (AIS) when including all time points and AIS scores at sampling ($P<0.0001$, ~~and $P=0.0061$, respectively,~~ Fig. 2C), but also when including only baseline samples and outcome AIS score (~~$P=0.0014$ and $P=0.0039$~~<0.0001~~, respectively~~, Fig. 2D). Similarly, CNTN-1 levels negatively correlated with clinical outcome ($P<0.0001$ for AIS at sampling and $P=0.0024$ for outcome AIS~~12, respectively~~, Fig. 2C-D). ~~At later time points~~Interestingly, NfL correlated positively with AIS outcome at all follow-up time points~~3w and 6w post-SCI~~, while GFAP remained positively correlated with clinical outcome up to 12w post-SCI (Supplementary Fig. 3). CNTN-1 maintained a negative correlation with ~~clinical~~ AIS outcome until 6~~3~~w post-injury ~~(Supplementary Fig. 2)~~. Correlations between baseline NfL, GFAP and CNTN-1 plasma levels ~~at baseline~~ and individual motor scores at clinical outcome were also considered (Supplementary Fig. 4~~3~~). Here, baseline NfL and GFAP levels showed a significant negative correlation with outcome motor score ($P=0.0$~~296~~002 and $P=0.00$~~66~~03, respectively), indicating that higher NfL and GFAP levels at baseline are correlated with lower outcome motor scores. For CNTN-1, ~~a trend towards~~ a positive correlation between baseline levels and outcome motor

score was indicated (P=0.0027~~529~~). ~~SCI patients were categorized based on disease outcome at clinical endpoint: AIS-A/B (motor-complete SCI) and AIS-C/D/E (motor-incomplete SCI).~~ Biomarker levels were similar in SCI patients with cervical or thoracic lesions within the same AIS class (data not shown). In conclusion, plasma NfL, GFAP and CNTN-1 levels were especially capable of identifying SCI patients with AIS-A or motor-complete outcomes when collected at baseline, while significant correlations with injury severity were maintained during follow-up. ~~Baseline plasma NfL and GFAP levels were increased in AIS-A/B patients compared with HC (31.8x and 216x, respectively, P<0.0001) but also with AIS-C/D/E patients (9.4x and 11.5x, respectively, P<0.0001) (Fig. 2A). In contrast, baseline CNTN-1 levels were decreased in AIS-A/B (2x) and AIS-C/D/E (1.5x) SCI patients compared to HC (P<0.0001), with no difference between the SCI groups (P=0.2167). When analyzing the different time points, higher NfL levels were demonstrated in AIS-A/B patients at baseline (P=0.0085) up to 3w post-SCI (P=0.0002) (Fig. 2B, Supplementary Tables 2-4). GFAP levels were higher in AIS-A/B patients only at baseline (P<0.0001). The longitudinal profile of CNTN-1 could not discriminate between patients who were AIS-A/B and AIS-C/D/E at outcome. Furthermore, NfL and GFAP levels positively correlated with injury severity (AIS) when including all time points and AIS scores at sampling (P<0.0001 and P=0.0061, respectively, Fig. 2C), but also when including only baseline samples and outcome AIS score (P=0.0014 and P=0.0039, respectively, Fig. 2D). Similarly, CNTN-1 levels negatively correlated with clinical outcome (P<0.0001 and P=0.0012, respectively, Fig. 2C-D). At later time points, NfL correlated positively with outcome at 3w and 6w post-SCI, while CNTN-1 maintained a negative correlation with clinical outcome until 3w post-injury (Supplementary Fig. 2). Correlations between NfL, GFAP and CNTN-1 plasma levels at baseline and individual motor scores at clinical outcome were also considered (Supplementary Fig. 3). Here, baseline NfL and GFAP levels showed a significant negative correlation with outcome motor score (P=0.0296 and P=0.0066, respectively), indicating that higher NfL and GFAP levels at baseline are correlated with lower outcome motor scores. For CNTN-1, a trend towards a positive correlation between baseline levels and outcome motor score was indicated (P=0.0529).~~

Plasma NfL, GFAP and CNTN-1 levels predict AIS conversion and motor score change in SCI patients

