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RESEARCH REPORT

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A longitudinal and cross-sectional study of plasma neurofilament light chain concentration in Charcot-Marie-Tooth disease

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Abstract

Advances in genetic technology and small molecule drug development have paved the way for clinical trials in Charcot-Marie-Tooth disease (CMT); however, the current FDA-approved clinical trial outcome measures are insensitive to detect a meaningful clinical response. There is, therefore, a need to identify sensitive outcome measures or clinically relevant biomarkers. The aim of this study was to further evaluate plasma neurofilament light chain (NFL) as a disease biomarker in CMT. Plasma NFL was measured using SIMOA technology in both a cross-sectional study of a US cohort of CMT patients and longitudinally over 6 years in a UK CMT cohort. In addition, plasma NFL was measured longitudinally in two mouse models of CMT2D. Plasma concentrations of NFL were increased in a US cohort of patients with CMT1B, CMT1X and CMT2A but not CMT2E compared with controls. In a separate UK cohort, over a 6-year interval, there was no significant change in plasma NFL concentration in CMT1A or HSN1, but a small but significant reduction in patients with CMT1X. Plasma NFL was increased in wild type compared to $GARS^{C201R}$ mice. There was no significant difference in plasma NFL in $GARS^{P278KY}$ compared to wild type mice. In patients with CMT1A, the small difference in cross-sectional NFL concentration vs

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healthy controls and the lack of change over time suggests that plasma NFL may lack sufficient sensitivity to detect a clinically meaningful treatment response in adulthood.

KEYWORDS
biomarkers, Charcot-Marie-Tooth disease, neurofilament

1 | INTRODUCTION

Charcot-Marie-Tooth disease (CMT) is one of the commonest inherited neurological diseases with a population prevalence of 1 in 3000.[1] With increased understanding of the genetic aetiology of CMT combined with advances in genetic therapies and small molecule drug development, the field is now entering an era where there are a number of promising therapies in the pipeline.[2]

Developing successful treatments in preclinical models of CMT is only part of the journey in delivering therapies to patients. For a treatment to be adopted in routine clinical practice, it will need to show efficacy in clinical trials. CMT provides particular difficulties when it comes to designing clinical trials.[3] CMT is usually a lifelong disease, and even in the rapidly progressive forms, the rate of progression is slower than for other diseases such as amyotrophic lateral sclerosis. It is widely assumed that a successful treatment would be one that stops the progression of the disease, and therefore, clinical trials need to be designed with outcome measures that are able to detect a slowing in the rate of progression. A number of CMT-specific clinical outcome measures have been designed that have been validated or are undergoing validation, including the CMT neuropathy score, CMT Functional Outcome Measure, CMT Health Index and CMT Peds.[4-9] In addition, biomarkers of disease progression, such as nerve and muscle MRI are also being developed as outcome measures for clinical trials.[10-12]

Neurofilaments are the most abundant cytoskeletal proteins in neurons of both the central and peripheral nervous systems.[13] It has been shown that plasma neurofilament light chain concentration (NFL) is increased in several neurological diseases, including CMT, where it also correlates with disease severity.[14-21] To be able to use a blood biomarker such as NFL in clinical trials, it is important to know how plasma concentrations vary over time. In this study, we replicate our previous cross-sectional work in another cohort of CMT patients, investigate the change in plasma NFL over time in patients with CMT and in two mouse models of the disease.

2 | METHODS

2.1 | GARS mouse models

The generation and characterisation of the GARS^{P278KY} and GARS^{C201R} mouse models have been described previously.[22,23] All experimental procedures were conducted in accordance with animal care protocols approved by the Institutional Animal Care and Use Committee at The Jackson Laboratory. Blood samples were obtained from 5, 7, 9 and 11-week-old wild-type and GARS mice (n = 3-7 per age group) using a lancet puncture of the submandibular vein.

2.2 | Participants

Blood samples were collected prospectively between January 2017 and May 2019, with informed consent, from 27 out of 75 CMT patients who had previously donated blood for a previous study.[21] In addition, blood from 49 patients with CMT, identified and evaluated in the Inherited Neuropathy Consortium (INC) clinic in the Department of Neurology at Iowa, was also collected.

