Neurobiology of Disease 220 (2026) 107306

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Spinal cord reactive-antibodies identified by serological antigen selection show prognostic value in traumatic spinal cord injury patients

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ARTICLE INFO

Keywords:
Spinal cord injury
Autoantibodies
Biomarker
Prognosis
cDNA phage display

ABSTRACT

Outcome prediction after traumatic spinal cord injury (SCI) remains challenging due to patient heterogeneity, highlighting the need for better prognostic tools. Neural tissue damage and blood–spinal cord barrier disruption expose the immune system to spinal cord proteins, eliciting autoantibody responses that may be beneficial or detrimental. This study aimed to identify the (auto)antibody profile of SCI patients, and examine the prognostic antibody biomarker potential.

Abbreviations: ACTB, beta actin; AEBP1, adipocyte enhancer-binding protein 1; AIS, American Spinal Injury Association impairment scale; ASIA, American Spinal Injury Association; AUS, Australia; BLAST, Basic Local Alignment Search Tool; cfu, colony forming units; CNS, central nervous system; CRMP2, collapsin response mediator protein 2; DAMPs, damage-associated molecular patterns; DEU, Germany; dpi, days post injury; ELISA, enzyme-linked immunosorbent assay; *E. coli*, *Escherichia coli*; ERO1a, endoplasmic reticulum oxidoreductase 1 alpha; ESP, Spain; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; FAIM2, Fas apoptotic inhibitory molecule 2; GFAP, glial fibrillary acid protein; h, hours; HC, healthy control; hSC, healthy spinal cord; IgG, immunoglobulin G; IgM, immunoglobulin M; IL, interleukin; iSC, injured spinal cord; ISNCSCI, International Standards for Neurological Classification of Spinal Cord Injury; IVIg, intravenous immunoglobulin G; KMT5B, lysine methyltransferase 5B; LEMS, lower extremity motor score; LT, light touch; MAG, myelin associated glycoprotein; MALDI/MS-MS, matrix assisted laser desorption/ionization mass spectrometry; MBP, myelin basic protein; min, minutes; MPBS, milk powder-phosphate buffered saline; MTRNR2L8, mitochondrially Encoded 16S RRNA; MYEOV2, myeloma overexpressed 2; NAWM, normal appearing white matter; NCBI, National Center for Biotechnology Information; OD, optical density; PBS, phosphate buffered saline; PBS-T, phosphate buffered saline-tween; PCR, polymerase chain reaction; PLP, proteolipid protein; PP, pin prick; PSMD4, 26S proteasome non-ATPase regulatory subunit 4; rpm, revolutions per minute; RT, room temperature; S100B, S100 calcium binding protein B; SAS, serological antigen selection; SCI, spinal cord injury; sec, seconds; t, timepoint; T, thoracic level; UEMS, upper extremity motor score; UH, Hasselt University; UH.SCI, Hasselt University.spinal cord injury; UHasselt, University Hasselt; USA, United States of America; YWHAZ, Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta..

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https://doi.org/10.1016/j.nbd.2026.107306
Received 19 December 2025; Received in revised form 1 February 2026; Accepted 1 February 2026
Available online 2 February 2026

A healthy and a SCI cDNA phage display library were screened for novel antibodies using SCI samples ($n = 12/11$). Antibody reactivity was validated using phage ELISA in 291 samples from 190 SCI patients collected at baseline (0–4 days post-injury [dpi]) and follow-up (15–30 dpi; 31–56 dpi). Correlations between antibody reactivity and clinical characteristics including SCI level, and American Spinal Injury Association impairment scale (AIS), were analysed. Immunofluorescent stainings were used to validate expression of two antigenic targets.

We identified antibodies against 6 novel autoantigens (University Hasselt [UH.] SCI.104/105/106/108/109/110). A panel of three antigens (UH.SCI.104/109/110) demonstrated increased antibody reactivity in 31.3% of SCI patients with AIS improvement versus 4.8% with no improvement, resulting in a positive likelihood ratio of 6.56. Patients with injuries above thoracic level 4 had significantly lower antibody reactivity against UH.SCI.105/110 compared to patients with lower lesions. Anti-UH.SCI.108/110 antibodies bound to astrocytes, in mouse spinal cord tissue and primary cell cultures, confirming disease-relevant reactivity.

Antibodies targeting the novel antigens demonstrated prognostic biomarker potential, supporting their future use in outcome prediction and patient stratification for SCI management and clinical trial design.

1. Background

Traumatic spinal cord injury (SCI) typically leads to loss of sensory function and varying degrees of paralysis. The severity of the injury is estimated by the American Spinal Injury Association (ASIA) impairment scale (AIS) of the International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) (Rupp et al., 2021). This scale consists of five classes, ranging from A to E, and is based on scoring preserved motor and sensory function. Although the AIS is currently the most used method to determine the severity of SCI, it is not always feasible to conduct this physical examination during the acute phase in unstable, intoxicated or sedated patients, and it also fails to capture patient heterogeneity within AIS classes (Krishna et al., 2014; Elizei and Kwon, 2017; ter Wengel et al., 2020). It thus remains challenging to provide an accurate early prognosis for individual SCI patients, highlighting the need for other prognostic methods that can be used as an alternative and/or in conjunction with the AIS to better predict long-term outcomes.

Traumatic SCI results in a variety of physiological responses, such as vascular disruption, neuronal cell death, axonal injury, glial activation and neuroinflammation (Alizadeh et al., 2019). The latter includes a humoral immune response, involving the production of (auto)antibodies that can persist for years after the injury (Mizrachi et al., 1983; Ankeny et al., 2006; Ankeny et al., 2009; Shnawa et al., 2022; Schwab et al., 2023). The current hypothesis on (auto)antibody production following SCI is that antigens may leak through the damaged blood-spinal cord barrier into the bloodstream and drain to secondary lymphoid organs, where they can cause B cell activation and (auto)antibody production (Ankeny et al., 2006; Ankeny et al., 2009; Raad et al., 2014; Ankeny and Popovich, 2010). Interestingly, autoantibodies have been suggested as potential prognostic biomarkers in SCI (Ydens et al., 2017). Autoantibodies directed against a variety of neural target antigens have already been described in SCI patients, including dorsal root ganglia neurons, myelin basic protein (MBP), myelin associated glycoprotein (MAG), ganglioside GM1, glial fibrillary acidic protein (GFAP), and collapsin response mediator protein 2 (CRMP2) (Mizrachi et al., 1983; Schwab et al., 2023; Ydens et al., 2017; Zajarías-Fainsod et al., 2012; Hayes et al., 2002; Davies et al., 2007; Schneider et al., 2022; Hergenroeder et al., 2016; Hergenroeder et al., 2018). We ourselves previously used an unbiased approach and identified antibody reactivity towards six novel antigens (Palmers et al., 2016). This method involved selecting antibody-antigen interactions in acute SCI using a cDNA phage display library derived from healthy human spinal cord material. A second unbiased approach, combining two-dimensional western blot and matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS-MS) identified antibodies in acute SCI patients that were reactive against non-native isoforms of 16 different proteins (Arevalo-Martin et al., 2018). However, the prognostic potential of these autoantibodies has remained largely unclear.

