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Original research

IgG₁ pan-neurofascin antibodies identify a severe yet treatable neuropathy with a high mortality

Janev Fehmi ,¹ Alexander J Davies,¹ Jon Walters,² Timothy Lavin,³ Ryan Keh,³ Alexander M Rossor,⁴ Tudor Munteanu,⁵ Norman Delanty,⁵ Rhys Roberts,⁶ Dirk Bäumer,⁶ Graham Lennox,⁷ Simon Rinaldi ^{1,8}

► Additional supplemental material is published online only. To view, please visit the journal online (<http://dx.doi.org/10.1136/jnnp-2021-326343>).

For numbered affiliations see end of article.

Correspondence to

Dr Simon Rinaldi, Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, OX1 2JD, UK; simon.rinaldi@nhs.net

Received 10 February 2021
Accepted 9 August 2021

ABSTRACT

Objectives We aimed to define the clinical and serological characteristics of pan-neurofascin antibody-positive patients.

Methods We tested serum from patients with suspected immune-mediated neuropathies for antibodies directed against nodal/paranodal protein antigens using a live cell-based assay and solid-phase platform. The clinical and serological characteristics of antibody-positive and seronegative patients were then compared. Sera positive for pan-neurofascin were also tested against live myelinated human stem cell-derived sensory neurons for antibody binding.

Results Eight patients with IgG₁-subclass antibodies directed against both isoforms of the nodal/paranodal cell adhesion molecule neurofascin were identified. All developed rapidly progressive tetraplegia. Cranial nerve deficits (100% vs 26%), autonomic dysfunction (75% vs 13%) and respiratory involvement (88% vs 14%) were more common than in seronegative patients. Four patients died despite treatment with one or more modalities of standard immunotherapy (intravenous immunoglobulin, steroids and/or plasmapheresis), whereas the four patients who later went on to receive the B cell-depleting therapy rituximab then began to show progressive functional improvements within weeks, became seronegative and ultimately became functionally independent.

Conclusions IgG₁ pan-neurofascin antibodies define a very severe autoimmune neuropathy. We urgently recommend trials of targeted immunotherapy for this serologically classified patient group.

channels at the node and juxtaparanode, respectively. This is ensured in part by cell adhesion molecules at the node (neurofascin-186 (NF186) and gliomedin) and paranode (contactin-1 (CNTN1), contactin-associated protein (Caspr1) and neurofascin-155 (NF155)).⁴

Pathology affecting the node, termed ‘nodo/paranodopathy’, has been linked to some forms of GBS,⁵ in which anti-ganglioside antibodies capable of inducing complement-mediated nodal injury are found.⁶ Recently, antibodies directed against nodal/paranodal proteins have been identified in patients meeting diagnostic criteria for CIDP.^{4 7–12}

Herein, we describe eight patients with a very severe neuropathy associated with ‘pan-neurofascin’ (panNF) IgG₁-subclass antibodies.

METHODS

From July 2017 to May 2020, we tested serum samples from 649 patients with suspected inflammatory neuropathies, and 210 controls, for IgG antibodies directed against nodal (NF186) and paranodal (NF155, CNTN1 and Caspr1) cell adhesion molecules, using a live, cell-based assay (CBA).¹² A standardised request form was used to collect clinical data. Further methodological details are given in the online supplemental appendix. The data that support the findings of this study are available from the corresponding author, on reasonable request.

RESULTS

Overall, 46 of 649 patients with suspected inflammatory neuropathies (7.1%) were positive for nodal/paranodal IgG-class antibodies. These antibodies were not detected in 210 controls (90 patients with other neurological diseases (20 with multiple sclerosis, 70 with antibody-positive central nervous system disorders) and 120 healthy individuals). Seropositive patients consisted of 17 (2.5%) with antibodies against NF155 alone, 1 (0.15%) with monospecific NF186 antibodies, 11 (1.6%) with CNTN1 antibodies alone, and 9 (1.3%) with CNTN1/Caspr1 complex antibodies. Patients with the latter two antibody specificities were included in previous studies.^{11 13 14}

Eight patients (1.2%) had IgG antibodies which cross-reacted with both the nodal/axonal NF186 and NF140 isoforms, and paranodal/glia NF155 isoform (subsequently termed ‘panNF’) (figure 1A and online supplemental figure 1). These antibodies

INTRODUCTION

Guillain-Barré syndrome (GBS) is characterised by flaccid limb weakness, suppressed deep tendon reflexes and a monophasic disease course reaching nadir within 4 weeks. Cranial nerve and autonomic dysfunction are common, and around 25% of affected individuals develop neuromuscular respiratory failure.¹ Demyelinating and axonal subtypes are defined by neurophysiology.² In chronic inflammatory demyelinating polyneuropathy (CIDP), disease activity and clinical progression continue for more than 8 weeks from onset.³

The node of Ranvier facilitates fast and efficient saltatory conduction along myelinated axons, which is reliant on the strict localisation of voltage-gated sodium channels and voltage-gated potassium



► <http://dx.doi.org/10.1136/jnnp-2021-326676>



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To cite: Fehmi J, Davies AJ, Walters J, et al. *J Neurol Neurosurg Psychiatry* Epub ahead of print: [please include Day Month Year]. doi:10.1136/jnnp-2021-326343

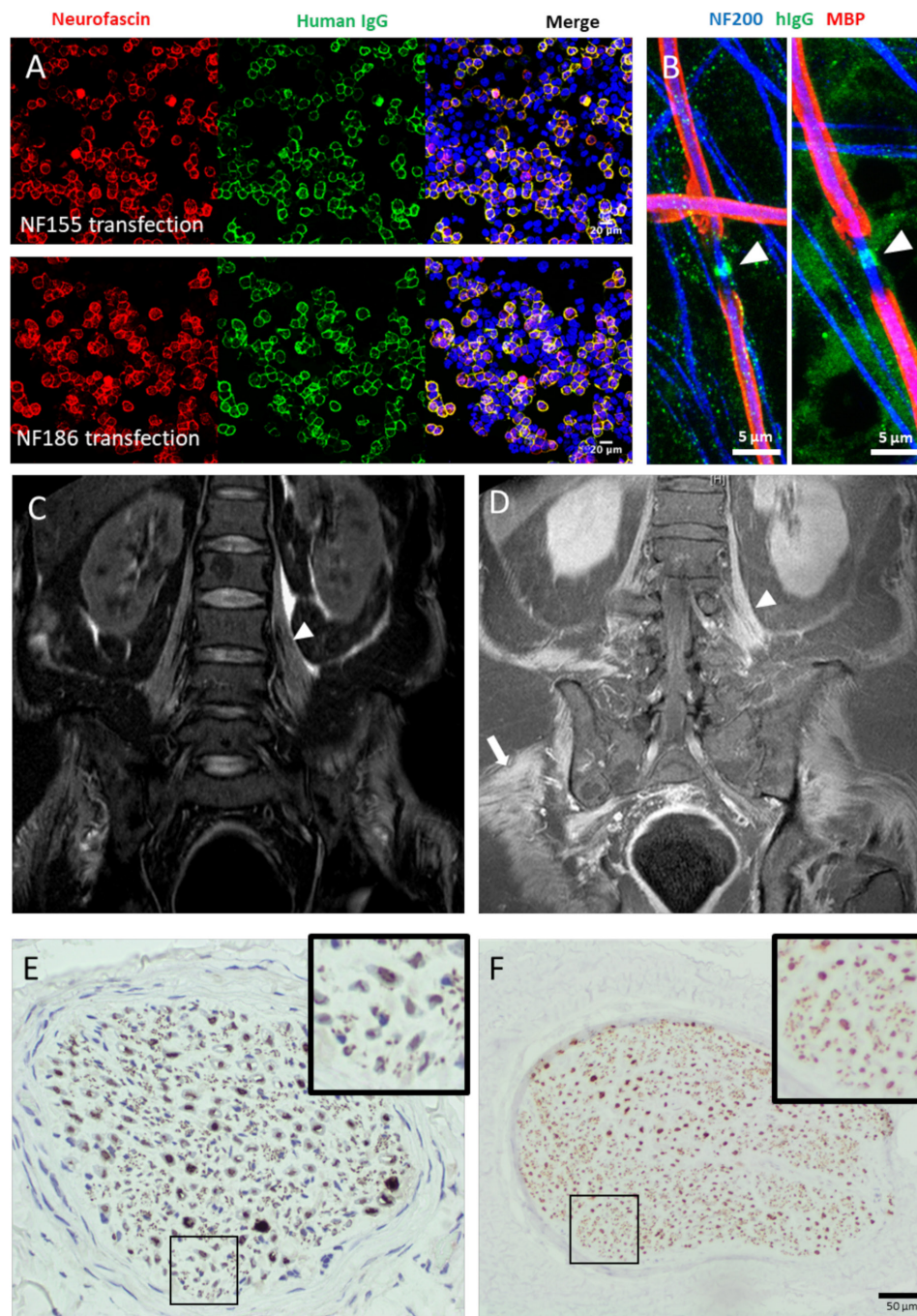


Figure 1 Serological, radiological and histological findings. (A) Cell-based assays using HEK293T cells transiently transfected to overexpress neurofascin-155 (NF155) (upper panels) or neurofascin-186 (NF186) (lower panels). Neurofascin (red) expression in the cell membrane is revealed by a commercial polyclonal antibody, and colocalises with human IgG (green) after exposure to acute-phase serum from the patients described in this series. (B) IgG (green) from two pan-neurofascin antibody-positive patient sera (left, P1; right, P6) is deposited at the node of Ranvier (arrowhead) after exposure to myelinating co-cultures. Axons (neurofilament-heavy, NF200, blue) were also observed weakly labelled with punctate IgG deposition in P1. Neither nodal or axonal labelling was observed in sera from healthy controls (data not shown). Myelin basic protein (MBP, red) defines the myelinated internode. (C,D) MRI of the lumbar spine, with coronal short tau inversion recovery (C) and post-contrast T1 (D) sequences, shows diffuse symmetric thickening and enhancement of lumbosacral plexus nerve roots (arrowhead), and enhancement of paraspinal, psoas, pelvic and proximal leg skeletal muscles (arrow). (E) Nerve biopsy from P6 stained for NFP shows reduced numbers of NFP positive axons (more clearly seen in the inset panels), and dense patches of staining, consistent with axonal degeneration. 'Near normal' NFP staining (F) is shown for comparison.

were exclusively IgG₁, while IgG₃ and/or IgG₄-subclass antibodies with panNF reactivity were not detected. In contrast, using the same assays, IgG₁ was exclusively detected in only two patients with NF155 monospecific antibodies, and in none of the CNTN1 or CNTN1/Caspr1-positive patients. IgG₄ was the

dominant subclass in the majority of patients in all the other antibody groups, though all other subclass antibodies could occasionally be detected at lower intensities (online supplemental table 1 and figure 1). All patients showed only one of the five distinct patterns of serological reactivity. Specifically, all eight