The disease severity, as measured using the Rasch modified CMT examination score, version 2[9] (hereto referred to as the weighted CMTES), was recorded at the same time that plasma was collected. The weighted CMTES is a validated outcome measure for assessing the severity of CMT. It is a composite score that includes the patient's symptoms and examination findings.[9] All patients underwent nerve conduction studies to confirm the presence of neuropathy; however, a weighted CMT neuropathy score (CMTNS) (which required neurophysiology at the same time as the clinical assessment) was only included if a nerve conduction study had been performed within 18 months of the blood sample.

2.3 | Blood sampling and sample collection and storage

All participants were evaluated in outpatient clinics, and blood samples were taken and processed within 1 hour. Blood was collected into EDTA-containing tubes and centrifuged at 20°C at 3500 rpm for 10 minutes. Plasma was then aliquoted and stored at −80°C.

2.4 | Standard protocol approvals, registrations, and patient consents

This study was approved by The National Hospital for Neurology and Neurosurgery Research Ethics Committee/Central London REC 3 09/H0716, and written informed consent was obtained from all participants in the study.

TABLE 1 Plasma NFL concentration in a US (Iowa) cohort of patients with CMT

	Number of patients	Age (mean, 95% CI)	Median [NFL] pg/ml (range)	*CMTES/[NFL] Spearman correlation co-efficient	*CMTNS/[NFL] Spearman correlation co-efficient	Ulnar CV/[NFL] correlation co-efficient	Ulnar CMAP/[NFL] correlation co-efficient
CMT1B	18	49.9 (37.5-60.9)	25.5 (6.7-52.4)	0.329, $P = .183$	0.208, $P = .517$	0.876, $P < .0001$	0.682, $P = .015$
CMT1X	18	47.4 (39.5-55.3)	18.3 (11.2-26.5)	−0.144, $P = .568$	−0.043, $P = .907$	0.258, $P = .471$	−0.322, $P = .364$
CMT2A	4	42 (28.8-55.2)	19.7 (15.6-23.7)	−0.8, $P = .2$	−0.896, $P = .104$	−0.5, $P = .667$	−0.5, $P = .667$
CMT2E	9	46.9 (35.9-57.8)	5.58 (3.84-17.6)	n/a	n/a	n/a	n/a
Controls	25	49 (44.2-53.8)	7.54 (4.52-15.8)	n/a	n/a	n/a	n/a

Abbreviations: CI, confidence interval; CMAP, compound muscle action potential; CMT, Charcot-Marie-Tooth disease; *CMTES, Rasch modified (weighted) CMT Examination Score; *CMTNS, Rasch modified (weighted) CMT Neuropathy Score; CV, conduction velocity; NFL, neurofilament light chain; [ ], concentration. All correlations refer to Spearman's correlation coefficient.

Institutional Review Board approval was also obtained from the University of Iowa, and written informed assent/consent was provided by participants under a protocol approved by the ethics board of the NIH Rare Diseases Clinical Research Network (Protocol INC6601).

2.5 | Simoa plasma NFL measurements

Plasma NFL concentration was measured using two highly correlated methods, employing the same antibodies: the in-house Simoa NFL assay that has been described in detail previously,[16] and the commercially available NF-Light assay (Quanterix, Billerica, MA). Samples were analysed 'blind' and in duplicate using one batch of reagents. For the UK samples, an aliquot of the original baseline sample was analysed in the same batch as the 6-year follow up sample.

2.6 | Statistical analysis

Statistical analysis was performed using SPSS version 27.00 (IBM, New York, USA) and GraphPad Prism 9.0 (GraphPad Inc., California, USA). Correlations were assessed using Spearman's correlation coefficient. Two-tailed paired *t*-tests were used to compare differences in plasma NFL concentration in patients with CMT at baseline and after 6 years. One-way ANOVA with post hoc Dunnett's two-tailed *t*-test was used to compare differences in age and plasma NFL between CMT subtypes and controls in the Iowa cohort.