In this study, we examined the utility of autoantibodies as prognostic biomarkers for traumatic SCI. For this purpose, we performed a large-scale, unbiased profiling of the antibody reactivity in SCI patients, against antigens in healthy and damaged human spinal cord tissues. We further analysed correlations between the antibody reactivity and clinical characteristics, aiming to identify prognostic autoantibody biomarkers.

2. Methods

2.1. Study cohort

For our Hasselt University (UHasselt) SCI cohort, blood samples from traumatic SCI patients were collected in Belgium and The Netherlands (UHasselt cohort, $n = 36$) at Jessa Hospital (Hasselt, Belgium), University Hospitals Leuven (Leuven, Belgium), Hospital Oost-Limburg (Genk, Belgium), Algemeen Ziekenhuis Herentals (Herentals, Belgium), Algemeen Ziekenhuis Turnhout (Turnhout, Belgium), Sint-Trudo Ziekenhuis (Sint-Truiden, Belgium), and Adelante Centre of Expertise in Rehabilitation and Audiology (Hoensbroek, The Netherlands). Written informed consent was obtained from all patients before inclusion. Patients with pre-existing autoimmune disorders were excluded from the study. This study was approved by the Hasselt University medical ethics committee and all the hospitals' ethical committees. Samples were collected at three time points, t0 (0–4 days post-injury [dpi]), t1 (15–30 dpi) and t2 (31–56 dpi). Plasma was isolated by centrifugation of the peripheral blood samples (lithium-heparin tubes) for 10 min (min) at 400*g* and then for 10 min at 1500 g. Purified plasma samples were aliquoted and stored at −80 °C. All samples were processed and stored via the University Biobank Limburg (UBiLim) (Linsen et al., 2019).

Additional samples were obtained from international cohorts. The Australian cohort (AUS; The University of Queensland, Brisbane, Australia) consisted of 17 SCI patients with plasma samples collected at the three timepoints (t0, t1 and t2). The German cohort (DEU; Center for Spinal Cord Injuries, Trauma Center Murnau, Murnau, Germany) contained 113 SCI patients with serum samples collected at t0 and t1/t2, while the Spanish cohort (ESP; Hospital Nacional de Paraplejicos, Toledo, Spain) contained 24 SCI patients with serum samples collected at t1/t2. Plasma and serum samples were received as aliquots and stored at −80 °C in UBiLim. At each participating centre, written informed consent and ethical approval was obtained before patient inclusion.

Initial AIS scores were collected at t0 for the UHasselt, AUS and DEU cohorts. Follow-up AIS were collected for the UHasselt cohort at a follow-up consultation (180–365 dpi), for the AUS cohort at discharge (77–305 dpi), and for the DEU and ESP cohorts between 106 and 134 dpi. AIS subscores (upper extremity and lower extremity motor scores [UEMS and LEMS], pinprick score [PP] and light touch score [LT]), collected at t0 and follow-up, were analysed by calculating the delta score between these timepoints. This data was available for 10 UHasselt, 16 AUS and 62 DEU patients. Finally, AIS class improvement was examined in the UHasselt, AUS and DEU cohorts and was defined as advancing to a less severe AIS class between the initial and follow-up AIS

classification. This data was available for 24 UHasselt, 13 AUS and 62 DEU patients.

Additionally, a cross-sectional collection of healthy control (HC) plasma samples ($n = 153$) was obtained via UBiLim, considering age- and sex-matching with SCI patients while excluding individuals with allergies, infections and autoimmune disorders. Purified plasma was processed as described above and stored at −80 °C by UBiLim.

Demographic and clinical information of all included SCI patients and HC is provided in Table 1.

Table 1
Demographic and clinical information of the SCI cohorts and HC.

	UHasselt	AUS	DEU	ESP	Total SCI	HC
N	36	17	113	24	190	153
Age (IQR)[†]	56 (24)	31 (40)	48 (32)	33 (17.5)	48 (33.3)	50 (37.5)
Male (%)[‡]	28 (77.8)	16 (94.1)	98 (86.7)	21 (87.5)	163 (85.8)	127 (83.0)
Initial AIS (%)[‡,§]						
A	9 (25.0)	8 (47.1)	24 (21.2)	0 (0.0)	41 (21.6)	NA
B	5 (13.9)	3 (17.6)	12 (10.6)	0 (0.0)	20 (10.5)	NA
C	6 (16.7)	3 (17.6)	7 (6.2)	0 (0.0)	16 (8.4)	NA
D	15 (41.7)	2 (11.8)	20 (17.7)	0 (0.0)	37 (19.5)	NA
UNK	1 (2.8)	1 (5.9)	50 (44.2)	24 (100)	76 (40.0)	NA
Follow-up AIS (%)[‡,¶]						
A	3 (8.3)	7 (41.2)	33 (29.2)	15 (62.5)	58 (30.5)	NA
B	2 (5.6)	1 (5.9)	11 (9.7)	2 (8.3)	16 (8.4)	NA
C	5 (13.9)	3 (17.6)	12 (10.6)	1 (4.2)	21 (11.1)	NA
D	9 (25.0)	5 (29.4)	45 (39.8)	6 (25.0)	65 (34.2)	NA
E	5 (13.9)	0 (0.0)	9 (8.0)	0 (0.0)	14 (7.4)	NA
UNK	12 (33.3)	1 (5.9)	3 (2.7)	0 (0.0)	16 (8.4)	NA
AIS improvement (%)[‡,††]	10 (41.7)	6 (37.5)	25 (40.0)	/	41 (40.2)	NA
Location SCI (%)[‡]						
Cervical	26 (72.2)	9 (52.9)	75 (66.4)	11 (45.8)	121 (63.7)	NA
Thoracic	8 (22.2)	7 (41.2)	28 (24.8)	12 (50.0)	55 (28.9)	NA
Lumbar	1 (2.8)	0 (0.0)	5 (4.4)	1 (4.2)	7 (3.7)	NA
UNK	1 (2.8)	1 (5.9)	5 (4.4)	0 (0.0)	7 (3.7)	NA

Abbreviations: *UHasselt*, Hasselt University cohort; *AUS,* Australian cohort; *DEU*, German cohort; *ESP,* Spanish cohort; *SCI,* spinal cord injury; *HC,* healthy control; *N*, number of individuals; *IQR,* interquartile range; *AIS,* American Spinal Injury Association (ASIA) impairment scale; *UNK*, unknown; *NA,* not applicable; *t0,* 0–4 days post injury.

[†] Age expressed as median years with interquartile range (IQR).

[‡] Expressed as the number of individuals and the percentage.

[§] Initial AIS was collected at t0 for the UHasselt, AUS and DEU cohorts, and was not available at t0 for the ESP cohort.

[¶] Follow-up AIS was collected for the UHasselt cohort at a follow-up consultation (180–365 dpi), for the AUS cohort at discharge (77–305 dpi), for the DEU and ESP cohorts between 106 and 134 dpi.

[††] AIS class improvement is defined as advancing to a less severe AIS class between the initial and follow-up AIS classification. Available for 24/37 UHasselt patients, 16/17 AUS patients, 62/113 DEU patients and no ESP patients.