Table 1 Clinical features of patients with pan-neurofascin (panNF) antibodies and comparison with patients with neurofascin-155 (NF155), CNTN1, CNTN1/Caspr1 antibodies, and seronegative cohorts

Clinical feature (n, %)	PanNF (n=8)	NF155 (n=15)	CNTN1 (n=11)	CNTN1/Caspr1 (n=8)	Seronegative (n=194)	OR vs NF155	95% CI	OR vs seronegative	95% CI
Initial clinical diagnosis of GBS	5/8 (63%)	3/15 (20%)	3/11 (27%)	4/7 (57%)	38/185 (21%)	6.7	1.1 to 34.5	6.5	1.6 to 24.9
Acute/subacute progression	8/8 (100%)	7/15 (47%)	4/11 (36%)	5/7 (71%)	56/184 (30%)	∞	2.0 to ∞	∞	4.8 to ∞
Ataxia	3/8 (38%)	7/15 (47%)	7/11 (64%)	5/7 (71%)	62/158 (39%)	0.7	0.1 to 3.4	0.9	0.2 to 3.7
Tremor	0/8 (0%)	5/15 (33%)	3/11 (27%)	3/7 (43%)	39/154 (25%)	0	0 to 1.3	0	0 to 1.4
Neuropathic pain	4/8 (50%)	1/15 (7%)	7/11 (64%)	5/7 (71%)	49/134 (37%)	14	1.3 to 180	1.7	0.5 to 6.2
Cranial nerve palsy	8/8 (100%)	5/15 (33%)	5/11 (45%)	1/7 (14%)	41/156 (26%)	∞	3.3 to ∞	∞	5.7 to ∞
Autonomic dysfunction	6/8 (75%)	0/15 (0%)	2/11 (18%)	0/2	9/71 (13%)	∞	6.1 to ∞	20.7	3.9 to 105.4
Respiratory involvement	7/8 (88%)	0/15 (0%)	3/11 (27%)	0/7	25/185 (14%)	∞	9.9 to ∞	44.8	7.3 to 506.3
Nephrotic syndrome	3/8 (38%)	0/15 (0%)	9/11 (82%)	0/7	5/147 (3%)	∞	1.9 to ∞	17	3.5 to 73.7
MRI plexus/root abnormalities	2/4 (50%)	2/7 (29%)	2/7 (29%)	5/6 (83%)	15/53 (28%)	6.7	1.1 to 34.5	6.5	1.6 to 24.9
Nadir mRS >4	8/8 (100%)	3/15 (20%)	4/11 (36%)	2/7 (29%)	38/185 (21%)				
						Significant vs NF155	P value	Significant vs seronegative	P value
Nadir mRS (median, range)	5.5 (5–6)	4 (2–5)	4 (2–6)	4 (4–5)	3 (1–5)	**	0.006	***	<0.001
CSF protein (g/L) (median, range)	0.48 (0.34–0.62)	1.65 (0.61–7.05)	2 (0.24–5.9)	2.7 (0.91–4.46)	0.87 (0.18–6.0)	***	<0.001	*	0.04

The 95% CI of the OR was calculated by the Baptista-Pike method. Nadir mRS and CSF protein were compared by a two-tailed Kruskal-Wallis test with Dunn's correction for multiple comparisons. The patients with CNTN1 and CNTN1/Caspr1 antibodies were included in previous studies.^{11,13}

*p<0.05, **p<0.01, ***p<0.001.

Caspr1, contactin-associated protein; CNTN1, contactin-1; CSF, cerebrospinal fluid; GBS, Guillain-Barré syndrome; mRS, modified Rankin Scale.

panNF-positive sera and all 17 NF155-positive sera were negative for CNTN1 and CNTN1/Caspr1 antibodies, all CNTN1 and CNTN1/Caspr1-positive patients were negative for both NF155 and NF186 antibodies, and all CNTN1/Caspr1-positive patients were also negative for CNTN1 antibodies.

As the clinical associations of IgG₁-subclass panNF antibodies have not been reported, we sought to assess the characteristics of this serologically defined cohort and compare these with seronegative patients and those seropositive for other nodal/paranodal antibodies, particularly focusing on those with NF155 monospecific antibodies only.

Clinical features

Clinical details were available for all eight panNF antibody-positive patients, 15 of 17 NF155, 11 of 11 CNTN1, 8 of 9 CNTN1/Caspr1-positive patients, and 194 of 603 seronegative cases (table 1). The median age of this patient cohort was 68.5 years (range 43–78), and the majority male (75%). Overall, there were significant differences between these groups in the frequency of patients initially diagnosed with GBS (p=0.03), experiencing tremor (p=0.03) or neuropathic pain (p=0.01), or developing cranial nerve palsies, autonomic dysfunction, respiratory involvement or episodes of acute deterioration (all p<0.001, multiple X² tests). There were no significant differences in the frequencies of patients with ataxia (p=0.26) or MRI plexus/nerve root abnormalities (p=0.09, X² tests).

PanNF antibody-positive patients were all very severely affected and had rapidly developed profound tetraplegia. Compared with seronegative patients, they were more likely to have presented following acute or subacute deterioration (OR ∞, 95% CI 4.8 to ∞), and to have received an initial clinical diagnosis of GBS (OR 6.5, 95% CI 1.6 to 24.9). Nadir modified Rankin Scale (mRS) scores (median 5.5, range 5–6) were significantly higher than those of the NF155 monospecific antibody-positive (median

4, range 2–5, p=0.005), CNTN1 antibody-positive (median 4, range 2–6, p=0.05) and seronegative patients (median 3, range 1–5, p<0.001), but non-significantly higher than those with CNTN1/Caspr1 complex antibodies (median 4, range 4–5, p=0.59, Kruskal-Wallis test with Dunn's correction for multiple comparisons) (table 1 and online supplemental figure 2A). Cranial nerve palsies (100%), autonomic dysfunction (75%) and respiratory compromise (88%) were also more frequent than in seronegative patients and those with other nodal/paranodal antibodies (table 1 and online supplemental table 2). A small number of patients (2 of 7) had evidence of papilloedema. Concurrent presentation with nephrotic syndrome was notable (38%) but not as frequent as reported in the CNTN1 antibody-positive group (82%).¹¹ Ataxia (3 of 8) and neuropathic pain (4 of 8) were occasional features in panNF-positive patients, although less common than in CNTN1 and CNTN1/Caspr1 antibody-positive patients. Clinical vignettes for patients 1, 5 and 6 are given in the online supplemental appendix. Patient 4 was described in a recent case report.¹⁵

Laboratory findings

During work-up of their neuropathy or shortly thereafter, two panNF antibody-positive patients were found to have an IgG-lambda paraprotein and were subsequently diagnosed with lymphoproliferative disorders (Hodgkin's lymphoma and chronic lymphocytic leukaemia). A third (P8—online supplemental table 2) was found to have a clonal urinary lambda light chain, without a serum paraprotein, that was not further investigated prior to his death. Three patients had features of nephrotic syndrome (peripheral oedema and hypoalbuminaemia) which had developed in parallel with their neuropathy, and, in two, urinary protein levels were analysed and nephrotic range proteinuria confirmed. All patients were otherwise negative for standard neuropathy screening bloods, including anti-GM1

and GQ1b-ganglioside antibodies. In panNF-positive patients, cerebrospinal fluid (CSF) protein was either normal or only marginally elevated at presentation (evaluated in all seven patients tested within 14 days of onset, median 0.51 g/L, range 0.34–0.62), non-significantly lower than that of the seronegative patients (median 0.87 g/L, range 0.18–6, $p=0.09$) and significantly lower than in all other seropositive cohorts ($p=0.007$ vs CNTN1 and $p<0.001$ vs NF155 or CNTN1/Caspr1, Kruskal-Wallis with Dunn's correction for multiple comparisons) (online supplemental figure 2D). CSF white cell counts were invariably normal (range 1–3/ μ L) with unremarkable cytology and flow cytometry.

Using the CBA, all patients had IgG₁-subclass antibodies reactive against both paranodal NF155 and nodal NF186 and NF140 isoforms, and were negative on IgG₂, IgG₃ and IgG₄-subclass-specific assays (online supplemental table 1 and online supplemental figure 1B). Endpoint titres ranged from 1:400 to 1:3200 (online supplemental figure 4A). Six of seven patients tested were also positive on a neurofascin ELISA, although the endpoint titres were consistently lower than those obtained by CBA. In contrast, ELISA appeared to be slightly more sensitive for the detection of CNTN1 antibodies (online supplemental figure 3C). Detection of subclass-specific antibodies by ELISA was also less sensitive than with CBA, with an IgG₁ signal above background only being detected in only two patient samples (online supplemental figure 4B).

Five of the seven panNF sera tested showed nodal binding, and two additional axonal binding, in live, myelinating co-cultures (figure 1B and online supplemental figure 4A) generated from human-induced pluripotent stem cell-derived neurons. These sera showed a pattern distinct from that seen with NF155 mono-specific sera (online supplemental figure 4A).¹⁶ No patients had IgG₄ panNF antibodies or developed these during follow-up.

The antigen specificity of panNF antibodies detected in patient sera was confirmed by pre-adsorption assays in CBA and myelinating co-cultures. Pre-incubation of sera with soluble NF155 or NF186 protein abrogated cell membrane IgG labelling in transiently transfected human embryonic kidney (HEK293) cells (online supplemental figure 1C,D), and nodal/axonal labelling in co-cultures (online supplemental figure 4).

ELISA was performed to assess whether panNF antibodies in the sera of the two patients with IgG-lambda paraproteins exclusively used lambda light chains. In each case, both kappa and lambda light chain-containing panNF antibodies were detected (data not shown).

Neurophysiology

Neurophysiological results were available for six of eight patients. In one, the nerves were inexcitable when first assessed 2 weeks after onset. In four of six, conduction slowing on initial studies was considered to indicate demyelination. However, five of six showed conduction block without temporal dispersion, suggestive of nodal pathology.⁵ In three, follow-up studies 3–4 weeks later revealed very reduced or unrecordable compound muscle action potentials and electromyographic findings consistent with severe axonal degeneration. Detailed neurophysiological results are given in online supplemental table 3.

Imaging

Four patients were examined by MRI. In one, symmetric enhancement and thickening of the lumbosacral plexus nerve roots, as well as enhancement of paraspinal, pelvic and proximal lower limb muscles, were observed. T2 hyperintensities

of the brachial plexus and L5–S2 roots but no thickening or enhancement were seen in another (figure 1C,D).