The predictive ability of the biomarkers for AIS conversion (i.e. change of AIS to a less severe injury grade) was studied in two groups of SCI patients: AIS converters (n=130) and non-converters (n=208) (Fig. 3). As the AIS scoring system cannot fully address the heterogeneity among SCI patients, SCI patients with a change in the AIS motor score of < 5 points (n=145) versus a change of ≥ 5 points (n=159) at outcome were additionally studied (Fig. 4). As expected from the correlations with clinical outcome (Fig. 2C-D), baseline NfL and GFAP levels were significantly higher in AIS non-converters than AIS converters (P = 0.0057 and P = 0.00431, respectively) and HC (P = 0.0003 andboth P < 0.0001), but also in AIS converters compared to HC (P=0.0035 and P<0.0001, respectively) (Fig. 3A). Baseline CNTN-1 levels only significantly differed between HC and both AIS non-converters (P <= 0.0005) and AIS non-converters (P < 0.0001) but were similar in the SCI groups (P = 0.22194521). More significant differences were found between the groups when analyzing clinical improvement using the more sensitive measure of motor score change. Here, baseline levels of all three markers, NfL, GFAP and CNTN-1, significantly differed between patients with a motor score change < and ≥ 5 (P=0.0001, P<0.0001, P=0.010, respectively) (Fig. 4A). Additionally, both SCI patients with a motor score change < 5 and ≥ 5 presented with higher NfL and GFAP and lower CNTN-1 levels compared to HC (NfL: P<0.0001 and P=0.0028, respectively; GFAP: both P<0.0001; CNTN-1: P<0.0001 and P=0.0113, respectively). Similar results were obtained when comparing SCI patients with a motor score change < 5 and ≥ 5 (Fig. 4A), with higher NfL and GFAP and lower CNTN-1 baseline levels in those patients with a motor score change < 5 compared to HC (NfL+GFAP+CNTN-1: P <0.0001) and for NfL and GFAP also compared to SCI patients with a motor score change ≥ 5 (NfL: P = 0.0002, GFAP: P = 0.0004). No differences were observed in NfL, GFAP or CNTN-1 between AIS converters and non-converters at follow-up time points (Fig. 3B, Supplementary Tables 5-7). When considering motor scores, SCI patients with a motor score change < 5 had significantly higher NfL levels at 6 and 12w post-injuryduring the entire follow-up compared to SCI patients with a motor score change ≥ 5 (Fig. 4B, Supplementary Tables 8-10). Furthermore, CNTN-1 levels were significantly lower in SCI patients with a motor score change < 5 at 3 and 6w post-injury than in those patients with a change ≥ 5.

ROC curve analysis (Fig. 3C, Supplementary Fig. 5) revealed that baseline NfL and, GFAP and CNTN-1 could predict AIS conversion with an AUC of 0.696788 (NfL) and 0.800767 (GFAP and CNTN-1). Hereby, higher NfL (OR for 100 unit increase: 2.761.47; 95% CI: 1.102-2.9629.58; *P*=0.031214) and GFAP (OR for 10,000 unit increase: 3.2230; 95% CI: 1.32-178.52; *P*=0.00206) and lower CNTN-1 (OR for 1000 unit decrease: 0.64; 95% CI: 0.32-1.03; *P*=0.071) levels were associated with the absence of AIS conversion. Although the sample size was smallerMore importantly, baseline NfL, GFAP and CNTN-1 levels could also predict motor score change with an AUC of 0.83844 (NfL), 0.83767 (GFAP) and 0.762822 (CNTN-1) (Fig. 4C, Supplementary Fig. 6). Combining all three biomarkers slightly further improved the predictive value for both AIS conversion (AUC 0.863813, *P*=0.0059, Fig. 3C) although this was not the case forand motor score change (AUC 0.85700, *P*=0.0017, Fig. 4C). Interestingly, combining only baseline NfL and GFAP resulted in a similar and even slightly higher predictive ability for motor score change (AUC 0.872, data not shown) but not for AIS conversion (AUC 0.775, data not shown). At later time points, the absence of AIS conversion could not be predicted using any of the biomarkersno significant discrimination was observed between AIS converters and non-converters (Supplementary Fig. 54). However, motor score improvement at clinical endpoint could be predicted using GFAP up to 3w post-SCI, using CNTN-1 up to 6w post-SCI and using NfL even up to 12w post-SCI (Supplementary Fig. 6).