3 | RESULTS

There has been recent interest in the potential use of plasma NFL as a biomarker of disease progression in CMT for use in clinical trials. We, therefore, sought to examine plasma NFL concentration in a cross-sectional cohort of CMT patients and longitudinally in a further cohort of patients with CMT and in two established mouse models of the disease.

3.1 | Plasma NFL concentration is increased in patients with CMT1B, CMT1X and CMT2A, but not CMT2E, compared with controls

We have previously demonstrated an increase in plasma NFL concentration in UK patients with CMT1A, CMT1X and HSN1.[21] We, therefore, sought to see if we could replicate this finding in an independent cohort of patients with CMT from the United States of America. The cohort of patients from Iowa comprised 18 patients with CMT1B, 18 with CMT1X, 4 with CMT2A and 9 with CMT2E and 25 controls (Table 1). There was no significant difference in the age of the patients with each type of CMT and controls (One-way ANOVA, $P = .931$) or the sex ratio (Chi-square, $P = .53$). Plasma NFL concentration was significantly increased in patients with CMT1B (ANOVA $P < .0001$, Dunnett's two-tailed *t*-test, $P < .0001$), CMT1X ($P = .001$) and CMT2A ($P = .048$) compared with controls but not in patients with CMT2E ($P = .939$) (Figure 1A and Table 1). In contrast to our previous study in a UK cohort, there was no correlation between plasma NFL and the weighted CMTES and CMTNS for any of the CMT subtypes included in the study (Figure 1B and Table 1). There was a significant correlation between plasma NFL in patients with CMT1B and the ulnar nerve Conduction Velocity (CV) (Spearman Rho = 0.876, $P < .0001$) and Ulnar Compound Muscle Action Potential (Rho = 0.682, $P = .015$) but not for patients with CMT1X or CMT2A (Table 1). There was no correlation between age of onset and plasma NFL in the CMT1B cohort (Pearson correlation coefficient, r = 0.44, $P = .11$).

3.2 | Plasma NFL changes with time in two mouse models of CMT

In order for plasma NFL to be of use as a biomarker of disease progression in CMT, it is necessary to know if the concentration changes with time. We have previously shown that in a mouse model of CMTX,[24] the concentration of plasma NFL rises rapidly between 2 and 3 months before falling by a third at 1 year. We, therefore, measured plasma NFL at 5, 7, 9 and 11 weeks in two mouse models of CMT2D (Figure 2). The $GARS^{C201R}$ mouse is a milder model with

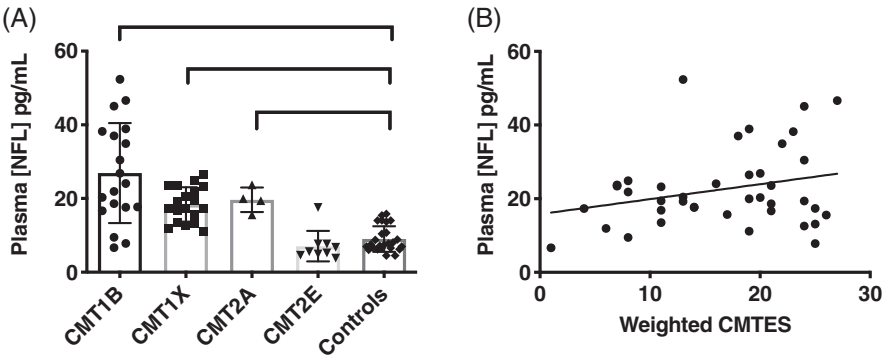

FIGURE 1 Plasma NFL concentration in a US cohort of CMT. (A) Plasma NFL concentration is significantly increased in patients with CMT1B, CMT1X and CMT2A compared with controls (ANOVA and post hoc Dunnett's two-tailed *t*-test, $^{*}P = .048$, $^{**}P = .001$, $^{***}P < .0001$). There is no difference in plasma NFL concentration as measured using SIMOA technology between patients with CMT2E and controls. Error bars = SEM. (B) Shows plasma NFL concentration plotted against the Rasch modified (weighted) CMTES for all patients with CMT1B, CMT1X and CMT2A. There is no significant correlation (Spearman Rho = 0.139, $P = .393$)