Histology

Nerve biopsy was performed in two patients. In both cases, this demonstrated axonal loss, without any features of cellular infiltration, inflammation, segmental demyelination, amyloid or vasculitis (figure 1E and online supplemental figure 5). Electron microscopy was performed in one patient and did not show any evidence of paranodal retraction/detachment. One patient had a necrotising myopathy on muscle biopsy with a normal creatine kinase.

Treatment and outcome

All patients received intravenous immunoglobulin (IVIg) 2 g/kg over 5 days. In six cases, this was not associated with any perceptible benefit. In two cases, there was a minor and/or transient neurological improvement. Six patients received at least one cycle of plasma exchange (PLEX), with three patients showing slight but non-sustained neurological recovery. Further detail and physician reported assessments of response are given in online supplemental tables 2 and 4, as well as treatment time lines in online supplemental figure 6. Four patients died. One suffered a cardiorespiratory arrest 8 days after presentation, and in the absence of recovery of cortical function, ventilatory support was withdrawn 10 days later. In another, recurrent pulmonary infections and the absence of any neurological recovery after IVIg and two cycles of PLEX led to the withdrawal of ventilatory support on day 108. A third patient, with comorbid metastatic breast cancer, declined artificial ventilation, having failed to respond to steroids, IVIg, PLEX and cyclophosphamide, and died on day 93. Most recently, during the COVID-19 pandemic, intensive care and mechanical ventilation were not deemed appropriate for one patient who was SARS-CoV-2 PCR negative, but developed increasing breathlessness and tachypnoea on day 12, and died 48 hours later. In both patients with nephrotic range proteinuria, this was still apparent when last measured shortly before their death.

After initial rounds of treatment with IVIg, PLEX and steroids, following which panNF antibodies were detected, the remaining four patients received rituximab 3–4 months (1 g repeated after 2 weeks) into their illness, following no or minimal and transient apparent responses to other therapies, as assessed by their treating physicians. All four patients were found to be panNF antibody positive from their initial rounds of therapy, prior to rituximab treatment being started. Two patients additionally received combination chemotherapy for newly diagnosed haematological disorders. In one (P4), this began concurrently with rituximab, and was started (P3) 5 months later in the other. All four rituximab-treated patients made progressive functional improvements, regained independent mobility and were ultimately discharged home, often via a rehabilitation facility. All showed improvements of at least 3 points on the mRS within 6 months of rituximab treatment, and two became asymptomatic by 9 months (online supplemental figure 2B). In the one rituximab-treated patient with nephrotic syndrome, the serum albumin normalised in parallel with neurological improvement. In all four patients, neurofascin antibodies were negative when retested 4–11 months after rituximab. One of these four patients, having returned almost to his baseline (mRS=1, minimal residual symptoms) had a return of motor and sensory symptoms, approximately

18 months after receiving a single cycle of rituximab. He developed marked arm weakness and became immobile over a few weeks, and redeveloped hypoalbuminaemia in parallel. At this stage, panNF antibodies were again positive, though at low titre (1:100 and 1:200). Again, IgG₁ was the dominant subclass. He was retreated with steroids and a second cycle of rituximab, and again improved, regaining independence.

DISCUSSION

This is the first reported series of patients with IgG₁-subclass, panNF antibodies. These antibodies are shown to be associated with an extremely severe and rapidly progressive neuropathy.

To date, antibodies directed against nodal and paranodal cell adhesion molecules have largely been described in patients meeting diagnostic for CIDP. In this context, IgG₄ antibodies have been reported as pathologically important markers of a clinically distinct CIDP subgroup, whereas patients with only non-IgG₄-subclass antibodies were found to be indistinguishable from seronegative individuals.¹⁷ In our series, patients with IgG₁-subclass panNF antibodies, who had significantly different clinical features and disease severity compared with identically identified seronegative controls, were more likely to have received an initial clinical diagnosis of GBS.

In the five panNF patients in our series diagnosed with GBS, there was no clinical basis to reclassify their neuropathy as CIDP. None of these patients had clinical evidence of progressive neuropathy beyond 4 weeks from onset. Three showed one or two transient fluctuations after treatment, though all within 8 weeks of onset.¹⁸ The three other patients met the clinical and electrodiagnostic criteria for definite CIDP,³ yet their neuropathy was likely to represent a nodo/paranodopathy rather than being primarily demyelinating.

At present, the distinction between GBS and CIDP can only be confidently drawn when ongoing deterioration is seen more than 8 weeks after onset.¹⁸ This criterion is of little to no use in informing therapeutic decisions during the early phases of a rapidly progressive, but potentially chronically persistent, and treatable autoimmune/inflammatory neuropathy. As the immunopathological process in GBS is typically conceptualised as monophasic and short lived, a diagnosis of GBS provides little impetus to give immunomodulatory therapy outside of the acute phase. We therefore believe that testing for nodal/paranodal antibodies is justified in any patient with a GBS-like presentation, and in particular in severe cases poorly responsive to standard immunotherapy. Of note, 36.6% of our nodal/paranodal antibody-positive cohort overall received an initial clinical diagnosis of GBS.

The improvements after rituximab seen here occurred several months into the illness, after responses to steroids, IVIg and/or PLEX were deemed inadequate. Our observations suggest that a more persistent autoimmune response can potentially drive ongoing axonal loss, and prevent recovery, in patients whose initial presentation nonetheless resembles GBS, and in whom clinical deterioration greater than 8 weeks from onset is not always apparent, preventing a diagnosis of CIDP. They also raise the possibility that treatment approaches that lead to more prolonged suppressions of antibody titres may be required for sustained neurological improvement in patients with nodal/paranodal antibodies.¹² Serological results and biomarkers of ongoing peripheral nerve injury may in future prove to have greater utility in guiding these treatment decisions and may ultimately supersede clinical categorisation. The earlier use of potent immunotherapies with longer duration

of action in such patients may be more effective in reducing long-term disability.

In this observational study, patients received multiple different therapies at different time points. We cannot, therefore, provide any clear evidence on the efficacy of any particular therapy in this setting. It is possible that the four patients who survived long enough to be treated with rituximab would have started to improve even if this therapy had not been given. We also cannot determine to what extent chemotherapy for the underlying haematological malignancies contributed to neurological recovery in two of the patients. Future trials should address whether the earlier use of targeted immunotherapy in such serologically defined cohorts could ameliorate the very severe disease course seen here.

Cancer is rarely associated with GBS/CIDP,¹⁹ though onco-neural antibodies have not previously been identified. The frequency of IgG/lambd paraprotein-associated lymphoproliferative disorders and solid organ malignancy (4 of 8 overall) in this cohort suggests that panNF antibodies may be responsible for some such cases.

Antibodies against the paranodal isoform NF155 have been linked to 'atypical CIDP'. NF155 monospecific seropositive individuals are often younger men with predominantly distal weakness, sensory ataxia and tremor. NF155 autoantibodies are predominantly of the non-complement-fixing IgG₄ subclass and the response to IVIg is typically poor.⁸ Antibodies against nodal isoforms of NF140/186 have been described in 12 patients so far. Four out of the five originally described patients were diagnosed with CIDP¹⁰ and had predominantly IgG₄-subclass antibodies, but three of four improved after IVIg. The remaining patient had IgG₃-subclass antibodies and did not improve after IVIg, but did so after steroids and PLEX. Similar to our cohort, two of five were found to have nephrotic syndrome. This complication appears less common than in patients with CNTN1 antibodies,¹¹ and may be explained by the expression of NF186 by glomerular podocytes as well as neurons.²⁰ A further five severely affected neurofascin IgG₃ seropositive patients with a reported poor response to standard therapies have more recently been described. In all but one case, antibodies cross-reacted with both NF140/186 and NF155 in CBAs, as in our cohort. One patient treated with rituximab subsequently improved, as did another, apparently spontaneously, starting 3 months into his illness.^{21–23} The patients with IgG₃ NF140/186 and panNF antibodies previously reported seem most similar to our cohort. The differences in the panNF antibody-subclass distribution detected in our study may be due to technical factors related to the diagnostic assays. The secondary antibodies used here were shown to recognise recombinant human IgG of the relevant subclass in ELISA, did not cross-react with any of the other subclasses and were all detected with the same tertiary antibody. Whereas most other studies used ELISA, we found CBAs to be more sensitive in the detection of panNF antibodies and to determine subclass (online supplemental figure 3). This has also been reported with other antigenic targets²⁴ and should stimulate calls for a multicentre, interlaboratory, blinded comparison study of solid-state and live cell-based nodal/paranodal antibody assays.

We have shown that the antibodies from these patients' sera specifically target three neurofascin isoforms, and that pre-adsorption of panNF sera with neurofascin isoforms 155 or 186 abrogates IgG binding to cells expressing NF186 or NF155, and nodes of Ranvier within a live neuronal culture. This suggests the panNF antibodies recognise an epitope common

to both isoforms. NF155, NF186 and NF140 are isoforms of the same protein that differ in the arrangement and composition of their fibronectin domains. Previous studies have shown that panNF antibodies specifically recognise the immunoglobulin domain shared between isoforms,^{10 23} in contrast to NF155 antibodies which require the third fibronectin domain unique to this isoform in order to bind.^{25 26}

Whether panNF antibodies are pathogenic or simply an epiphenomenon remains to be determined. The predominance of the IgG₁ subclass in this panNF patient cohort suggests complement may play a potential role in antibody pathogenicity. This important question should be addressed in future studies.

In summary, the observations herein provide further rationale for nodal/paranodal antibody testing in GBS-like presentations. They highlight the importance of testing against *both* glial and neuronal neurofascin isoforms (to distinguish panNF from NF155 monospecific antibodies) and determining the IgG subclass, as this may also influence the clinical phenotype and response to treatment. We advocate that such testing should increasingly form part of the routine diagnostic process. If it is not feasible to test nodal/paranodal antibodies in all GBS-like presentations, we believe antibody testing could be prioritised for those with severe disease, especially if there are no signs of response to the first round of treatment, and certainly if there is evidence of nephrotic syndrome or any suggestion of a more chronic autoimmune neuropathic process.

The possible benefit of rituximab in patients with panNF antibodies reported here should be evaluated in a well-conducted clinical trial, the design of which must consider the potentially grave outcome in this serologically defined cohort.