2.2. Spinal cord cDNA phage display libraries

Two cDNA phage display libraries, a healthy spinal cord (hSC) and an injured spinal cord (iSC) library, were used to screen for novel antigenic targets. The hSC cDNA phage display library was previously generated from human spinal cord RNA (Ydens et al., 2017; Palmers et al., 2016). The iSC cDNA phage display library was newly constructed using lesion tissue of a young (20–30 years old) male SCI patient, with trauma due to a fall from a height and AIS A. The tissue was provided by

This tissue was provided by Prof. Dr. Rainer Abel (Klinikum Bayreuth GmbH, Bayreuth, Germany). Tissue was collected during surgery, which took place at 1 day post-injury and was stored thereafter at −80 °C. Thereafter, the iSC cDNA phage display library was constructed as previously described, with minor adjustments (Supplemental methods) (Vandormael et al., 2024; Vandormael et al., 2017; Mazón-Cabrera et al., 2023).

2.3. Serological antigen selection (SAS)

To screen for novel autoantibodies, pools of 11–12 plasma samples from SCI patients with AIS classes A, B or C at baseline (t0, 0–4 dpi) and t1 (15–30 dpi) from the UHasselt and AUS cohorts were used for SAS (Supplementary Fig. 1 and Supplementary Table 1). Plasma samples at t1 were used during positive selection rounds to select for specific enrichment of SCI antibody-antigen interactions, while t0 samples were used in negative selection to remove baseline antibody-antigen interactions (Supplementary Fig. 2). The two cDNA phage display libraries were used in two separate SAS procedures. As preparation for the SAS, SCI plasma samples of t0 and t1 were pooled separately (1/100 dilution of each sample) and preabsorbed against *Escherichia coli* (*E. coli*) and M13 bacteriophage proteins to remove antibodies reactive against bacterial and phage proteins. In addition, plasma pools were depleted of immunoglobulin M (IgM) with anti-IgM agarose beads (Sigma, Hoeilaart, Belgium) according to the manufacturer's instructions. SAS was performed as previously described (Vandormael et al., 2024). During negative selection, the t0 plasma pool (1/100 dilution of each sample in 2% [*w/v*] skimmed milk powder-phosphate buffered saline [MPBS]) was pre-incubated with the hSC or iSC cDNA phage display library (1×10^{13} colony forming units [cfu]) rotating for 1 h at room temperature (RT). Immunoglobulin G (IgG) capturing beads (protein G resin; Genscript, Rijswijk, The Netherlands) were blocked with phage extract (100 μg/ml in 2% MPBS) for 1 h, while rotating at RT. Complexes between phage and t0 antibodies were bound to the IgG capturing beads during a 10 min rotating incubation, after which the supernatant containing unbound phage was used for positive selection. During positive selection, the phage-containing supernatant was added to the t1 plasma pool (1/100 of each sample in 2% MPBS) and incubated for 1 h, while rotating at RT. Complexes between phage and t1 antibodies were captured using the IgG beads during a 30 min rotating incubation at RT. Next, the beads were washed 10 times with 0.1% PBS-Tween (PBS-T) and 10 times with PBS. Finally, selected phage were eluted from the beads with triethylamine (100 mM, pH 12) for 10 min while rotating and thereafter with glycine (100 mM, pH 2) for 10 min while rotating, and neutralised with Tris-HCl (1 M, pH 7.4). Eluted phage were amplified for the next SAS round by infection of TG1 *E. coli* bacteria at 37 °C for 30 min and cultured overnight on 2× yeast extract tryptone agar plates (16 g/l bacto-tryptone, 10 g/l yeast extract, 5 g/l NaCl and 15 g/l bacto-agar) containing glucose (2%, VWR, Leuven, Belgium) and ampicillin (0.1 μg/ml, Roche). These steps were repeated for the next 3 SAS rounds. The phage input was produced by scraping the bacteria of the amplification plates and infection of TG1 *E. coli*. The second, third and fourth round each started with two negative selections followed by one positive selection. After the fourth SAS round, single colonies were picked from the agar plates for PCR analysis as described in the supplemental methods with the addition of a first step at 94 °C for 10 min to release the DNA from the bacteria. Finally, the purified PCR products were sequenced by

Sanger sequencing. After SAS, amino acid sequences of the identified clones were obtained by analysing the sequences with our in-house DNAnalyzer software and were compared with the RefSeq Select protein database on National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) protein algorithm (Quaden et al., 2020). The identified antigens received a code consisting of University Hasselt-spinal cord injury (UH-SCI.) followed by a number, for easy reporting.

2.4. Phage ELISA

To assess antibody reactivity against the identified antigens, phage ELISA was performed. ELISA was conducted using pooled and individual samples (Supplementary Fig. 1 and Supplementary Table 1). Phage ELISA was performed as previously described (Vandormael et al., 2024; Mazón-Cabrera et al., 2023). For each sample, the optical density (OD) signal was measured of the antigen-expressing phage and of an empty phage which did not express an antigen. The antibody reactivity was determined by calculating the ratio of the two signals ((OD antigen-expressing phage)/(OD empty phage)). All samples were set-up in duplicate and tested two or three times in independent ELISA plates, to obtain a coefficient of variation below 20% between all duplicates and repeats. For each antigen, a cut-off was calculated to examine antibody reactivity in AIS class improvement, based on the average antibody reactivity at t1 of AIS non-improvers plus three times the standard deviation.

2.5. Immunofluorescence staining

Anti-UH-SCI.108 and UH-SCI.110 antibodies were purified from a SCI patient and HC, respectively, using affinity chromatography with the aminolink plus coupling resin (UH-SCI.108) or high capacity streptavidin agarose resin (UH-SCI.110) (Thermo Fisher Scientific, Zaventem, Belgium) according to the manufacturer's instructions (Supplemental methods). The affinity purified antibodies were used for immunofluorescence staining on mouse SCI spinal cord tissue and primary mouse astrocytes (Supplemental methods). For the mouse SCI tissue, experiments were approved by the local ethical committee of Hasselt University and performed in accordance with the guidelines described in Directive 2010/63/EU on the protection of animals used for scientific purposes. Mouse spinal cord tissue was collected at 28 dpi from a 12-week old female mouse with a severe T8 contusion injury (85 kilodyne, Infinite Horizon Impactor (Precision Systems and Instrumentation, Lexington, Kentucky, United States of America [USA])). Additionally, mouse astrocytes were extracted from postnatal mouse pups (day 0) and cultured in a mixed glial cell culture, as described previously (Vangansewinkel et al., 2023).

2.6. Statistical analysis

Statistical analysis was performed using SAS (version 9.4, Cary, North Carolina, USA) and GraphPad prism (version 10.2.1, GraphPad Software, Boston, Massachusetts, USA). Linear mixed models were used to analyse antibody reactivity between consecutive timepoints in the follow-up SCI samples. The model included the timepoint as fixed effect and the patient code as random effect, while age and sex were added as covariates. The same mixed model was used to compare the antibody reactivity across the three SCI timepoints and HC samples. In addition, this mixed model was used to compare the antibody reactivity among the AIS classes (initial and follow-up). Mixed models were chosen due to missing data at some timepoints. Differences in antibody reactivity at t1 were analysed between groups of SCI patients with injuries above versus below and at the fourth thoracic level and between AIS class improvers and non-improvers (i.e. advancing to a less severe AIS class between the initial and follow-up assessments). The t1 timepoint was selected to allow the study of both novel and existing antibody responses and was expected to more accurately represent patient responses in comparison to acute timepoints after the injury. The *t*-test was used for normally distributed data, while the Mann-Whitney test was applied for non-normally distributed data, respectively. Finally, correlations between antibody reactivity and AIS subscores (UEMS, LEMS, PP and LT) were examined using Pearson correlation coefficients for normally distributed data and Spearman correlation for non-Gaussian distributions. These statistical tests were performed at all timepoints. A *P*-value smaller than 0.05 ($P < 0.05$) was considered significant.