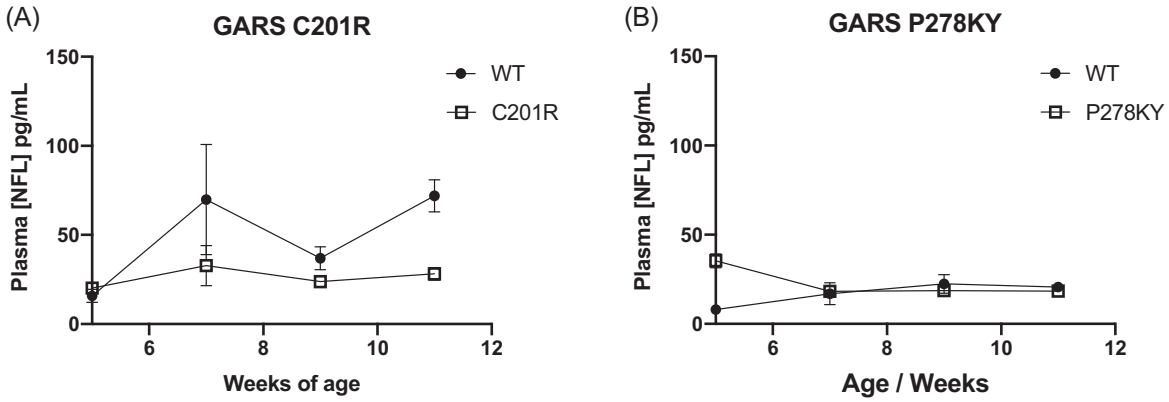

FIGURE 2 Changes in plasma NFL concentration over time in two mouse models of CMT2D (serial measurements in the same mice at four time points). ((A) $GARS^{C201R}$ (n = 7), WT, wild type (n = 5), (B) $GARS^{P278KY}$ (n = 7), WT (n = 3). Error bars = SEM

normal life expectancy in contrast to the $GARS^{P278KY}$ mouse, which has a background-dependent reduced life expectancy of less than 6 months.[22] Plasma NFL was increased in wild type compared with $GARS^{C201R}$ mice at 5, 7 and 11 weeks, although the difference only reached statistical significance at 11 weeks (Mann–Whitney *U*-test, $P = .01$, Figure 2A). Plasma NFL concentration was also increased in wild type compared with $GARS^{C201R}$ mice at 11 weeks in an unrelated colony in the United Kingdom (see Figure S1). Plasma NFL was increased in the $GARS^{P278KY}$ mouse compared with wild type at 5 weeks, although this did not reach significance. There was no difference in plasma NFL at 7, 9 and 11 weeks consistent with the early axon loss in these mice, followed by very slow progress after 6-8 weeks of age (Figure 2B).[22]

3.3 | Plasma NFL is stable in CMT1A and HSN but not CMT1X over a 6-year period

Repeat blood samples were collected from 27 patients with CMT after a 6-year time interval (CMT1A = 10, CMT1X = 6, HSN1 = 6, SPTLC2 = 2, CMT2A = 1, CMT4C = 1, CMT4B1 = 1). The mean increase in the weighted CMTES over this time period was +2.3. Unlike the US cohort, there was a significant correlation between 6-year follow up plasma NFL and weighted CMTES (Spearman Rho = 0.53, $P = .004$) (Figure 3A). There was no significant difference in plasma NFL over 6 years for all CMT patients, (mean change = −3.17 pg/mL, SD = 8.07, paired *t* test $P = .05$) (Table 2). An analysis of follow up plasma NFL for the three major CMT subtypes revealed no significant change over 6 years in CMT1A (mean change = −2.44 pg/mL, SD = 11.5, $P = .52$, Figure 4A) and HSN1 (mean change = −0.69 pg/mL, SD = 1.18, $P = .21$, Figure 4C) but a significant reduction in CMT1X (mean change = −3.28 pg/mL, SD = 2.13, $P = .01$, Figure 4B). Plotting the 6-year change in plasma NFL against the 6-year change in the weighted CMTES showed no significant correlation (CMT1A, Spearman Rho = −0.2, $P = .6$; CMT1X Rho = −0.7247, $P = .12$; HSN1 Rho = 0.25, $P = .63$) (Figure 3B-D).