Discussion

In this study, plasma NfL, GFAP and CNTN-1 were assessed longitudinally in SCI patients with different clinical outcomes. This allowed us to determine their long-term kinetic profiles and to evaluate their prognostic ability in a longitudinal fashion. Different kinetics were observed with GFAP reaching peak levels at baseline, NfL at 3w, and CNTN-1 being decreased at baseline. NfL and GFAP at baseline could identify SCI patients with AIS-A class at outcome and could discriminate between SCI patients with a motor-complete and motor-incomplete outcome. ~~NfL and GFAP at baseline discriminated between motor-complete (AIS-A/B) and motor-incomplete (AIS-C/D/E) SCI patients at outcome.~~ Notably, NfL levels remained elevated in outcome AIS-A and motor-complete patients even ~~at 3w~~up to 12w post-SCI, and in SCI patients with a motor score change < 5 even up to ~~12w~~ 18w post-SCI, highlighting its value not only in acute but also in subacute and more chronic phases. Baseline levels of all three markers correlated with clinical outcome (AIS grade and motor score). Moreover, combined analysis of plasma NfL, GFAP and CNTN-1 demonstrated good predictive ability for AIS conversion (AUC 0.863~~≈0.8~~) and motor score change (AUC ~~0.822-0.867~~0.857). In combination with their different temporal profiles, this emphasizes the usefulness of measuring each marker following traumatic SCI.

This study is the first to longitudinally compare these biomarkers in SCI patients over a longer period with longer clinical follow-up. Previous research focused on measuring serum GFAP and/or NfL levels in acute SCI patients within a limited timeframe, ranging from 12h up to 7d post-SCI in one study[14] and from 1-4d post-SCI in two other studies.[15,16] Different technologies have been used for biomarker analysis, including electrochemiluminescence[14,15] and single molecule array.[16] Still, baseline plasma levels of NfL and GFAP measured in our study aligned with these previous analyses in serum. Clinical correlations with AIS motor scores, total AIS grade or AIS conversion at 6 months post-injury have already been reported.[14-16] We now provide the necessary longer follow-up of NfL and GFAP levels up to 4.5 months, examining their association with clinical outcome including a more sensitive measure (motor score improvement) over an extended period of more than one year. This is crucial for understanding the time frame in which these markers provide prognostic information.

GFAP and NfL levels peaked at 0w and 3w post-SCI, respectively. The distinct kinetics of NfL and GFAP over longer periods may be attributed to differences in their cellular expression (GFAP in astrocytes, NfL in neuronal cells), CNS specificity (GFAP) versus enrichment (NfL)

or increase with aging or other physiological or pathological processes. Additionally, NfL has been reported to exhibit a longer half-life (weeks) than GFAP (days).[25,26] GFAP had an estimated half-life of ~~5.17~~6.4d in our study, although additional time points should be considered for more precise estimation. Determining the exact half-life of NfL was not possible in this study.