3. Results

3.1. Screening for novel SCI antibodies against healthy and injured spinal cord antigens resulted in six novel antigens with higher antibody reactivity in pooled SCI samples

To identify novel autoantibodies with reactivity towards human spinal cord antigens, two human cDNA phage display libraries were used: a healthy (hSC) and an injured (iSC) cDNA phage display library. The previously created hSC cDNA phage display library had a diversity of 2.44×10^6 displayed antigens, and a good representation of the antigens present in a healthy human spinal cord (Ydens et al., 2017; Palmers et al., 2016). In order to fully represent the antigens present in injured spinal cord tissue after a SCI, the iSC cDNA phage display library was newly generated from lesioned human spinal cord tissue ($n = 1$). The iSC cDNA phage display library had a final diversity of 6.92×10^5 displayed antigens with cDNA inserts ranging between 6 and 7500 base pairs. These two cDNA phage display libraries were used separately to screen for antibody reactivity in pooled SCI plasma samples ($n = 10/11$, respectively) using SAS. Selected SCI patients were mostly male (10/11, 91%), with an average age of 48 years (Supplementary Table 1) and AIS scores A (6/11, 54.5%), B (3/11, 27.3%) or C (2/11, 18.2%). Sequencing analysis of a selection of phage clones after SAS identified 88 uniquely displayed novel antigens.

Antibody reactivity against each of these 88 antigens was initially measured in pools of SCI and HC plasma samples (6 pools of 5–6 samples each; Supplementary Fig. 1 and Supplementary Table 1) using phage ELISA. This led to the selection of six antigens, namely University Hasselt-Spinal Cord Injury (UH-SCI.)104, UH-SCI.105, UH-SCI.106, UH-SCI.108, UH-SCI.109 and UH-SCI.110, with exclusive antibody reactivity in SCI pools and not in HC pools (Fig. 1). For five of the antigens (UH-SCI.104/105/106/108/109), antibody reactivity was exclusive to one SCI pool, while UH-SCI.110 presented high antibody reactivity in two SCI pools (Fig. 1). Of the remaining 82 antigens, 63 demonstrated low reactivities in both SCI and HC pools (data not shown), whereas 19 antigens had high antibody reactivity in both groups (Supplementary Fig. 3). Overall, this first assessment of the antibody reactivity resulted in six antigens of interest with antibody reactivity observed exclusively in SCI pools.

3.2. The six novel antigens showed a high degree of homology with human proteins

To investigate the targets of the identified antibody responses, the amino acid sequences of the six selected antigens with SCI reactivity were compared to human protein sequences using BLAST (Table 2). All of the identified antigens were non-physiological peptides, originating from non-coding cDNA sequences (UH-SCI.104/106/108/109) or out-of-frame cloning of coding sequences (UH-SCI.105/110), and therefore showed only partial homology to human proteins. Some of the identified peptides partially corresponded with proteins that are typically highly expressed in CNS cells (e.g. oligodendrocytes and bipolar cells), such as Shinbaro2 domain-containing adapter protein F (UH-SCI.105), RNA-binding protein 6 and arf-GAP with GTPase, ANK repeat and PH domain-containing protein 2 (UH-SCI.106) and collagen alpha-5(IV) chain (UH-SCI.109) (Uhlén et al., 2015; Thul et al., 2017). The other

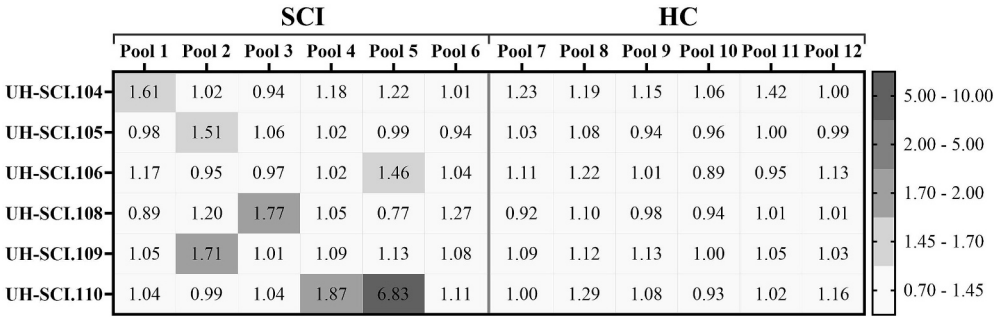

Fig. 1. Six UH-SCI antigens with higher antibody reactivity in SCI pools compared to HC pools. Antibody reactivity is expressed as the ratio of the antibody reactivity against the specific antigen-expressing phage over the reactivity against the control empty phage. Cut-off for positivity is set at 1.45, as shown in legend. Pools 1–6 are composed of SCI patients (Pool 2 $n = 5$; Pools 1, 3, 4, 5 and 6 $n = 6$; sample at t1), pools 7–12 are made up of age-and sex-matched HC (Pool 8 n = 5; Pools 7, 9, 10, 11 and 12 n = 6). Legend on the side indicates the reactivity level.
Abbreviations: *SCI,* spinal cord injury; *HC,* healthy control, *UH-SCI,* University Hasselt Spinal Cord Injury biomarkers; *t1,* 15–30 days post injury.

Table 2
Sequence and homology of the six UH-SCI antigens.

Antigen	Length[†]	Sequence[‡]	Protein info[§]	Homology[¶]
104	10	(S)TEDNFIPLN	Histone-lysine *N*-methyltransferase (KMT5B)	6/7 (86%)
			Coatomer subunit delta isoform 4	6/8 (75%)
105	16	(S)TARPPEEYEKAERLG	Endoplasmic reticulum oxidoreductase-1 like protein alpha	8/10 (80%)
			Shinbaro2 domain-containing adapter protein F	9/16 (56%)
106	14	(S)TRERERESWQDIW	Pre-mRNA 3′-end-processing factor FIP1	7/7 (100%)
			Arf-GAP with GTPase, ANK repeat and PH domain-containing protein 2	8/10 (80%)
			RNA-binding protein 6	9/15 (50%)
108	8	(D)KMQNTEE	Interleukin-32	6/7 (86%)
			RNA polymerase-associated protein LEO1	7/10 (70%)
109	21	(R)PPSLPGEPSGQGGEWWSQSL	Collagen alpha-5(IV) chain	9/11 (82%)
			Tyrosine-protein kinase lck	8/11 (73%)
			Chain A, proto-oncogene tyrosine-protein kinase LCK	8/11 (73%)
			Interleukin 15 receptor alpha	7/8 (88%)
110	14	(S)MAGGTESRDEDKT	Ubiquilin-3	9/12 (75%)
			Microspherule protein 1	8/11 (73%)

[†] Length is expressed as the number of amino acids.
[‡] Amino acid sequence of the displayed peptides on the phage surface. Amino acid between brackets is created by the fusion of the cDNA insert and the display vector.
[§] Top human proteins showing homology with the antigen using the RefSeq Select protein database on NCBI with the BLAST protein algorithm.
[¶] Number of amino acids of the antigen corresponding with the homology top hits and the homology percentage.

matches had moderate to low expression within the CNS. Although none of these homology hits have been studied in the context of SCI, these peptides are interesting antigenic candidates that could be involved in post-SCI processes and autoantibody responses.