4 | DISCUSSION

Clinical trials in CMT require the development and validation of clinical outcome measures and biomarkers that are sufficiently sensitive to detect a modest reduction in the rate of progression. This study

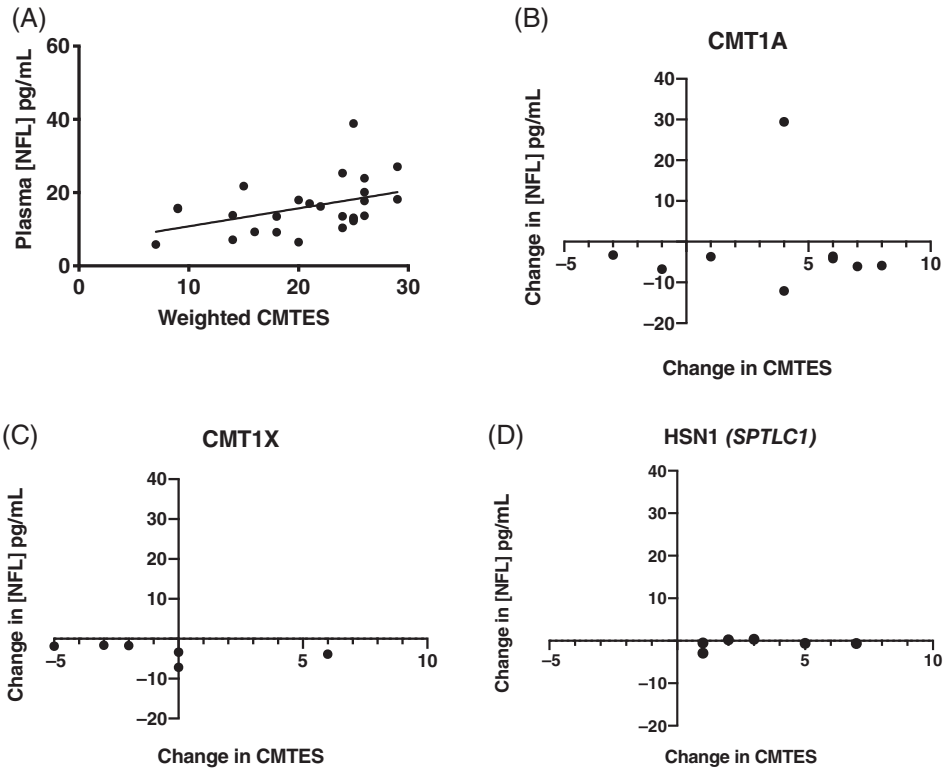

FIGURE 3 Plasma NFL concentration and disease severity in CMT. (A) shows a significant correlation between plasma NFL concentration and the weighted CMTES for the pooled 6-year follow up samples of all UK CMT patients in the study (Spearman Rho = 0.53, $P = .004$). (B-D) shows the change in plasma NFL concentration plotted against the change in the weighted CMTES over a 6-year period for patients with CMT1A, CMT1X and HSN1 using the repeat baseline measurement. There is no significant correlation between the change in plasma NFL concentration and the weighted CMTES for any of the three CMT subtypes

TABLE 2 Six-year follow up data of plasma NFL concentration and disease severity in CMT