At baseline, GFAP had the highest predictive ability for ~~both~~ AIS conversion (AUC 0.767~~8~~, narrowest 95% CI). When analyzing ~~and~~ the lack of motor score change, NfL and GFAP had superior predictive ability (AUC 0.8~~4~~67, narrowest 95% CI) over CNTN-1. This was also reflected in the slightly higher predictive value of combined NfL and GFAP analysis, without CNTN-1, for motor score change (AUC 0.872). ~~However~~Moreover, the ~~increase in NfL~~differences in NfL, GFAP and CNTN-1 levels ~~up to 12w~~at follow-up time points between~~in~~ subgroups of SCI patients with different clinical outcome suggested ~~its~~their potential suitability also in subacute and chronic SCI stages, and that their measurement does not have to be performed in strict time-windows. Indeed, NfL levels ~~at 3w~~up to 12w post-SCI could still discriminate between motor-complete and motor-incomplete SCI patients, while NfL levels up to 1~~8~~2w post-injury could discriminate between SCI patients with a motor score change < 5 and those with a motor score change ≥ 5. The usefulness of these markers in subacute and chronic SCI stages was further demonstrated by their predictive ability for the lack of motor score change at follow-up time points, even up to 12w post-SCI for NfL. Thus, all three markers ~~NfL~~ offer~~s~~ an extended prognostic window for predicting neurological outcome. This is particularly valuable when blood samples cannot be collected or stored during the acute SCI stage. Moreover, despite advancements in imaging techniques, delayed SCI diagnosis still occurs, especially in busy trauma centers.[17,18] Our study emphasizes the utility of NfL, GFAP and CNTN-1 measurements for prognosis, even if SCI diagnosis is not made during the acute stage. The association of NfL and GFAP levels with age in HC could indicate elevated neuronal injury with aging. This aligns with previous reports[27-29] where increased NfL levels during aging were linked with brain volume changes[27].

The decrease in CNTN-1 immediately post-SCI corresponded to reduced CSF and serum CNTN-1 levels seen in patients with multiple sclerosis and chronic inflammatory demyelinating neuropathy.[20-22] In these studies, it was hypothesized that decreased CNTN-1 release in body fluids could function as a compensatory mechanism to restore axonal function or that it could reflect reduced axonal density.[20] The latter seems more likely in our SCI cohort as CNTN-1 levels were decreased already at baseline. CNTN-1 could~~did not~~ discriminate

between motor-complete and motor-incomplete SCI patients at clinical endpoint, ~~but~~ negatively correlated with outcome AIS and predicted ~~AIS conversion and~~ motor score change ~~with high discriminating ability (AUC 0.8 and 0.822, respectively),~~ both at baseline and follow-up time points. ~~Furthermore, CNTN-1 levels at baseline up to 6w post-injury discriminated between SCI patients with a motor score change < 5 and those with a motor score change ≥ 5.~~ Thus, CNTN-1 also presents with prognostic biomarker potential in the acute and subacute stage, reflecting damage at the level of axonal function, possibly at the interface of axons with myelin, between axons or at the initial segment of the axons.

A limitation of this study was the limited number of included SCI patients. ~~As a consequence, AIS grades could not be evaluated separately but were combined into a motor-complete (AIS-A/B) and motor-incomplete (AIS-C/D/E) outcome group.~~ Large-cohort~~Future~~ studies are needed in order to study~~should analyse~~ the prognostic potential of these neurological biomarkers ~~in relation to~~within the separate AIS grades. Still, the AIS system cannot fully capture the heterogeneity within the SCI patient population. Therefore, the predictive potential of baseline NfL, GFAP and CNTN-1 was also investigated in function of a motor score change < 5 and ≥ 5. ~~However, paired baseline and follow-up motor scores were only available for 14 out of 22 SCI patients. Therefore, only exploratory analyses were performed for these data and these results should be treated with caution and validated in larger cohorts.~~ In addition, the presence of concurrent TBI was not sufficiently documented in the available clinical data. The potential presence of TBI in some of the included SCI patients can therefore not be excluded. Moreover, the longitudinal relationship between plasma biomarker levels and MRI analysis of neuronal and axonal injury and lesion extent should be assessed for a more detailed analysis of injury severity and outcome. Additionally, more information on the efflux mechanism from the spinal cord and clearance from the plasma/serum of these biomarkers is needed.

In conclusion, this study highlights the usefulness of GFAP, CNTN-1 and especially NfL as ~~a~~ prognostic biomarkers over a longer time period up to 182w post-SCI, ~~while GFAP and CNTN-1 displayed prognostic potential when collected at baseline~~with the highest predictive ability for clinical outcome when analyzed together. These plasma biomarkers are thus a promising tool supporting informed decision-making during initial treatment and rehabilitation as well as patient stratification in clinical trials, reducing sample size and maximizing therapeutic efficacy.