Author affiliations

¹Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, UK

²Department of Neurology, Morriston Hospital, Swansea, UK

³Manchester Centre for Clinical Neuroscience, Salford Royal NHS Foundation Trust, Salford, UK

⁴MRC Centre for Neuromuscular Disease, National Hospital for Neurology and Neurosurgery, London, UK

⁵Department of Neurology, Beaumont Hospital, Dublin, Ireland

⁶Department of Clinical Neurosciences, Cambridge Institute for Medical Research, Cambridge, UK

⁷Department of Neurology, Great Western Hospital, Swindon, UK

⁸Department of Neurology, Oxford University Hospitals NHS Foundation Trust, Oxford, UK

Acknowledgements The 'near normal' nerve biopsy image was kindly supplied by Dr Monika Hofer, Consultant Neuropathologist, John Radcliffe Hospital, Oxford. The rabbit anti-Caspr antibody used to stain paranodes in myelinating co-cultures was a kind gift from Dr Manzoor Bhat, UT Health Science Center, San Antonio.

Contributors SR designed and conceived the study. Patient data and samples were contributed by SR, JW, TL, RK, AMR, TM, ND, RR, DB and GL. Patient samples analysed by JF using CBA and ELISA, and by JF and AJD using myelinated co-cultures. SR and JF wrote the original manuscript. SR, JF and AJD revised the work critically for important intellectual content. All authors approved the final manuscript.

Funding We acknowledge funding support from the GBS/CIDP Foundation International (Benson Fellowship awarded to JF, grant 1709HM001/SB17) and the Medical Research Council (UK) (grant MR/P008399/1 supports SR and AJD).

Disclaimer The funders had no role in the design of this study or in the interpretation or presentation of its results.

Competing interests JF has received research grants from the Guarantors of Brain and GBS/CIDP Foundation International. TL has received speaker's honoraria from CSL Behring and Akcea. AJD, JW, RK, AMR, TM, ND, RR, DB and GL report no disclosures or conflicts of interest. SR has received speaker's honoraria from Fresenius, Alnylam and Excemed, and payments to provide expert medicolegal advice on inflammatory neuropathies and their treatment. He has received complimentary registration and prize money from the Peripheral Nerve Society, and a travel bursary from the European School of Neuroimmunology. He is a member of the GBS and

Associated Inflammatory Neuropathies (GAIN) patient charity medical advisory board. He runs a not-for-profit nodal/paranodal antibody testing service at the Nuffield Department of Clinical Neurosciences, John Radcliffe Hospital, Oxford, UK, in partnership with Clinical Laboratory Immunology of Oxford University Hospitals.

Patient consent for publication Not required.

Ethics approval This study involves human participants and was approved by the South Central Oxford—a Research Ethics Committee (approval number 14/SC/0280).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

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ORCID iDs

Janev Fehmi <http://orcid.org/0000-0002-2329-5708>

Simon Rinaldi <http://orcid.org/0000-0003-2039-3457>

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Supplementary methods

Nodal/paranodal antibody cell-based assays

All sera were initially screened using a live, transiently transfected cell-based assay (CBA), following previously described methods with slight modification.[1] Human embryonic kidney 293T ("HEK") cells were plated at a density of 75,000 cells/13mm coverslip, and mono-transfected with either neurofascin-155 (NF155, *NFASC*) (RC228652, Origene), neurofascin-186 (NF186, courtesy of Jerome Devaux, University of Marseille), or co-transfected with contactin-1 (*CNTN1*, EXA1153-MO29 Genecopoeia, Maryland, US) and contactin-associated protein ('1') (*CASPR1*, EXMO417-MO2 Genecopoeia, Maryland, US), diluted in Jet-PEI transfection reagent (101-10; Polyplus). Patient sera were screened using a dilution to 1:100 in DMEM/1% bovine serum albumin (BSA) for incubation with live neurofascin transfected cells (1h at 37°C), then titred out to 1:6400. Co-incubation with commercial chicken anti-neurofascin primary antibody, (1:1000) (Cat no. AF3235; R&D Systems, Bio-Techne) was used to confirm successful transfection and to assess for co-localisation with any bound human IgG. Cells were then fixed with 4% paraformaldehyde and secondary antibody incubation was with goat anti-human IgG-Fc specific-Alexa Fluor 488 (1:750) (Cat no. H10120; Life Tech) and goat anti-Chicken Alexa Fluor 546 1:1000 (Cat no. A11040; Life Tech). To determine antibody subclass unconjugated mouse anti-human IgG subclass 1-4 antibodies were used at 1:100 (Cat nos. I2513, I25635, I7260 I7385; Sigma-Aldrich, Merck) with the fluorescently tagged tertiary antibody goat anti-mouse Alexa Fluor 488 (1:750) (Cat no. A11029; Life Tech).

ELISA

ELISA was performed as previously described.[2] In brief, Nunc Maxisorp ELISA plates (Fisher Scientific) were coated with 100 µl/well of PBS containing human recombinant neurofascin-155 (NF155) (8208-NF, R&D systems), NF186 (TP329070, OriGene Technologies) or CNTN1 (10383-H08H, Sino Biological Inc) at 1 mg/ml. Blank wells were incubated with carrier only. After overnight incubation at 4°C, the coating solution was tipped off and wells were blocked with 5% milk for 1h at room temperature. Sera were incubated for 1h at room temperature, and initially screened at 1:100 dilution in 5% milk. Following a wash step through 5 changes of PBS, anti-human IgG (Fc-specific) peroxidase-conjugated anti-human IgG (A0170, Sigma) was applied at 1:3000 in 5% milk and incubated for 1h at room temperature, then washed as before. The detection reaction was performed using o-Phenylenediamine dihydrochloride (OPD, Sigma), applied for 20 minutes in the dark, then terminated with 4M H₂SO₄. Optical densities were measured at 492nm. Wells with ODs greater than 0.1 above uncoated control wells were considered positive. The end-point titre of positive samples was then assessed by serial doubling dilution from 1:100 to 1:6400. The end-point titre was defined as the highest dilution with an OD greater than 0.1 above the uncoated control. To assess the specificity of the subclass specific secondary antibodies used in the CBA, recombinant human IgG subclass proteins (HCA049, HCA50, HCA178, HCA193, HCA194, HCA195, Bio-Rad) were coated as above, and then exposed to the unconjugated mouse anti-human IgG subclass 1-4 antibodies for 1h at room temperature (IgG1 1:1000, IgG2 1:250, IgG3 1:100, IgG4 1:250). Following a wash step, a HRP anti-mouse-IgG antibody (A4416, Sigma) was applied at 1:1000 for 1h, washed off, and binding detected as before. A standard curve was created for each subclass antibody using duplicate wells coated with the recombinant subclass proteins on the same plate as patient sera, and the limit of the blank (highest detectable analyte in duplicate blank wells) and limit of detection (lowest analyte concentration distinguishable from the blank) for each subclass were calculated.[3] Optical densities 5 standard deviations of a low concentration analyte above the limit of the blank were considered positive.

Collection of clinical data

A request form (over page) was sent to all clinicians submitting samples for nodal/paranodal antibody testing and initial information was requested before test results were known. Clinical data was obtained from all 8 patients with pan-neurofascin antibodies, 15/17 with neurofascin-155 antibodies, and 194/606 seronegative individuals. Two attempts were made to collect missing data by re-contacting referring clinicians directly. All patients without clinical data returns following these efforts were omitted from the subsequent analysis. Follow up times varied from 3 years to 2 months for the antibody positive cohort (average 19 months), with the shortest follow up for the surviving pan-neurofascin patient being 9 months. Nodal/paranodal and seronegative neuropathy patients seen in Oxford were recruited to an observational study. This study was approved by the NHS National Research Ethics Service Committee (South Central – Oxford A, 14/SC/0280). The use of de-identified clinical data from other patients to audit the relative value of available and novel methods to

determine autoantibodies, based on the gold standard of immune-mediated clinical phenotypes, was approved by the Oxford University Hospitals NHS Trust clinical audit leads (ID 5106).

Analysis of clinical associations

Contingency data for the presence or absence of 10 core clinical features (diagnostic category, onset/progression, ataxia, tremor, neuropathic pain, cranial nerve palsy, autonomic dysfunction, respiratory involvement, nephrotic syndrome, and MRI abnormalities) were analysed by Chi-square tests without family-wise correction for error rate. For subsequent comparisons between panNF positive patients, NF155 antibody positive patients, and seronegative patients, Fisher's exact test was used. Point estimates of the odds ratio are reported, with 95% confidence intervals calculated by the Baptista-Pike method. The widths of the intervals have not been adjusted for multiplicity and as such the inferences drawn may not be reproducible. The nadir modified Rankin score and CSF protein level at diagnosis were compared by the Kruskal-Wallis test with Dunn's correction for multiple comparisons.

Preparation and immunofluorescence of myelinating co-cultures

Sera were assessed for topographical binding using myelinated co-cultures. These were generated using sensory neurons derived from induced pluripotent stem cells (iPSC) according to a previously described protocol.^[4] iPSCs were differentiated into neurons over 10 days, and myelinated with rat Schwann cells after four weeks to establish mature myelinated co-cultures. For immunolabelling, these were incubated with the patient's sera, diluted at 1:100, for 1h at 37°C, washed in 3 changes of DMEM/HEPES, then fixed with 2% PFA before incubation with secondary antibody goat anti-human IgG AF488 (Cat no. A11013; ThermoFisher Scientific). The cultures were then permeabilised with ice-cold methanol before co-staining was used to localise specific peripheral nerve subdomains. This involved either chicken anti-Neurofilament 200 (1:10,000) (Cat no. 4680; Abcam) if axons were being labelled, or rabbit anti-Caspr (1:1000, gift from Dr Manzoor Bhat, UT Health Science Center San Antonio) if paranodes were labelled, and rat anti-myelin basic protein (1:500) (Cat no. 7349; Abcam). The secondary antibodies combinations then used were biotinylated goat anti-chicken IgY (1:500) (Cat no. BA9010; Vector lab) and goat anti-rat IgG Alexa Fluor 546 (1:1000) (Cat no. A11081 Life Tech), followed by Streptavidin pacific blue (1:500) (Cat no. S11222 Life Tech), or goat anti-rabbit IgG Alexa Fluor 546 (1:400) (Cat no. A11010; ThermoFisher Scientific) and biotinylated goat anti-rat IgG (1:1000) (Cat no. BA9400; Vector lab), followed by Streptavidin pacific blue. Fluorescence images of IgG nodal labelling in myelinating cultures were acquired on a laser scanning confocal microscope (LSM 700, Zeiss) using the x63 or x20 objectives. 10-15 z-sections at 0.5 µm interval were exported as maximum projection images. Brightness and contrast were adjusted for presentation.

Pre-adsorption

Pre-adsorption assays were performed by first incubating sera with 1µg of the same recombinant NF155 or NF186 protein used in the ELISA, at 4°C overnight on a rotating mixer. Pre-incubated sera was then used in the myelinating co-culture and cell-based assays in the same way as previously described for non-pre-adsorbed sera.