CMT subtype	Mean age (years)	Number of participants	Median baseline	Median 6-y follow up	Mean nfl change (95% CI)	2-sided paired *t* test	Mean change in *CMTES
CMT1A	46.0	10	19.03 (9.47-31.4)	14.72 (6.46-32.4)	−2.44 (−10.67-5.80)	$P = .52$	+3.56
CMTX1	48.0	6	18.98 (8.79-28.7)	14.54 (7.14-25.3)	−3.28 (−1.04-−5.51)	$P = .01$	−0.67
HSN1 (SPTLC1)	42.3	6	18.0 (13.0-24.4)	17.9 (12.3-23.9)	−0.69 (−1.93-0.55)	$P = .21$	+3.167
HSN1 (SPTLC2)	50	2	12.9 (7.90-18.0)	9.68 (5.84-13.5)	−3.24 (−18.3-11.8)	n/a	+3.5
CMT2A	20	1	24.9	13.8	−11.1	n/a	+1
CMT4C	49	1	48.7	27.1	−21.6	n/a	+5
CMT4B1	32	1	16.5	18.2	1.67	n/a	−1
All CMT	46.2	27	18.6 (7.90-48.7)	15.6 (5.84-38.9)	−3.17 (−6.37-0.02)	$P = .05$	+2.3

Abbreviations: CI, confidence interval; CMT, Charcot-Marie-Tooth disease; *CMTES, Rasch modified (weighted) CMT Examination Score; HSN, hereditary sensory neuropathy.

provides further evidence on plasma NFL as a biomarker in CMT. In a *post hoc* analysis of the Phase 3 study of Patisiran (APOLLO) in patients with hereditary transthyretin-mediated (hATTR) amyloidosis, there was a significant reduction in plasma NFL in those patients randomised to Patisiran compared with placebo, validating the use of plasma NFL as a biomarker for this subtype of inherited peripheral neuropathy. Nevertheless, the concentration of plasma NFL in hATTR patients prior to treatment (69.4 pg/mL) was significantly higher than in our cohort of patients with CMT (18.6 pg/mL).[25]

In our previous single UK centre study, we demonstrated that plasma NFL was increased in several forms of CMT compared with age and sex-matched controls.[21] The current study replicates those findings in a separate cohort from the Iowa group in the United States. In this cohort, plasma NFL was increased in all the subtypes of CMT examined except for CMT2E due to mutations in the neurofilament light chain gene (*NEFL*). Interestingly, the concentrations in this group were lower than controls, although this did not reach statistical significance. The reasons for this are not clear, but the finding is concordant with a previous report demonstrating reduced NFL expression in cutaneous nerve fibres of patients with CMT2E.[26] An alternative explanation may be due to alteration of the NFL epitope recognised by the antibody used in the Simoa analysis as a result of neurofilament aggregations induced by the point mutation.[27]

It is often assumed that the rate of axonal degeneration in genetic peripheral neuropathy such as CMT is constant. This is an important assumption to test, because if the rates of axonal degeneration

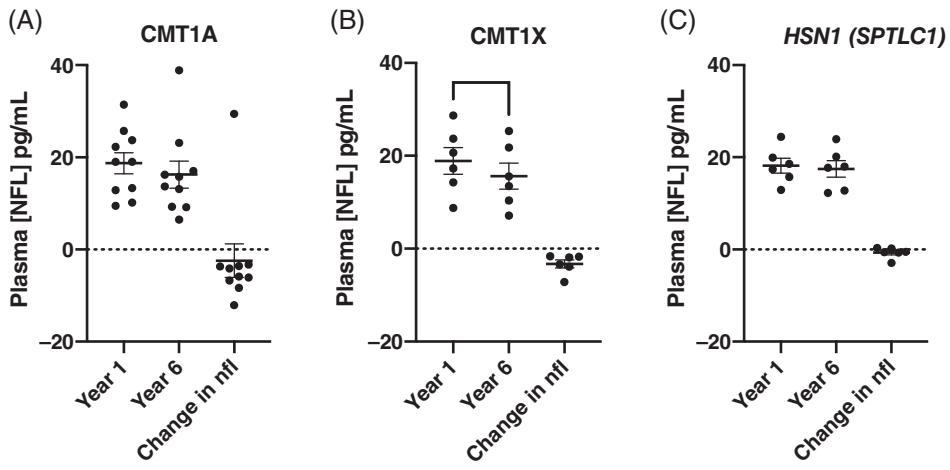

FIGURE 4 Plasma NFL concentrations for patients with CMT1A, CMT1X and HSN1 at baseline and 6 years and the mean paired difference. There is a significant reduction in plasma NFL concentration in patients with CMT1X over a 6-year period (**$P = .01$, error bars = SEM)