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Author contributions

J.F., S.I.t.V., C.C.M.v.L.-G., B.D., J.D., D.P., E.M.J.C., C.E.T, S.B. and V.S. contributed to study concept and design. J.F. and S.I.t.V. contributed to data acquisition and analysis. J.F, C.E.T. and V.S. contributed to drafting of the text and figures.

Potential conflicts of interest

Nothing to report.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Ahuja CS, Wilson JR, Nori S, et al. Traumatic spinal cord injury. Nat Rev Dis Primers 2017 Apr 27;3:17018
2. Lammertse D, Dungan D, Dreisbach J, et al. Neuroimaging in traumatic spinal cord injury: an evidence-based review for clinical practice and research. J Spinal Cord Med 2007;30(3):205-214

3. Kirshblum S, Snider B, Rupp R, Read MS, International Standards Committee of A, IscoS. Updates of the International Standards for Neurologic Classification of Spinal Cord Injury: 2015 and 2019. Phys Med Rehabil Clin N Am 2020 Aug;31(3):319-330
4. Ditunno JF, Jr., Young W, Donovan WH, Creasey G. The international standards booklet for neurological and functional classification of spinal cord injury. American Spinal Injury Association. Paraplegia 1994 Feb;32(2):70-80
5. Kirshblum S, Snider B, Eren F, Guest J. Characterizing Natural Recovery after Traumatic Spinal Cord Injury. J Neurotrauma 2021 May 1;38(9):1267-1284
6. Krishna V, Andrews H, Varma A, Mintzer J, Kindy MS, Guest J. Spinal cord injury: how can we improve the classification and quantification of its severity and prognosis? J Neurotrauma 2014 Feb 1;31(3):215-227
7. Holmstrom U, Tsitsopoulos PP, Holtz A, et al. Cerebrospinal fluid levels of GFAP and pNF-H are elevated in patients with chronic spinal cord injury and neurological deterioration. Acta Neurochir (Wien) 2020 Sep;162(9):2075-2086
8. Kwon BK, Stammers AM, Belanger LM, et al. Cerebrospinal fluid inflammatory cytokines and biomarkers of injury severity in acute human spinal cord injury. J Neurotrauma 2010 Apr;27(4):669-682
9. Kwon BK, Streijger F, Fallah N, et al. Cerebrospinal Fluid Biomarkers To Stratify Injury Severity and Predict Outcome in Human Traumatic Spinal Cord Injury. J Neurotrauma 2017 Feb;34(3):567-580
10. Pouw MH, Kwon BK, Verbeek MM, et al. Structural biomarkers in the cerebrospinal fluid within 24 h after a traumatic spinal cord injury: a descriptive analysis of 16 subjects. Spinal Cord 2014 Jun;52(6):428-433
11. Khalil M, Teunissen CE, Otto M, et al. Neurofilaments as biomarkers in neurological disorders. Nat Rev Neurol 2018 Oct;14(10):577-589
12. Abdelhak A, Foschi M, Abu-Rumeileh S, et al. Blood GFAP as an emerging biomarker in brain and spinal cord disorders. Nat Rev Neurol 2022 Mar;18(3):158-172
13. Teunissen CE, Khalil M. Neurofilaments as biomarkers in multiple sclerosis. Mult Scler 2012 May;18(5):552-556
14. Kuhle J, Gaiottino J, Leppert D, et al. Serum neurofilament light chain is a biomarker of human spinal cord injury severity and outcome. J Neurol Neurosurg Psychiatry 2015 Mar;86(3):273-279