Request Form for Paranodal / Nodal Antibody Testing

Clinical Data

Date of neuropathy onset:

Age at diagnosis:

☐ Prodromal illness/trigger (please specify):

Start date for prodrome/trigger:

Initial diagnosis☐ GBS ☐ Typical CIDP ☐ MMN☐ Atypical CIDP / Other (specify):Current diagnosis☐ GBS ☐ CIDP ☐ MMN☐ Atypical CIDP / Other (specify):**If the current diagnosis is GBS, CIDP, or MMN please answer the following (tick all that apply):**Clinical course☐ Relapsing-remitting☐ Progressive☐ MonophasicOnset / progression☐ Acute (<4 weeks)☐ Subacute (4-8 weeks)☐ Chronic (>8 weeks)Weakness (Yes/No) (Tick all that apply)☐ Arms☐ Proximal☐ Asymmetric☐ Distal☐ Symmetric☐ Legs☐ Proximal☐ Asymmetric☐ Distal☐ SymmetricSensory deficit (Tick all that apply)☐ Arms☐ Vibration ☐ Pinprick ☐ JPS☐ Legs☐ Vibration ☐ Pinprick ☐ JPS☐ Ataxia☐ Tremor☐ Neuropathic pain

0 /10 Severity (1-10)

Reflexes☐ Absent☐ Decreased☐ Normal☐ Brisk☐ Cranial nerve involvement (specify)☐ Autonomic involvement (please specify)Respiratory involvement☐ Current☐ Previous☐ NoneEvidence ofnephrotic syndrome☐ Proteinuria (level:)☐ Hypoalbuminaemia (nadir level:☐ Oedema☐ Not assessed

g/L)

☐ NoneSeverityModified Rankin score (at nadir): 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐

Investigations

☐ CSF (at diagnosis) Date:
 Protein: g/L WCC: RCC:
 OCBs: (select from drop down menu) Other:

Neurophysiology

Overall impression: ☐ Demyelinating ☐ Axonal ☐ Mixed Other (specify)

☐ Motor involvement (describe core features):
☐ Sensory involvement (describe core features):

Other Antibodies

Gangliosides ☐ Positive ☐ Negative ☐ Not done
 Anti-MAG ☐ Positive ☐ Negative ☐ Not done

Paraprotein ☐ Positive ☐ Negative ☐ Not done
☐ IgG ☐ IgM ☐ IgA ☐ Kappa ☐ Lambda
 Level: g/L

Imaging

MRI lumbar roots ☐ Abnormal ☐ Normal ☐ Not done
 Specify:

Treatment and Outcome

			Response			
Trialled			Good	Partial	None	Worse
IVIg	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Steroids	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Plasma Ex.	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Other (specify)			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Other (specify)			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Current Disease Activity

1. Cure: ≥5 years off treatment ☐ B. Abnormal examination, stable/improving
☐ A. Normal examination
2. Remission: <5 years off treatment ☐ B. Abnormal examination, stable/improving
☐ A. Normal examination
3. Stable active disease: ≥1 year, on treatment ☐ B. Abnormal examination, stable/improving
☐ A. Normal examination
4. Improvement: ≥3 months <1 year, on Treatment ☐ B. Abnormal examination, stable/improving
☐ A. Normal examination
5. Unstable active disease: abnormal examination with progressive or relapsing course*
☐ A. Treatment naïve or <3 months
☐ B. Off treatment
☐ C. On treatment

Modified Rankin score (at best post treatment):
 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐

	Dominant subclass				Subclasses detected			
	PanNF (n=8)	NF155 (n=17)	CNTN1 (n=11)	CNTN1/Caspr1 (n=9)	PanNF (n=8)	NF155 (n=15)	CNTN1 (n=11)	CNTN1/Caspr1 (n=9)
None	0	1	0	0	-	-	-	-
IgG1	8	4	1	2	8	11	8	7
IgG2	0	0	0	0	0	10	6	4
IgG3	0	0	0	0	0	1	4	1
IgG4	0	9	6	5	0	14	11	8
IgG4=1	0	2	4	1	-	-	-	-
IgG4=2	0	1	0	1	-	-	-	-

Supplementary Table 1 - Subclass distribution of pan-neurofascin versus neurofascin-155, contactin-1 and contactin1/caspr1 complex reactive antibodies

In all cases, IgG1 was the only subclass detected for pan-neurofascin antibodies. In contrast, with other antibody groups, IgG4 was most often the dominant subclass detected, but IgG1 and to a lesser extent IgG2 were also frequently observed.

Age at onset / Gender	Clinical									Investigations				Treatment (response)					Outcome
	Initial Clinical Diagnosis	Motor/Sensory	CN palsy	Autonomic	Ataxia	Pain	Associated conditions	ITU / MV	Nadir mRS	Para-protein	NCS (Overall impression)	Nerve biopsy	Anti-NF155/186 Titre (CBA)	Steroids	IVIg	PLEX	RTX	Other	
P1	GBS	M&S	✓ ^a	✓	×	✓	Nephrotic syndrome	✓	6	ND	Inexcitable	×	1:6400/1:3200	×	✓	✓	×	×	Death (Day 110)
P2	CIDP	Motor only	✓ ^b	✓	×	✓	Breast Cancer	✓ (declined MV)	6	ND	Demyelinating	×	1:3200/1:3200	✓	✓	✓	×	CYC	Death (Day 93)
P3	CIDP	M&S	✓ ^c	×	×	×	Hodgkin lymphoma	✓	5	IgG-Lambda	Demyelinating	Axonal loss	1:3200/1:1600	✓	✓	✓	✓	ChIVPP	mRS 2 (1 year post RTX)
P4	Atypical MN	Motor only	✓ ^d	×	×	×	CLL	×	5	IgG-Lambda	Axonal	×	1:800/1:1800	✓	✓	✓	✓	CYC FDB	mRS 0 (9 months post RTX)
P5	GBS	M&S	✓ ^e	✓	✓	×	No	✓	5	ND	Demyelinating	×	1:400/1:800	✓	✓	✓	✓	×	mRS 0 (9 months post RTX)
P6	GBS	M&S	✓ ^f	✓	✓	✓	Nephrotic syndrome	✓	5	ND	Mixed	Axonal loss	1:3200/1:6400	✓	✓	✓	✓	×	mRS 1 (18 months post RTX)
P7	GBS	M&S	✓ ^b	✓	×	×	No	✓	6	ND	×	×	1:800/1:800	×	✓	×	×	×	Death (Day 18)
P8	GBS	Motor only	✓ ^g	✓	✓	✓	Nephrotic syndrome	×	6	ND ^h	×	×	1:400/1:400	×	✓	×	×	×	Death (Day 14)

Supplementary Table 2 - Clinical features of pan-neurofascin antibody positive patients

Key:

- ✓ Given but no response
- ✓ Given but only partial and/or transient response
- ✓ Given with good response
- ×

×

^a II-VII, IX-XII including optic disc swelling

^b Facial diplegia, subtle gaze palsy

^c Bilateral III, IV and VI nerve palsies, facial diplegia

^d Mild bulbar weakness

^e Facial diplegia, dysphonia

^f Facial diplegia, complete ophthalmoplegia, optic disc swelling with reduced visual acuity, bulbar dysfunction

^g Facial diplegia

^h Clonal urinary lambda light chain

ND: Not detected

CN: Cranial Nerve

CLL: Chronic Lymphocytic Leukaemia

MV: Mechanical ventilation

mRS: modified Rankin Score

CYC: Cyclophosphamide

FDB: Fludarabine

IVIg: Intravenous Immunoglobulin

PLEX: Plasma Exchange

ChIVPP: Combination chemotherapy for Hodgkins Lymphoma

(Chlorambucil, Vinblastine, Procarbazine, Prednisolone)

Patient	Time from symptom onset	SNAPs	Motor Studies											EMG	Neurophysiologist's report	
			Prolonged distal motor latency		Slowed conduction velocity			Abnormal F-waves			Conduction block		TD			CMAP
			>110 %	>150 %	<90%	<85%	<70%	>120 %	>150 %	absent	>0.7	>0.5				
P1	2 weeks	Absent UL preserved sural												Globally absent	No signs of acute denervation	Inexcitable
	4 weeks	Globally absent												Globally absent	Positive sharp waves and fibrillations	Inexcitable
	8 weeks	Globally absent												Globally absent	Florid denervation with no recordable motor units	Inexcitable
P2	4 weeks	Marginal reductions in amplitude and velocity	1		3	3	1	2			3	2		<50% LLN x 1	ND	Demyelinating
P3	3 weeks ^a	Marginal reductions in amplitude and velocity	2		4	3				8	7	4		<80% LLN x1	Relative paucity of active denervation changes	Demyelinating
	6 weeks ^a	Absent UL SNAPs												Globally absent	Profuse acute denervation	Severe axonal loss
P4	4 weeks	Normal	4		2	2		2		6	4	4		<80% LLN x1		Conduction block
P5	Day 5	Mildly reduced UL amplitudes	3	2	2	1					1			<80% x6 <50% x 3	No spontaneous or voluntary activity	Demyelinating
	2 weeks		2											<80% x1 <50% x 1	Normal	Subtle demyelinating features, some improvement
	6 weeks	Absent / reduced	4	4	1	1	1	1	1	1				<20% x4 <10% x3	Pronounced active denervation	Secondary axonal loss
P6	3 weeks	Absent / reduced	3	2	2	2	1			1	2	1		<50% x5 <10% x4 NR x3	No spontaneous activity	Demyelinating
	5 weeks	Globally absent	1	1										<10% x4 NR x2	Profuse positive sharp waves and fibrillations	Severe axonal loss