change with time, it may affect the timing of, or the ability to detect a significant alteration in NFL concentration in a clinical trial. To explore this further, we examined plasma NFL in two different mouse models of CMT2D, which are known to show progressive neurodegeneration over the examination period.[28] In the more severe GARS^{P278KY} mouse, plasma NFL was highest at 5 weeks before falling to normal levels suggesting an early window of opportunity for treatment. Plasma NFL for the GARS^{C201R} mouse was similar to the baseline wild type concentration; however, the wild type mice showed significantly increased plasma NFL at multiple later time points. The cause for this difference is unknown but raises concern about the suitability of plasma NFL as a biomarker of axonal degeneration for trials in mouse models of this subtype of the disease. We originally speculated that the elevated plasma NFL was due to haemolysis of samples, which can result in spuriously elevated NFL concentrations (NFL is expressed in red blood cells[29]); however, the replication of this result in a separate colony at a different time point would argue against this, although it remains a possibility.

In this study, we were also able to collect paired blood samples on 27 patients from our original CMT cohort after a 6-year interval.[21] Our analysis shows no statistically significant change in plasma NFL over 6 years for patients with either CMT1A or HSN1, although there was a trend towards a reduction in CMT1A, but this was not significant. On the other hand, there was a statistically significant, albeit small, reduction in plasma NFL in patients with CMT1X over 6 years, although the number of patients was small (n = 6). Large cross-sectional studies of patients with different CMT subtypes and ages spanning all decades will be invaluable in identifying the age of maximal axonal degeneration and the window of opportunity for maximum therapeutic effect.

The standardised response of the mean (SRM) is a measure of the responsiveness of an outcome measure to detect the change and is calculated by dividing the mean change by the SD of the change. In CMT1A, version 1 of the CMT Neuropathy Score has an SRM of 0.13 to detect a 50% slowing of disease progression over 24 months. This would equate to 7700 patients with CMT1A required in each arm of a placebo-controlled trial to detect a 50% change in disease progression with a significance level of 0.05% with 80% power.[11] For calf muscle MRI fat fraction with baseline fat fraction >10%, the SRM is 2.19 over 12 months, equating to a requirement of 11 patients in each arm of a trial.[3] We have previously demonstrated a mean difference in plasma NFL concentration between patients with CMT1A and controls of 10 pg/mL. If one uses the SD of the mean change in NFL at 6 years in this study as a measure of the intra-subject variability, one can estimate an SRM for plasma NFL in CMT1A. For a 50% drop in plasma NFL concentration (5 pg/mL) and an SD of 11.51, the estimated SRM for plasma NFL is 0.04, which is significantly worse than version 1 of the CMTNS (which has been shown to be underpowered for use as a primary outcome measure in clinical trials in CMT1A).[30] This suggests that due to the small increase in plasma NFL in CMT1A compared with controls and the significant intrasubject variation that it is unlikely to be suitable as a primary outcome measure in CMT1A for this age group. On the other hand, plasma NFL increases with age,[31] and it is noteworthy that the mean age of the CMT cohort was 46 years. As the rate of axonal degeneration in CMT may vary, with higher rates theoretically possible at a younger age, it remains a possibility that plasma NFL may still have a role as a biomarker for clinical trials at earlier time points.

In summary, this study provides additional data on the use of plasma NFL as a biomarker in CMT. We have replicated our previous findings of increased concentrations in patients with CMT compared with controls, and we have shown that in mouse models of the disease, concentrations can vary over the lifetime of the animal and that in humans, the change in concentration may vary according to subtype. We have also shown pilot data that NFL is unlikely to be suitable as a primary outcome measure in patients with CMT1A. We are currently exploring NFL vs a number of other clinical, plasma and MRI biomarkers in longitudinal studies of CMT1A, CMT1B, CMT2A and CMT1X.

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CONFLICT OF INTEREST

HZ has served at scientific advisory boards for Alector, Eisai, Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics, Nervgen, AZTherapies and CogRx, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Programme (outside submitted work).

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