3.3. Antibody reactivity correlated with AIS conversion and neurological injury level

Next, a large-scale cohort study was done to validate the antibody reactivity against the six identified antigens in SCI patients, and to study the prognostic potential of these antibodies. Individual samples from 190 SCI patients (longitudinal cohort) and 153 HC (cross-sectional cohort) were included (Table 1, Supplementary Fig. 1 and Supplementary Tables 1–2). Over time, there was no significant change in mean antibody reactivity towards the six antigens in the SCI cohort, although many of the antibody patterns from individual SCI patients showed an increasing trend from t0 to t1 (Fig. 2). In contrast to the antibody reactivity analysis in pooled samples (Fig. 1), this large-scale screening of individual samples did not show differences in antibody reactivity for any of the six antigens between HC and SCI patients (Supplementary Fig. 4). These findings suggest that these are pre-existing IgG autoantibodies. Further, there were no significant differences in antibody reactivity between the different SCI cohorts (data not shown).

To analyse the prognostic potential of the antibodies, antibody reactivity against the six antigenic targets at t1 was correlated with clinical parameters, taking into account the variability in patient demographics. When comparing SCI patients who showed AIS improvement with SCI patients who did not show AIS improvement (Supplementary Table 2), the percentage of anti-UH-SCI.104 antibody-positive SCI patients was significantly higher in the group of AIS improvers compared to AIS non-improvers (18.2% versus 0.0%, $P < 0.01$; Table 3). Combining three antigens into a panel (UH-SCI.104/109/110) resulted in antibody reactivity to at least one of these antigens in 31.3% of the AIS improvers compared to only 4.8% of the AIS non-improvers ($P < 0.01$). This furthermore led to a positive likelihood ratio of 6.56, that highlights the prognostic biomarker potential for this panel of anti-UH-SCI.104/109/110 antibodies. Only two SCI patients demonstrated antibody reactivity towards more than one antigen; one patient to UH-SCI.105/108 and the other patient to UH-SCI.104/105/109/110. Antibody reactivity was not different between the AIS classes (Supplementary Fig. 5) and did not correlate with the AIS subscores (UEMS, LEMS, PP and LT). Additionally, the observed antibody reactivity was not dependent on age and sex.

Finally, antibody reactivity at t1 was compared between patients with a SCI above or below the fourth thoracic (T4) level (Supplementary Table 2). Anti-UH-SCI.105 and anti-UH-SCI.110 antibody reactivity was significantly higher in SCI patients with low thoracic and lumbar injuries compared to patients with lesions above T4 ($P_{UH\text{-}SCI.105} < 0.05$; $P_{UH\text{-}SCI.110} < 0.001$; Fig. 3).

Taken together, these results indicate the prognostic biomarker potential of these novel antibodies, since the percentage of patients with antibody reactivity against the panel of 3 antigens (UH-SCI.104/109/

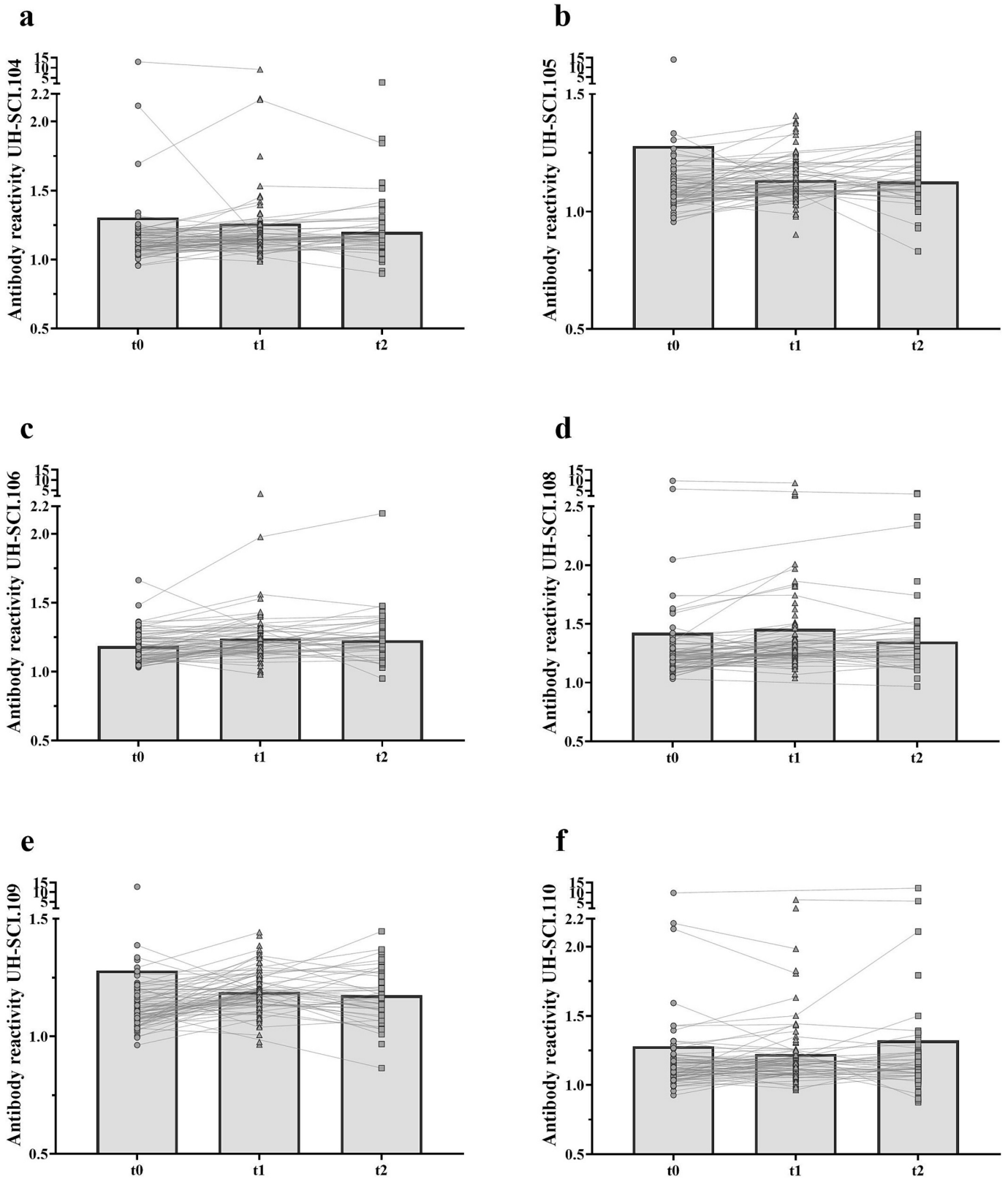

Fig. 2. Timeline of antibody reactivity in SCI patients against the six UH-SCI antigens.
Antibody reactivity is expressed as the ratio of the antibody reactivity against the specific antigen-expressing phage over the reactivity against the control empty phage. Bars display the mean of the antibody reactivity at each time point, including t0 ($n = 76$), t1 ($n = 121$) and t2 ($n = 95$). Each point represents an individual patient's sample and connected points are samples of the same patient at multiple timepoints.
Abbreviations: *UH-SCI*, University Hasselt-Spinal Cord Injury antigen; *dpi,* days post injury; *t0,* 0–4 dpi; *t1,* 15–30 dpi; *t2,* 31–56 dpi.