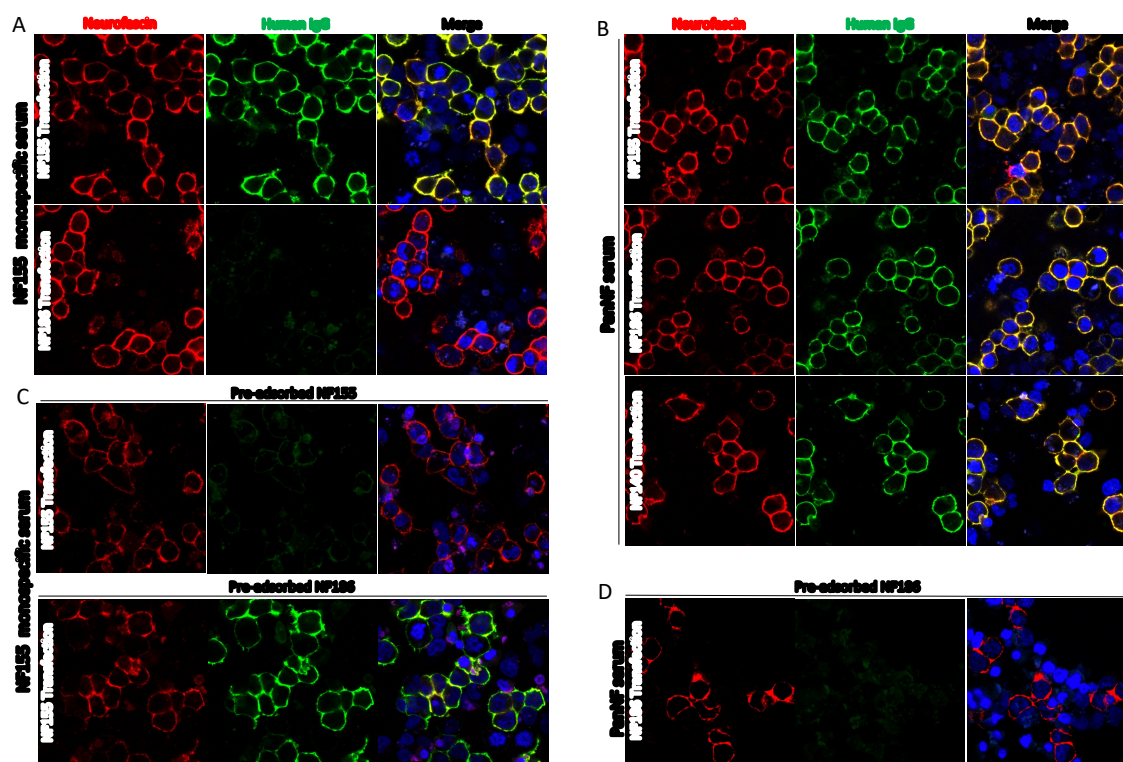
Supplementary Table 3 - Detailed neurophysiological findings

Numbers refer to how many individual nerves met the criteria stated. SNAP – sensory nerve action potential, CMAP – compound muscle action potential, ND – not done. ^a Studies in this patient were performed 3 and 6 weeks after an acute neurological deterioration, which itself occurred after 4 months of insidiously progressive left leg weakness. Note the frequent presence of conduction block, absence of temporal dispersion, and frequent switch from a demyelinating/AIDP to axonal pattern on repeat testing, suggestive of nodal pathology (“nodopathy”) [7].

	Treatment response (% of treated, n)															
	Steroids				IVIg				Plasma exchange				Rituximab			
Antibody (n)	Total	Good	Partial	None/ deteriorated	Total	Good	Partial	None/ deteriorated	Total	Good	Partial	None/ deteriorated	Total	Good	Partial	None/ deteriorated
PanNF (8)	4	0	0	0	8	0	25% (2)	75% (6)	6	0	50% (3)	50% (3)	4	100% (4)	0	0
NF155 (17)	16	19% (3)	37% (6)	44% (7)	15	6% (1)	27% (4)	67% (10)	8	0	25% (2)	75% (6)	6	50% (3)	33% (2)	17% (1)
CNTN1 (11)	11	36% (4)	27% (3)	36% (4)	8	0	50% (4)	50% (4)	4	0	0	100% (4)	4	50% (2)	25% (1)	25% (1)
CNTN1/Caspr1 (9)	8	13% (1)	50% (4)	37% (3)	8	0	13% (1)	87% (7)	4	0	50% (2)	50% (2)	6	67% (4)	33% (2)	0
Seronegative (194)	62	8% (5)	34% (21)	58% (36)	83	8% (7)	43% (36)	48% (40)	27	30% (8)	30% (8)	41% (11)	1	0	0	100% (1)

Supplementary Table 4 - Physician reported treatment response by serological group

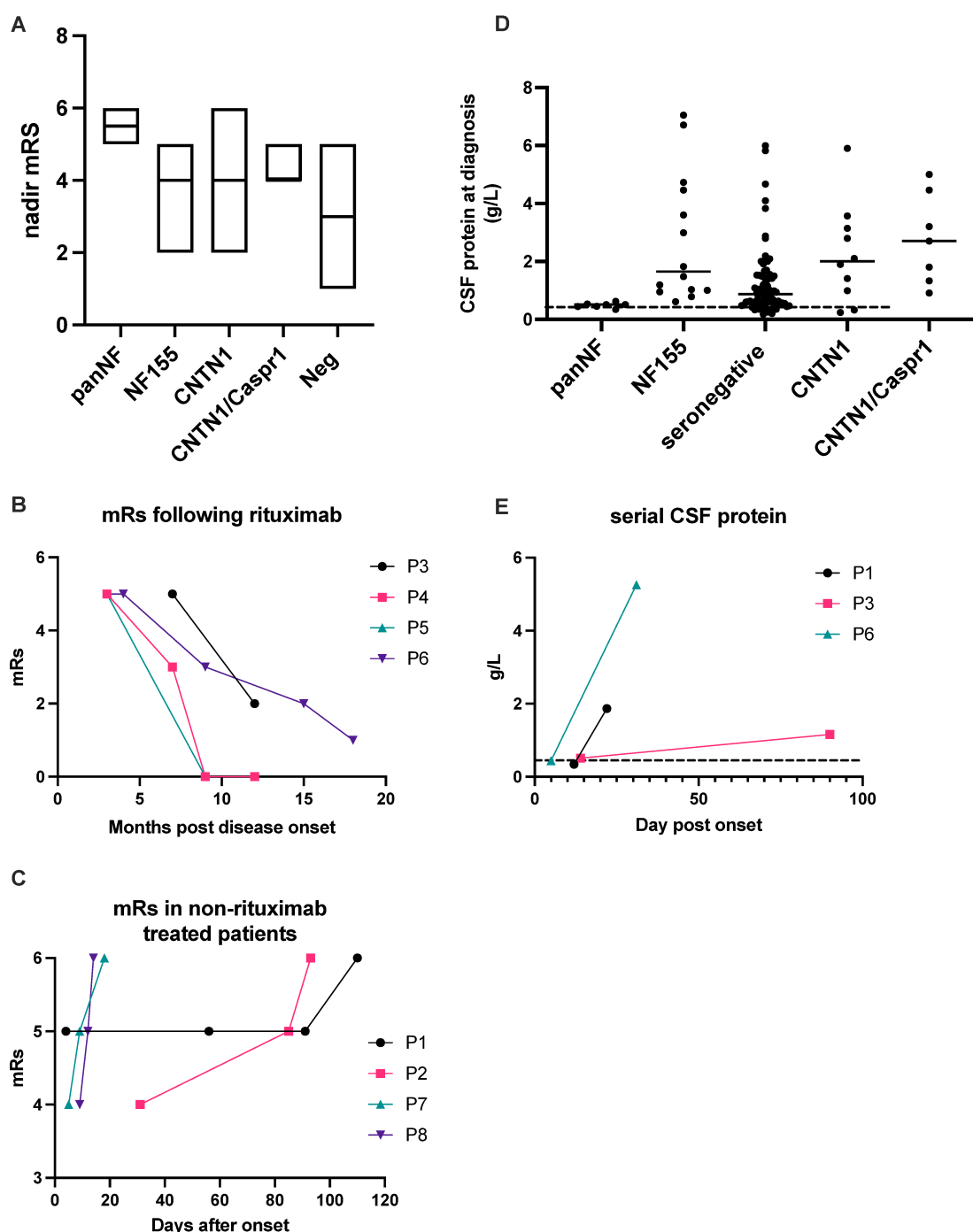
IVIg – intravenous immunoglobulin. panNF – pan-neurofascin antibody positive, NF155 – neurofascin-155 antibody positive



Supplementary Figure 1 - Comparison between neurofascin-155 monospecific and pan-neurofascin serum reactivity on cell-based assays

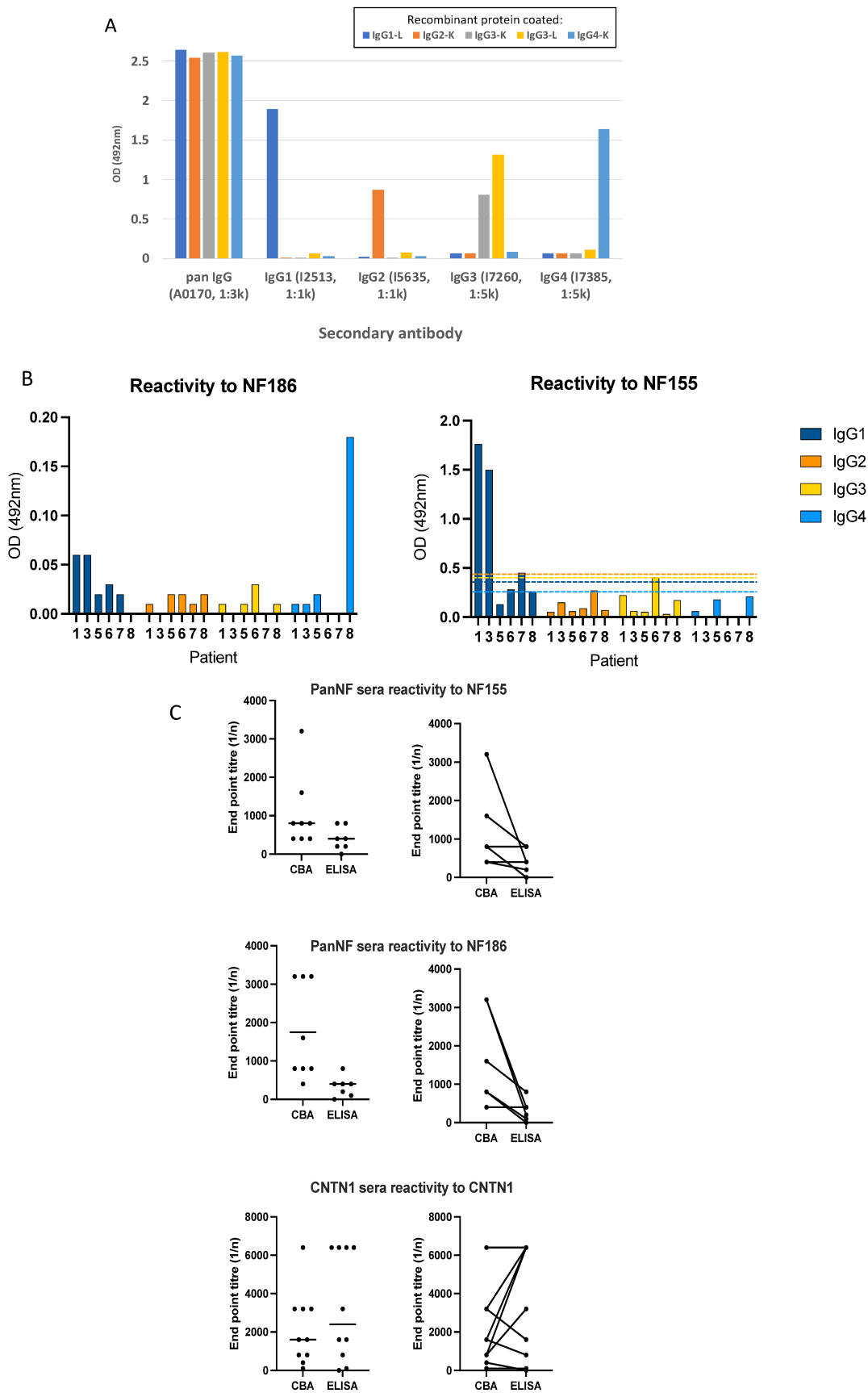
(A) Neurofascin (NF)-155 monospecific serum contains IgG (green) which reacts exclusively with the membrane of NF155 and not NF186 transfected cells (commercial neurofascin antibody, red). IgG4, IgG2 and IgG1 subclass antibodies are present in this example (bottom panel). (B) PanNF sera (patient 6) contains IgG reactive against NF155, NF186, and NF140 transfected cells. Reactivity to all three neurofascin isoforms was found in all patients. Only IgG1 subclass antibodies with this reactivity are detected (bottom panel).