15. Leister I, Altendorfer B, Maier D, et al. Serum Levels of Glial Fibrillary Acidic Protein and Neurofilament Light Protein Are Related to the Neurological Impairment and Spinal Edema after Traumatic Spinal Cord Injury. J Neurotrauma 2021 Dec;38(24):3431-3439
16. Stukas S, Cooper J, Gill J, et al. Association of CSF and Serum Neurofilament Light and Glial Fibrillary Acidic Protein, Injury Severity, and Outcome in Spinal Cord Injury. Neurology 2023 Jan 4
17. Eckert MJ, Martin MJ. Trauma: Spinal Cord Injury. Surg Clin North Am 2017 Oct;97(5):1031-1045
18. Levi AD, Hurlbert RJ, Anderson P, et al. Neurologic deterioration secondary to unrecognized spinal instability following trauma--a multicenter study. Spine (Phila Pa 1976) 2006 Feb 15;31(4):451-458
19. Colakoglu G, Bergstrom-Tyrberg U, Berglund EO, Ranscht B. Contactin-1 regulates myelination and nodal/paranodal domain organization in the central nervous system. Proc Natl Acad Sci U S A 2014 Jan 21;111(3):E394-403
20. Chatterjee M, Koel-Simmelink MJ, Verberk IM, et al. Contactin-1 and contactin-2 in cerebrospinal fluid as potential biomarkers for axonal domain dysfunction in multiple sclerosis. Mult Scler J Exp Transl Clin 2018 Oct-Dec;4(4):2055217318819535
21. van Lierop ZY, Wieske L, Koel-Simmelink MJ, et al. Serum contactin-1 as a biomarker of long-term disease progression in natalizumab-treated multiple sclerosis. Mult Scler 2022 Jan;28(1):102-110
22. Wieske L, Martin-Aguilar L, Fehmi J, et al. Serum Contactin-1 in CIDP: A Cross-Sectional Study. Neurol Neuroimmunol Neuroinflamm 2021 Jul;8(5)
23. Andreasson U, Perret-Liaudet A, van Waalwijk van Doorn LJ, et al. A Practical Guide to Immunoassay Method Validation. Front Neurol 2015;6:179
24. van Lierop Z, Verberk IMW, van Uffelen KWJ, et al. Pre-analytical stability of serum biomarkers for neurological disease: neurofilament-light, glial fibrillary acidic protein and contactin-1. Clin Chem Lab Med 2022 May 25;60(6):842-850
25. Thelin EP, Zeiler FA, Ercole A, et al. Serial Sampling of Serum Protein Biomarkers for Monitoring Human Traumatic Brain Injury Dynamics: A Systematic Review. Front Neurol 2017;8:300
26. Barry DM, Millecamps S, Julien JP, Garcia ML. New movements in neurofilament transport, turnover and disease. Exp Cell Res 2007 Jun 10;313(10):2110-2120

27. Khalil M, Pirpamer L, Hofer E, et al. Serum neurofilament light levels in normal aging and their association with morphologic brain changes. Nat Commun 2020 Feb 10;11(1):812
28. Shir D, Graff-Radford J, Hofrenning EI, et al. Association of plasma glial fibrillary acidic protein (GFAP) with neuroimaging of Alzheimer's disease and vascular pathology. Alzheimers Dement (Amst) 2022;14(1):e12291
29. Ward MD, Weber A, Merrill VD, Welch RD, Bazarian JJ, Christenson RH. Predictive Performance of Traumatic Brain Injury Biomarkers in High-Risk Elderly Patients. J Appl Lab Med 2020 May 1;5(3):608

Figure legends

Figure 1. Plasma levels of NfL, GFAP and CNTN-1 over time and in correlation with age in SCI patients and HC. (**A**) NfL, GFAP and CNTN-1 plasma levels are shown in HC (*n* = 22) and over time in SCI patients (*n* = 3722) at five time points post-injury (0 (0-4 days), 3, 6, 12 and 18w). Individual measurements are shown for each included subject. Bars indicate the mean concentration for each group or time point, which is also indicated below the graph. § indicates the statistical significance of SCI patients at baseline (0w) compared to HC (Mann-Whitney U test), and * indicates the statistical difference in biomarker levels over time in the SCI cohort (linear mixed model). The linear mixed model included age and time point as fixed effects, and subject as a random effect. Multiple comparisons were done using Tukey HSD all pairwise comparisons test. (**B**) Correlation of the plasma NfL, GFAP or CNTN-1 levels and age in HC (*n* = 22) and SCI patients (*n* = 3722). Spearman's correlation coefficients (r) and *P*-values are indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** or §§§§ $P < 0.0001$