Table 3
Antibody positivity to UH-SCI antigens and panel in AIS improvers and non-improvers.

UH-SCI antigen	AIS improvers (%)[†]	AIS non-improvers (%)[‡]	LR+	P-value
UH-SCI.104	6/33 (18.2)	0/42 (0.0)	NA	*0.0055***
UH-SCI.105	1/32 (3.1)	1/43 (2.3)	2.03	>0.999
UH-SCI.106	0/31 (0.0)	2/43 (4.7)	0.0	0.510
UH-SCI.108	2/32 (6.3)	2/42 (4.7)	2.17	>0.999
UH-SCI.109	2/32 (6.3)	0/42 (0.0)	NA	0.184
UH-SCI.110	4/32 (12.5)	2/43 (4.7)	4.26	0.392
Panel UH-SCI. 104/109/110	10/32 (31.3)	2/42 (4.8)	6.56	*0.0033***

Abbreviations: *UH-SCI,* University Hasselt-Spinal Cord Injury antigen; *AIS,* American Spinal Injury Association (ASIA) impairment scale; *LR+,* positive likelihood ratio; *NA,* not applicable; *t1,* 15–30 days post injury.
[†] $n = 31$–33.
[‡] $n = 42$–43.
** $P \leq 0.01$.

110) was higher in AIS improvers compared to AIS non-improvers.

3.4. Human antibodies against UH-SCI.108 and 110 bind to astrocytes

To elucidate the rationale behind our finding of increased circulating levels of the novel identified antibodies in SCI, and to confirm their spinal cord reactivity, we performed immunofluorescence stainings for the presence of two of the corresponding antibody targets in disease-relevant tissue and cells. More specifically, anti-UH-SCI.108 and anti-UH-SCI.110 antibodies were purified from human plasma and used for stainings on mouse SCI tissue and mouse astrocytes. Both antibodies selectively bound to astrocytes in mouse SCI tissue (Fig. 4 A-D). Co-staining of anti-UH-SCI.108 or anti-UH-SCI.110 antibodies on the mouse SCI tissue with the astrocyte marker GFAP suggested the presence of UH-SCI.108 and UH-SCI.110 antigens within the astrocyte cytoskeleton (Fig. 4 B and D). Astrocyte-specific binding of anti-UH-SCI.108/110 antibodies was confirmed in a mixed glia cell culture (>95% pure culture, Fig. 4 *E*-H), in which only astrocytes were stained by both of the antibodies. Thus, anti-UH-SCI.108 and anti-UH-SCI.110 antibody reactivity is directed against astrocytes within the injured spinal cord, confirming their spinal cord reactivity and potential pathological relevance.

4. Discussion

A large-scale, unbiased profiling of the antibody reactivity in SCI patients was performed in order to identify novel antibody biomarkers with prognostic potential. We identified antibodies against six novel antigens with increased antibody reactivity in pooled SCI samples compared to HC. Interestingly, a panel of three antibodies, anti-UH-SCI.104/109/110, showed prognostic potential, as 31.3% of patients with AIS improvement were positive for antibody reactivity, compared to only 4.8% of patients without AIS improvement, yielding a positive likelihood ratio of 6.56. We further showed that anti-UH-SCI.105 and anti-UH-SCI.110 antibody reactivity was linked to the neurological level of the injury. Finally, anti-UH-SCI.108 and anti-UH-SCI.110 antibodies bound to astrocytes, both in mouse SCI tissue and primary astrocytes, confirming their spinal cord reactivity and potential pathological relevance.

The SAS technology was used to study the antibody response post-SCI. In addition to an existing hSC cDNA phage display library, we generated a novel, SCI-specific library from mRNA isolated directly from SCI lesion tissue. This approach provides an improved in vitro representation of the spinal cord antigens expressed after injury. As the tissue was collected one day post-injury, the resulting antigen repertoire is highly relevant for characterizing the antibody response induced by SCI. To our knowledge, this is the first study to use human SCI-derived antigens for a comprehensive screening of the antibody repertoire in SCI patients.

Our SAS screenings using both the hSC and iSC cDNA phage display libraries resulted in the identification of 88 novel antigenic targets. From these, we identified six antibodies with increased reactivity in pooled

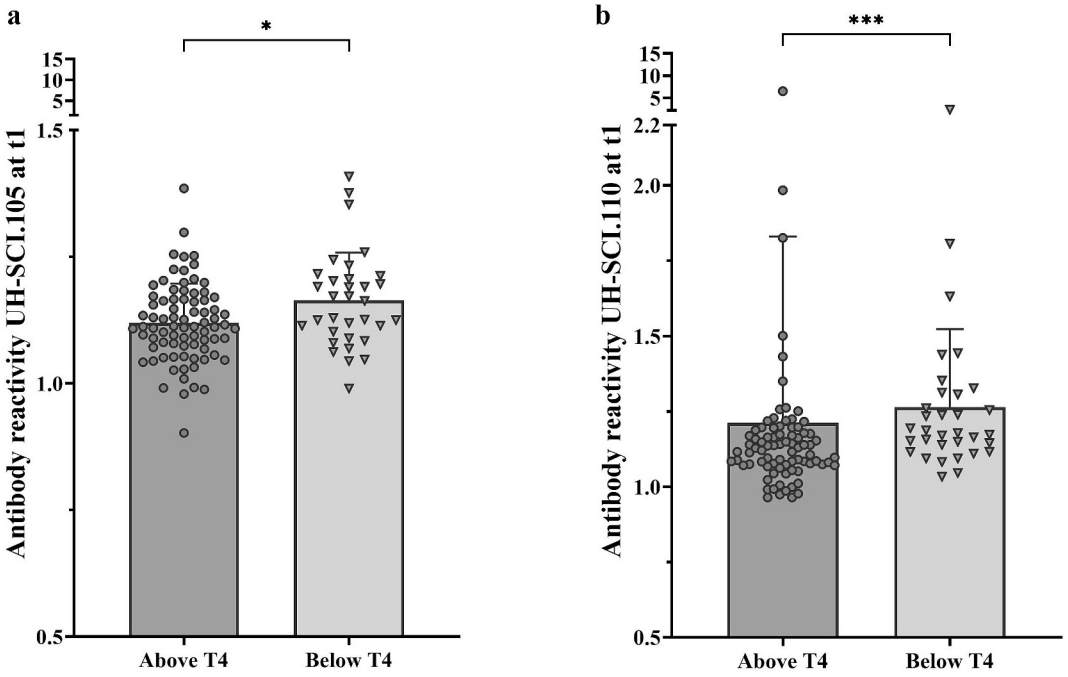

Fig. 3. Antibody reactivity against UH-SCI.105 and 110 in SCI patients with lesions above and below T4. Antibody reactivity is expressed as the ratio of the antibody reactivity at t1 against the specific antigen-expressing phage over the reactivity against the control empty phage. Bars display the mean and standard deviation of the antibody reactivity at t1 of SCI patients with a lesion above T4 ($n = 80$ for UH-SCI.105 and 81 for UH-SCI.110) and below T4 ($n = 33$). Each point represents an individual patient's sample. *$P \leq 0.05$; ***$P \leq 0.001$.
Abbreviations: *UH-SCI,* University Hasselt-Spinal Cord Injury antigen; *t1,* 15–30 days post injury; *T4,* fourth thoracic level.