Pre-adsorption of NF155 monospecific serum with NF155 protein, but not NF186 protein, abrogates IgG binding to NF155 transfected cells (C), in contrast to panNF sera, for which no IgG labelling to NF186 transfected cells can be seen after pre-adsorption with NF186 protein (D). Same results are obtained using NF155 transfected cells and pre-adsorption with NF155 protein.



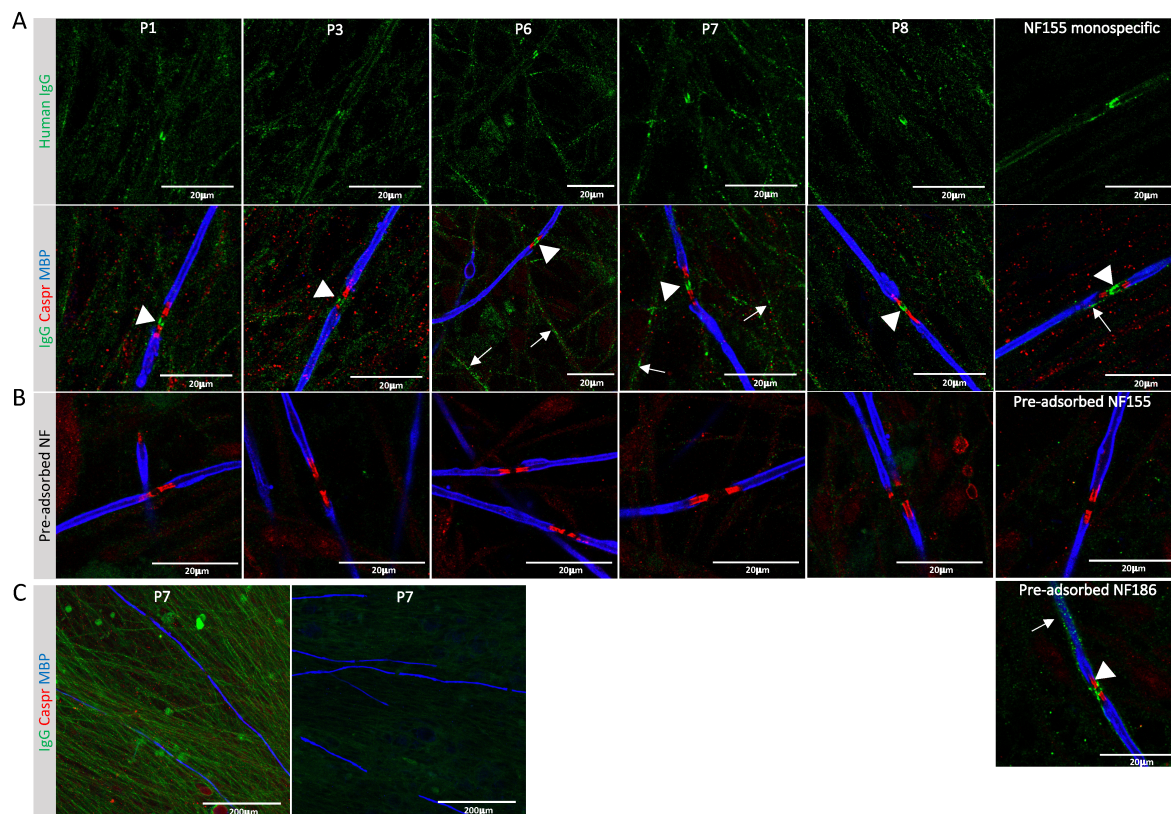
Supplementary Figure 1 - Modified Rankin scores and CSF protein levels

(A) The median nadir modified Rankin score (mRs) in the pan-neurofascin (panNF) antibody group was significantly higher than that in both the neurofascin-155 (NF155) antibody positive and seronegative groups. (B) Change in mRs in the 4 rituximab treated patients. The first datapoint for each patient represents the time of the first dose of rituximab. (C) Change in disability of non-rituximab treated patients. (D) CSF protein in the panNF group was either normal or only marginally elevated at diagnosis, and significantly lower than in both NF155 antibody positive and seronegative patients. (E) When performed, repeat CSF sampling showed abnormally increased protein levels at later time-points. Dashed lines indicate the upper limit of normal for CSF protein (0.45 g/L).



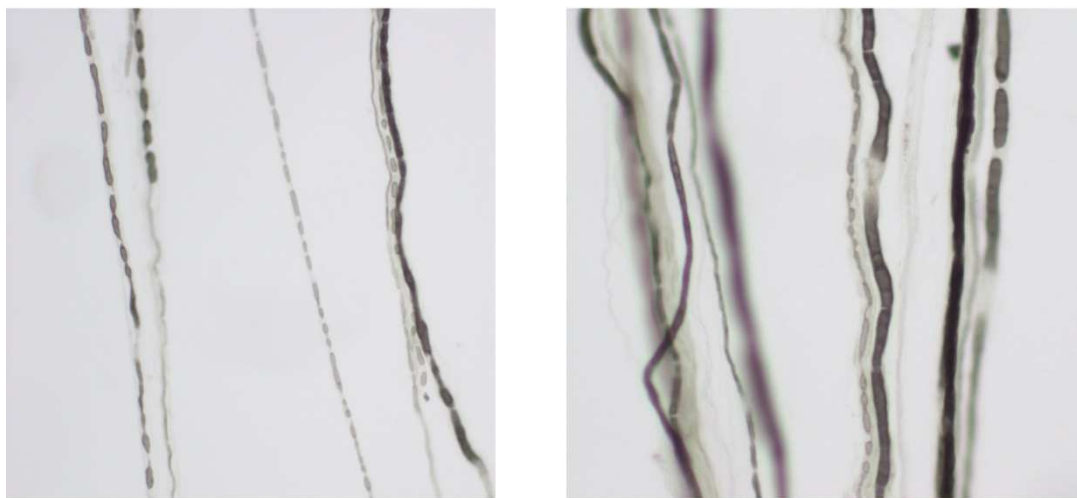
Supplementary Figure 3 - Subclass specificity and comparison between CBA and ELISA

(A) A pan-IgG secondary antibody detects all 5 human IgG recombinant proteins similarly. The subclass antibodies produce a signal only in wells coated with recombinants of their specified subclass. (B) Sera from patients in this cohort are tested for antibody subclass reactivity to either NF186 or NF155. Cut-off optical density values for each subclass are indicated by the dotted lines (IgG1 0.35, IgG2 0.42, IgG3 0.40, IgG4 0.26), thus P1, 3 and 7 are positive for IgG1 antibodies only by ELISA. All other sera and subclass combinations result in OD results below the limits of detection. (C) End point titres on the cell-based assay (CBA) are consistently higher than by ELISA for both NF155 and NF186 antibodies but ELISA is at least equally as sensitive to the CBA for CNTN1 positive sera. The panels on the left-hand side show the individual data points and mean titre, the panels to the right show the same data with lines linking individual patients' results across the 2 assays.



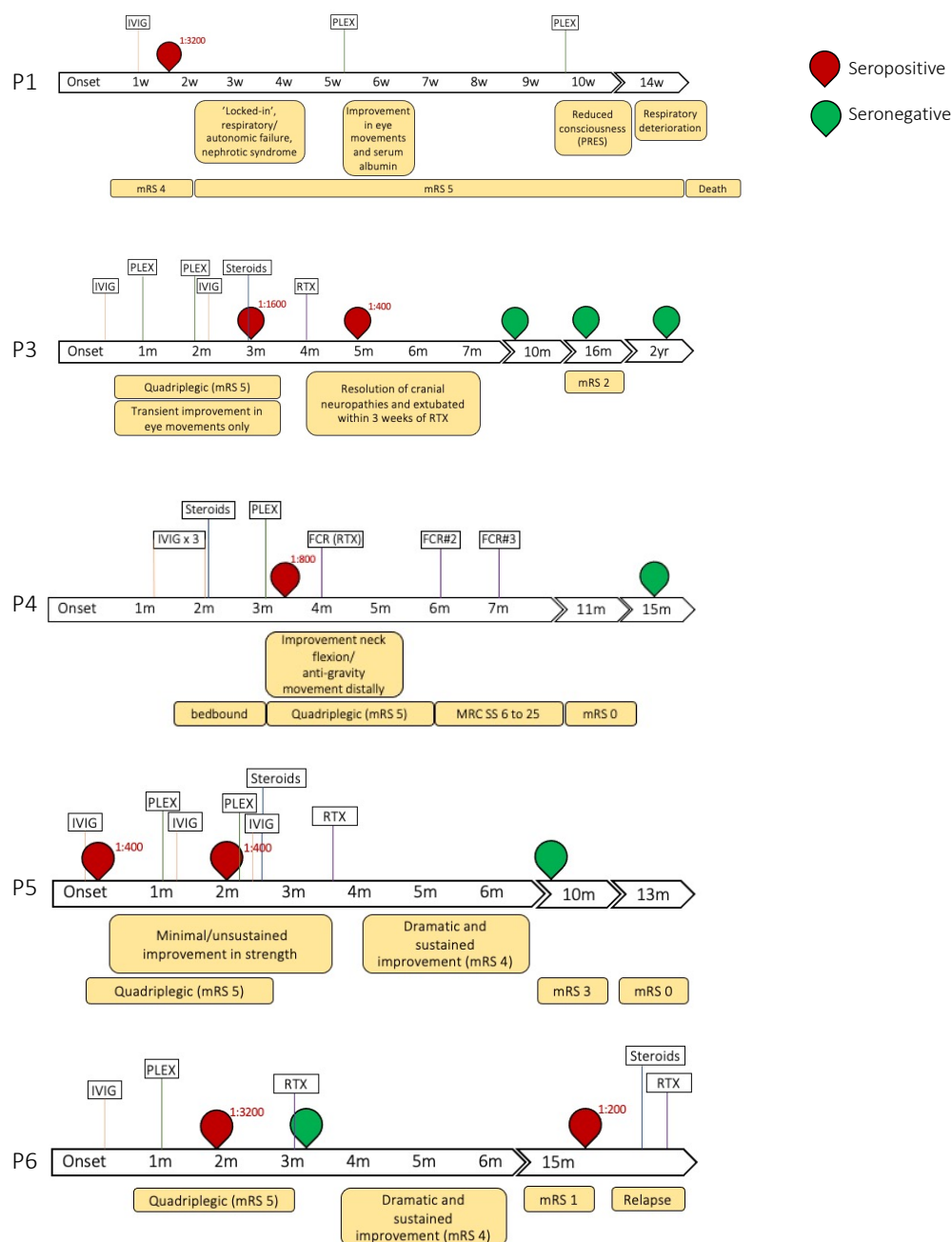
Supplementary Figure 4 – Pan-neurofascin serum is specific to NF155 and NF186 in live myelinating co-cultures