Figure 2. Plasma levels of NfL, GFAP and CNTN-1 in function of AIS ~~categories~~scores. (**A**) NfL, GFAP and CNTN-1 plasma levels at baseline (0w, 0-4 days) in ~~motor-complete~~ SCI patients (*n* = 34) with outcome(AIS-A (n = 10),~~or~~ AIS-B (n = 4), AIS-C (n = 7), AIS-D (n = 8) or AIS-E (n = 5) ~~endpoint~~ score~~, *n* = 6), motor-incomplete SCI patients (AIS-C, AIS-D or AIS-E endpoint score, *n* = 14)~~ and HC (*n* = 22). Mean (bars) ± SD is depicted. Differences in baseline biomarker levels between HC, AIS-A/B/C/D/E ~~and AIS-C/D/E~~ SCI patients were determined by ANOVA. § indicates statistical significance compared to HC, * indicates statistical significance compared to AIS-A and # indicates statistical significance compared to AIS-B. (**B**) NfL, GFAP and CNTN-1 plasma levels are shown over time in ~~AIS-A/B versus AIS-C/D/E~~ SCI patients with AIS-A (n = 10), AIS-B (n = 4), AIS-C (n = 7), AIS-D (n = 8) or AIS-E (n =5) at outcome. Mean ± SD is shown. Linear mixed models were used to determine differences between NfL, GFAP and CNTN-1 levels over time between ~~AIS-A/B and AIS-C/D/E~~ SCI patients of the different AIS scores, including patient as a random effect, and time point, AIS ~~category~~ score and their interaction as fixed effects. (**C**) Correlation of NfL, GFAP or CNTN-1 levels in all included SCI samples and AIS score at sampling timepoint. (**D**) Correlation of baseline NfL, GFAP or CNTN-1 levels and AIS score at clinical endpoint. Spearman's correlation coefficients (r) and *P*-values are indicated. */§/# $P < 0.05$, **/§§ $P < 0.01$, ***/§§§ $P < 0.001$, ****/§§§§ $P < 0.0001$

Figure 3. Plasma levels of NfL, GFAP and CNTN-1 in function of AIS conversion. (**A**) NfL, GFAP and CNTN-1 plasma levels at baseline (0w, 0-4 days) in SCI patients with (n = 130) or without (n = 208) AIS grade conversion and in HC (n = 22). Mean (bars) ± SD is depicted. Differences in baseline biomarker levels between HC, AIS converters and AIS non-converters were determined by ANOVA. (**B**) NfL, GFAP and CNTN-1 plasma levels are shown over time in SCI patients with and without AIS conversion. Mean ± SD is depicted. Linear mixed models were used to determine differences in NfL, GFAP or CNTN-1 plasma levels over time between AIS converters and non-converters, including patient as a random effect, and time point, AIS conversion and their interaction as fixed effects. (**C**) Receiving operating characteristic (ROC) curve analysis of NfL, GFAP, CNTN-1 or combined NfL + GFAP + CNTN-1 for the prediction of AIS conversion at baseline (0w, 0-4 days post-SCI). Area under the curve (AUC) ± standard error and 95% confidence intervals (95% CI) are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

Figure 4. Plasma levels of NfL, GFAP and CNTN-1 in function of motor score change. (**A**) NfL, GFAP and CNTN-1 plasma levels at baseline (0w, 0-4 days) in SCI patients with a motor score change < 5 (n = 145) or ≥ 5 (n = 159) and in HC (n = 22). Mean (bars) ± SD is depicted. Differences in baseline biomarker levels were determined by ANOVA. (**B**) NfL, GFAP and CNTN-1 plasma levels are shown over time in SCI patients with a change in motor score < 5 or ≥ 5. Mean ± SD is depicted. Linear mixed models were used to determine differences in NfL, GFAP or CNTN-1 plasma levels over time between the two SCI groups, including patient as a random effect, and time point, motor score change category and their interaction as fixed effects. (**C**) Receiving operating characteristic (ROC) curve analysis of NfL, GFAP, CNTN-1 or combined NfL + GFAP + CNTN-1 for the prediction of motor score improvement at baseline (0w, 0-4 days post-SCI). Area under the curve (AUC) ± standard error and 95% confidence intervals (95% CI) are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$