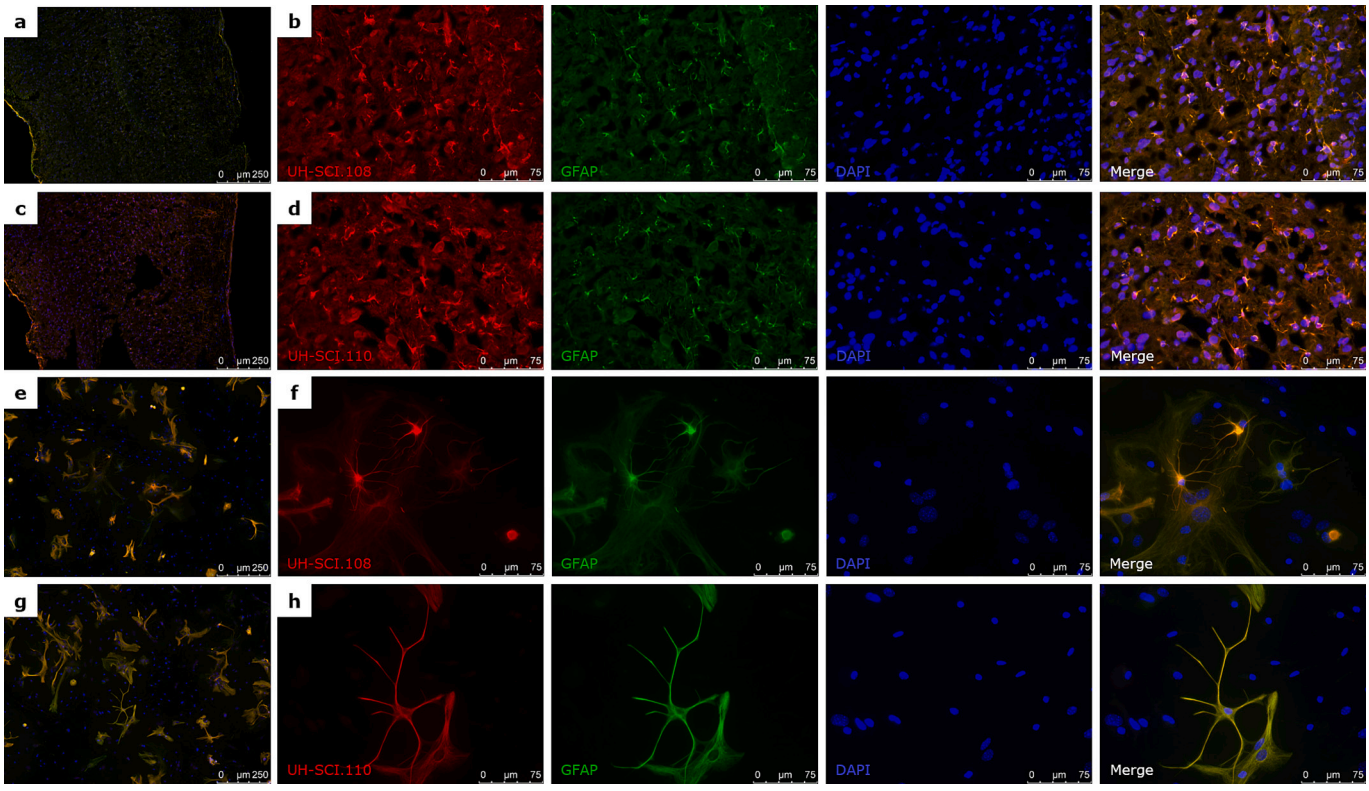

Fig. 4. Immunofluorescent staining of anti-UH-SCI.108 and 110 antibodies on mouse SCI tissue and mouse primary astrocytes. (A-D) Mouse SCI tissue (28 dpi, rostral to lesion) was stained with human purified antibodies reactive against UH-SCI.108 (A 20×; B 40×) or UH-SCI.110 (C 20×; D 40×) and anti-GFAP antibody to also stain for astrocytes. (*E*-H) Mouse primary astrocytes were stained with human purified antibodies reactive against UH-SCI.108 (E 20×; F 40×) or UH-SCI.110 (G 20×; H 40×) and anti-GFAP antibody.
Abbreviations: *dpi,* days post injury *UH-SCI,* University Hasselt-Spinal Cord Injury antigen; *DAPI,* 4′,6-diamidino-2-phenylindole; *GFAP,* glial fibrillary acidic protein.

SCI plasma samples as compared to HC samples. All six identified candidates were non-physiological peptides, due to out-of-frame cloning of coding sequences (UH-SCI.105/110) or due to originating from non-coding cDNA sequences (UH-SCI.104/106/108/109). Currently, their in vivo antigens are unknown. These peptides probably form mimotopes, which are short peptide sequences that mimic the epitopes against which the antibodies were originally generated. Antibodies reactive against such mimotopes can still be interesting biomarkers when correlated with disease-specific clinical parameters. Other studies in our research group have also shown that selection of mimotopes during antibody profiling can lead to potential biomarkers (Vandormael et al., 2017; Mazón-Cabrera et al., 2023; Quaden et al., 2020). Several human proteins displayed high degrees of homology with the UH-SCI antigens, but these proteins have not been studied in the context of SCI. However, many of them have been linked to other CNS disorders, such as lysine methyltransferase 5B (KMT5B) (UH-SCI.104) in glioma, endoplasmic reticulum oxidoreductase 1 alpha (ERO1a) (UH-SCI.105) in stroke, IL-15 signalling receptor chain (UH-SCI.110) in MS, interleukin-32 (IL-32; UH-SCI.108) in MS, myasthenia gravis and neuromyelitis optica (Shi et al., 2024; Ren et al., 2023; Laurent et al., 2021; Morsaljahan et al., 2017; Wang et al., 2013; Na et al., 2011). Therefore, these proteins could also be relevant in the context of SCI pathology, especially since antibodies against UH-SCI.104 and 110 were linked with disease outcome.

The antibody reactivity of SCI patients against all 6 antigens remained relatively constant over time. Since these antibodies were also present in HC, these results suggest that the autoantibodies are pre-existing and natural, and not SCI-specific. Other studies have demonstrated the presence of natural antibodies after SCI as well. In SCI mice, pre-existing antibodies were identified based on the presence of natural IgM antibodies towards damage-associated molecular patterns (Narang et al., 2017). Moreover, when examining blood samples of SCI patients, natural antibodies against 16 antigens were identified with these antigens being involved in cytoskeleton organisation, energy metabolism, and other processes (Arevalo-Martin et al., 2018). The exact roles of these antibodies within SCI remains to be elucidated.

Since the goal of this study was to determine the prognostic biomarker potential of autoantibodies in SCI patients, we examined correlations between the antibody reactivity at t1 and clinical characteristics. Antibodies against UH-SCI.104, 109 and 110 had the largest discriminative ability between patients with or without AIS improvement. A biomarker panel combining the three antibodies resulted in a sensitivity of 31.3%, specificity of 95.2% and a positive likelihood ratio of 6.56 for AIS improvement. This new antibody biomarker panel thus holds promise for the clinical prognosis of SCI patients.