In myelinating co-cultures, high magnification confocal microscopy (x63 objective) reveals (A) IgG (green) is deposited at the node of Ranvier (arrowhead), flanked by paranodes labelled with Caspr (red), in patients 1, 3 and 8, with additional IgG labelling along axons (marked by arrow, axons not co-labelled in these images) in patients 6 and 7. There was no IgG binding observed for P2 and P5, and no sera was available for P4 for testing on cultures. Incubation of cultures with NF155 monospecific sera reveals IgG deposition over the outer surface of the myelin sheath (MBP, blue - arrow) and at the node. Nodal IgG staining was abrogated following pre-adsorption of panNF sera with either neurofascin 155 or 186 protein (pre-adsorption with NF186 shown in figure) (B). Axonal IgG labelling was also reduced following pre-adsorption in P6 and P7, best appreciated using lower magnification (x20 objective) (C). In contrast IgG labelling from NF155 monospecific sera is only abrogated when sera is pre-adsorbed with NF155, and is retained following pre-incubation with recombinant NF186 protein (far right column).



Supplementary Figure 5 - Teased nerve fibres

The Teased nerve fibres from the sural nerve biopsy of patient 6 show myelin ovoids consistent with axonal degeneration (left panel) and occasional short internodes (right panel).



Supplementary Figure 6 – Treatment timelines

Treatments are indicated by flags at timepoints after onset of symptoms (m – months, w – weeks). Functional status and recorded responses are noted in yellow boxes. Serological status, with antibody titre if positive, are indicated by red and green markers (see key in figure), and included where these were serially performed.

ChlVPP – Chlorambucil, Vinblastine, Procarbazine, Prednisolone (started for Hodgkins Lymphoma) FCR – Fludarabine, Cyclophosphamide and rituximab (started for CLL in absence of haematological indication as felt to be driving panNF antibody production), mRS – modified rankin score, PRES – Posterior Reversible Encephalopathy Syndrome

Case Vignettes

Patient 1

This woman in her 6th decade of life with a prior medical history of hypertension and Barret's oesophagus presented to her local hospital 4 days after developing distal upper and lower limb paraesthesia, and 1 day after first noticing bilateral leg weakness. On the day of her admission she had developed a severe, occipital headache. An initial CT brain scan showed sub-arachnoid haemorrhage, largely focussed over the left hemispheric convexity and particularly prominent in the left Sylvian fissure. She was transferred to the regional neurosciences centre the following day after further progression in her symptoms. By day 7 from the initial onset of symptoms she had proximal greater than distal weakness of all 4 limbs (and an MRC sum score of 30/60) with additional neck flexion weakness, but remained fully conscious with no cognitive deficits. She was now globally areflexic, with a normal sensory examination, and had a markedly labile blood pressure suggestive of autonomic dysfunction. At this stage she was bedridden but there was no evidence of cranial nerve involvement or neuromuscular respiratory failure. Further imaging showed no evidence of a cerebral aneurysm and no progression in the sub-arachnoid haemorrhage. A clinical diagnosis of GBS was made and she was commenced on intravenous immunoglobulin (IVIg, 2 g/kg given over 5 days). Unfortunately, she continued to progress. By day 10, her vital capacity, which had been normal (3.2L) on admission, had fallen to 0.69L, and she was intubated, ventilated, and transferred to ITU. Her serum albumin, which had been low normal (31 g/L) on admission, fell progressively to a nadir of 12 g/L by day 13. She developed worsening peripheral oedema and was found to have nephrotic range proteinuria. An opinion was sought from the nephrology team who advised against a renal biopsy. Her limb weakness progressively worsened, and by day 14 there was no detectable movement in any limb (MRC sum score 0/60). She was first noticed to have cranial nerve deficits on day 10, when bilateral lower-motor neuron facial weakness was observed. By day 14 she had complete paralysis of the entire cranial musculature with complete external and internal ophthalmoplegia. Throughout this time, she continued to demonstrate evidence of cardiovascular autonomic failure with an extremely labile blood pressure (systolic measurements ranging from 113 to 258) and episodes of tachy- and brady-cardia. Despite multiple anti-hypertensives, at some points including intravenous labetalol, her BP remained difficult to control with frequent hypertensive episodes. A repeat CT on day 14 demonstrated maturation of the SAH, with no other changes and no evidence of further bleeding or hydrocephalus. On day 16 she was noted to have bilateral swollen optic discs. A lumbar puncture on day 17 revealed an opening pressure >40cm CSF, with a CSF WCC of 2 and RCC of 1680. CSF protein was normal at this point (0.34g/L), although was later elevated at 1.87g/L on repeat testing on day 27. In view of the raised ICP and papilloedema, a lumbar drain was placed. Multiple further CT scans on days 18, 23, 25, 36 and 46 showed only progressive resolution of the SAH, with no hydrocephalus or evidence of developing intra-cranial pathology. Electrophysiological testing on day 18 showed that only sural responses were obtainable, and all other sensory and motor nerves were unresponsive. On day 24 the patient had a brief cardiac arrest when her tracheostomy tube temporarily blocked. Following 5 days of plasma exchange starting on day 40, she was able to move her eyes vertically to command and was able to use this movement to communicate. Her pupillary light responses also returned. On day 45 she experienced an episode of desaturation, bradycardia and brief (5s) asystole, in the context of a left, lower lobe pneumonia. Serial EEG recordings up to day 60 were either normal or showed findings consistent with mild sedation. On day 67, the patient was noted to be consistently less responsive, and was no longer able to move her eyes to communicate. Repeat CT imaging showed features suggestive of posterior reversible encephalopathy syndrome (PRES). Repeat EEG showed encephalopathic features. A further cycle of PLEX was started on day 69 but no clinical improvement was apparent. During both cycles of PLEX the serum albumin levels normalised but then quickly fell. When nerve conduction studies were repeated on day 88 all nerves were unresponsive. Repeat CSF analysis at this point was acellular and the CSF protein had normalised (0.258g/L). Further CT scans on day 89 and 96 showed improvement in the PRES changes, which were "barely visible" on the final images. There was an improvement in the background EEG between days 91 and 104, with better defined cortical rhythms, but no EEG response to verbal, tactile or noxious stimuli. Nephrotic range proteinuria (10g/L) persisted throughout this period. A further deterioration in respiratory function occurred on day 102, in the context of a further episode of pulmonary infection, with pseudomonas detected by broncho-alveolar lavage. On day 108, following discussion with the patient's family, respiratory support was withdrawn, and the patient died soon after.

Patient 5

This gentleman in his 7th decade of life presented 2 weeks after a flu-like illness with acute and progressive symmetrical predominantly distal sensory loss and weakness, areflexia, limb ataxia and profound sensory gait ataxia, rendering him immobile. He promptly developed facial diplegia, dysphonia, autonomic dysfunction (tachycardia with multiple premature ventricular contractions on ECG, diaphoresis) and respiratory failure requiring mechanical ventilation on the intensive care unit by day 2 of his admission. He had an iron deficiency anaemia (Hb 10.3 g/dL) with a normal OGD (unfit for colonoscopy), and was negative for anti-ganglioside antibodies, anti-MAG antibodies, paraneoplastic serology and autoimmune screen. A CT chest/abdomen/pelvis, CT head and unenhanced MRI whole spine, were unremarkable.

Despite making a prompt recovery after commencing IVIg, allowing extubation after 48 hours, he rapidly re-deteriorated and required further re-intubation within 4 weeks following a *Klebsiella pneumoniae*. He had plasma exchange followed by IVIg at 1 and 2 months, followed by methylprednisolone and a tail of oral steroids with minimal, unsustained improvement in strength following each plasma exchange cycle. Finally, almost 3 months after admission to ITU he received rituximab and within weeks started to show dramatic and sustained improvement. By 6 months following rituximab he had regained independent mobility (modified Rankin score 3). Repeat panNF antibody testing at this point was negative.

Patient 6

A middle aged gentleman presented with progressive ascending bilateral symmetrical paraesthesia over a few days, followed by weakness in the same distribution, areflexia, and neuropathic pain. Other than a non-specific flu-like respiratory and gastrointestinal infection 6 weeks prior there was no more recent preceding illness. Objectively, all sensory modalities were affected, he had a severe sensory ataxia and developed quadriplegia with multiple cranial nerve abnormalities (II, III, IV, VI, VII, IX and X). He developed respiratory failure requiring ventilation on the intensive care unit. There was evidence of peripheral oedema with hypo-albuminaemia (19) although a urinary protein was not measured. Serology for HIV, Lyme, a paraprotein and anti-ganglioside antibodies were within the normal range and a CSF taken 4 days after admission revealed a normal protein and cell count. Nerve conduction studies initially performed 23 days after admission showed a diffuse, non-length dependent sensorimotor peripheral neuropathy with occasional conduction slowing and prolonged DMLs, possibly reflecting a demyelinating disorder. A subsequent study performed after another 15 days showed significant deterioration with no recordable sensory or motor potentials and features of severe axonal loss on needle EMG. An MRI of the brain was unremarkable, and lumbar roots only suggesting an S1 root impingement. The patient continued to deteriorate despite IVIg commenced 6 days into admission, and only sustained partial improvement with steroids, deteriorating further with PLEX at 1 month. Following treatment with rituximab, started 3 months after initial presentation, there was a clear and significant clinical improvement. PanNF antibodies were negative when retested at this point.

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