There is an urgent need for novel prognostic measures in SCI clinical care, since the current methods are unable to accurately predict the prognosis of individual patients. The AIS classification system artificially reduces the heterogeneity of the patient population and studies have shown that clinical improvement still takes place within AIS groups, which is not reflected by a change to a less severe AIS class (Spiess et al., 2009). Therefore, the newly identified anti-UH-SCI antibodies could be potential biomarker candidates that can be stably measured over time, are able to discriminate between AIS class improvers and non-improvers at around three weeks post-injury and allow objective interpretation. This was already shown in three independent international cohorts (from Australia, Germany and Spain). In a follow-up study, further validation of the antibody biomarkers is needed to additionally examine correlations with AIS improvement within each separate AIS class. By integrating these biomarkers with other tools, such as imaging and AIS classification, the prognostic sensitivity could be further increased, enabling more refined stratification of SCI patients. More specifically,

this biomarker panel may help identify patient subgroups most likely to benefit from specific therapies and support the monitoring of treatments. Thus, ultimately, this will aid with clinical trial enrolment and improve the interpretation of therapeutic outcomes, with the final goal of advancing towards effective treatments for SCI.

The increased presence of anti-UH-SCI.104, 109 and 110 antibodies in patients with AIS improvement indicates that these novel antibodies could have a beneficial effect, although the advantageous mechanisms are currently unknown. To date, most studies have focused on the neurotoxic potential of SCI-induced autoantibodies. Administration of these antibodies to naïve mice resulted in glial reactivity and neuronal loss, and also induced paralysis and a necrotic inflammatory lesion when injected directly into the uninjured spinal cord (Ankeny et al., 2006; Ankeny et al., 2009). In human studies, a correlation was found between the presence of anti-GFAP and anti-CRMP2 antibodies and neuropathic pain development (Hergenroeder et al., 2018). Similarly, a recent study demonstrated antibody reactivity against dorsal root ganglia neurons which was associated with earlier and more frequent use of the neuropathic pain treatment gabapentinoids (Schwab et al., 2023). Further, anti-GM1 IgG levels were higher in patients with urinary tract infections and pain complications (Davies et al., 2007). However, beneficial effects of antibodies have also been indicated in the context of SCI therapies, for example by the use of intravenous immunoglobulin G (IVIg), which is composed of pooled antibodies of healthy individuals and is used as a treatment for several autoimmune neurological diseases (Willis et al., 2023). Animal studies have shown that injection of IVIg in a SCI mouse model has beneficial immunomodulatory effects (Willis et al., 2023; Nguyen et al., 2012; Brennan et al., 2016). As opposed to the antibody mixture used in IVIg, in the current study, individual antibodies were examined. In addition, immunoglobulins have been shown to be important in wound healing, and as such they can also be involved in post-SCI clearing processes (Nishio et al., 2009). Future research can now focus on uncovering the biological relevance of these anti-UH-SCI antibodies.

The location of the injury within the spinal cord was also shown to be an important determinant in antibody responses, as anti-UH-SCI.105 and anti-UH-SCI.110 antibody reactivity was reduced in patients with a SCI above T4. The level of the spinal cord that is damaged by the injury is highly determinative of the affected limbs and organs. The T4 level is the most caudal innervation level of the celiac ganglion which innervates the spleen (Noble et al., 2018). High thoracic SCI has been shown to cause SCI-induced immunodeficiency syndrome due to loss of supraspinal sympathetic flow of the spinal preganglionic neurons that innervate the spleen (Schwab et al., 2014; Kopp et al., 2023). Further, mice with a high SCI showed reduced antibody responses against specific antigens and more apoptotic B cells were found in their spleens (Lucin et al., 2007; Oropallo et al., 2012). The reduced anti-UH-SCI.105/110 antibody reactivity in patients with a high SCI is thus similar to these mouse studies, although the exact explanation for this is currently unknown and remains to examined.

There were some limitations in the set-up of our study. Firstly, the iSC cDNA phage display library was generated using spinal cord tissue of a single SCI patient. It is possible that not all spinal cord tracts or structures, and thus antigens, are equally represented in this library. However, the significance and added value of using spinal cord lesion tissue to identify novel antigens outweighs this limitation. Secondly, due to the cloning method of the cDNA phage display library construction, a bias towards cloning and expression of the C-terminal ends of proteins, different conformations and protein folding are possible (Vandormael et al., 2017). Thirdly, no significant differences were observed in antibody reactivity between separate AIS classes and no correlations were found with the AIS delta subscores, potentially due to limited patient numbers for some of the AIS classes. Future validation should include additional patients from each AIS group to examine the AIS improvement more specifically per AIS class. Further, for the analysis of AIS improvement, we compared an initial AIS grade, collected at 0–4 dpi, with a follow-up AIS grade, collected at different moments for each cohort. As such there is some variation in the time between the two AIS determinations when comparing the UH, AUS and DEU cohorts. Additionally, the initial AIS grade might not always be highly reliable, since it is sometimes assessed in unstable patients. Therefore, care must be taken when interpreting these data. Further, some patients were lost to follow-up, therefore, there is a lack of follow-up samples and data for some individuals. Finally, future studies should be focused on characterizing the antigenic target(s) and antibodies binding to UH-SCI targets, and on determining how these interactions influence SCI pathology and outcomes.

5. Conclusion

To conclude, antibodies against six novel antigens were identified by screening SCI patients using an unbiased approach. A panel of three of these antibodies, anti-UH-SCI104/109/110, demonstrated prognostic biomarker potential related to AIS improvement. Antibody reactivity was linked to a more beneficial injury outcome, as 31.3% of patients who showed AIS improvement were antibody positive, compared to only 4.8% of patients without AIS improvement. This led to a positive likelihood ratio of 6.56 for this biomarker panel. Combining these antibody biomarkers with the AIS and imaging results may further refine patient stratification for prognostic purposes. This integrative approach will also improve clinical trial inclusion and monitoring, ultimately facilitating the development of new therapies for SCI.

Ethics approval and consent to participate

The study was conducted in accordance with the Helsinki Declaration. The study involving human participants was reviewed and approved by the Medical Ethics Committee UHasselt and the local ethical committees of collaborating institutions. All patients/participants provided their written informed consent to participate in this study.

Funding

This study was supported by Hasselt University and grants from the Research Foundation Flanders (Fonds voor Wetenschappelijk Onderzoek, FWO 1S33521N) and from the Wings for Life Spinal Cord Research Foundation (WFL-BE-20/20). Work in the laboratory of MJR was supported by SpinalCure Australia.

Authors' contribution

AP, PV, JF and VS were involved in the study concept and design. CCMvLG, EMJC, DP, EB, JD, BD and SB recruited the patients and collected blood samples for the UHasselt cohort. MJR included and collected plasma samples and clinical data from the AUS cohort. AAM, DGO, LG, OM, IL recruited patients and collected sera and clinical data for the ESP and DEU cohorts. AP acquired the data. AP, JF, TV and VS analysed and interpreted the data. AP and JF drafted the manuscript and all authors revised the manuscript critically for important intellectual

content. All authors read and approved the final manuscript.

Declaration of competing interest

All authors declare to have no competing interests.

Acknowledgements

We would like to thank Igna Rutten, Kim Ulenaers and Ellen Sleurs (Hasselt University, Biomedical Research Institute) for excellent technical support and help with the sample and data collection, and Anna Ivanova (BioStat, Data Science Institute, Hasselt University, Belgium) for help with data analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2026.107306.

Data availability

Data will be made available on request.

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