Received: 28 June 2019 | Revised: 18 July 2019 | Accepted: 22 July 2019

DOI: 10.1111/ane.13152

ORIGINAL ARTICLE

Acta Neurologica Scandinavica | WILEY

Plasma neurofilaments correlate with disability in progressive multiple sclerosis patients

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Funding information
Fondazione Italiana Sclerosi Multipla, Grant/Award Number: 2016/R/20

Objectives: Cerebrospinal fluid (CSF) and blood neurofilaments (NFLs) are markers of axonal damage and are being investigated, mostly in relapsing-remitting (RR) MS, as a marker of disease activity and of response to treatment, while there are less data in progressive MS patients. Primary aim was to measure NFL in plasma samples of untreated patients with primary (PP) and secondary (SP) progressive MS and to correlate them with disability, disease severity, and prior/subsequent disability progression.

Materials and Methods: Neurofilament concentrations were measured using SIMOA (Single Molecule Array, Simoa HD-1 Analyzer; Quanterix).

Results: Neurofilament concentrations were measured on plasma samples of 70 progressive (27 PP and 43 SP), 21 RRMS patients, and 10 HCs. Longitudinal plasma NFL (pNFL) concentrations (median interval between sampling: 25 months) were available for nine PP/SP patients. PNFL concentrations were significantly higher in PP/SP compared to RRMS patients. They correlated with EDSS and MS Severity Score values. There was no difference in pNFL levels between PP/SP patients with EDSS progression in the preceding year (14% of patients) or during a median follow-up of 27 months (41%). In the longitudinal sub-study, pNFL levels increased in all patients between sampling by a mean value of 23% while EDSS mostly remained stable (77% of cases).

Conclusion: In PP/SP progressive MS patients, pNFL levels correlate with disability and increase over time, but are not associated with prior/subsequent disability progression, as measured by EDSS, which may not be a sufficiently sensitive tool in this context.

KEYWORDS
biomarkers, multiple sclerosis, primary progressive, secondary progressive, serum neurofilaments, single molecule array

1 | INTRODUCTION

Neurofilaments are the major structural proteins of neurons and are released in significant quantity following axonal damage or neuronal degeneration. Disruption to the axonal membrane releases neurofilaments into the interstitial fluid and eventually into cerebrospinal fluid (CSF) and blood. In multiple sclerosis (MS), the neurofilament light chain (NFL) is considered a marker of disease activity and CSF-NFL

have been noted to be increased during relapses[1,2] and to be related to magnetic resonance imaging (MRI) activity, brain atrophy[1,3-5] neurological disability[2,6] and with conversion to MS in patients with clinically isolated syndrome (CIS).[1,2] Furthermore, a reduction in CSF-NFL occurs following MS treatments such as natalizumab, mitoxantrone, fingolimod, and rituximab.[4,7,8]

Serum (sNFL) levels are significantly lower compared to those in CSF and, due to the suboptimal sensitivity of serum assays available until a short while ago, they showed a weak correlation with CSF levels or they were below the detection limit of the ELISA assay.[9]

The development of an immunoassay based on the Single Molecule Array (SIMOA) technique, however, now allows quantification down to subfemtomolar concentrations (0.1 pg/mL) of the analyte[10] and recent studies using this technique showed correlations between sNFL and clinical and MRI activity in MS patients, as well as reductions following immunomodulatory/immunosuppressive treatment.[11-14]

Most studies using SIMOA have focused on blood NFL levels as a marker of inflammation and of subclinical disease activity in patients with relapsing-remitting (RR) MS, while there are less data on blood NFL levels in patients with primary/secondary progressive MS and on their correlation with disability/disability progression.

1.1 | Objective

Our primary aim was to measure NFL concentrations in plasma samples, which had been previously collected and stored, of untreated patients with primary (PP) and secondary (SP) progressive MS, and to retrospectively correlate them with disability, disease severity, and prior/subsequent disability progression. Secondary aims were to compare plasma NFL (pNFL) levels in progressive patients with those of untreated patients with RRMS and with those of healthy controls (HC), and to assess pNFL variations over time in a subgroup of progressive patients who had undergone more than one sampling.

2 | METHODS AND MATERIALS

2.1 | Patients

In the present study, we analyzed clinical data and plasma samples, which had been aliquoted and stored away at −80°C after collection, of patients enrolled in a previous study. Inclusion criteria were age ≤75 years and diagnosis of MS according to the 2010 revised McDonald Criteria.[15] Furthermore, patients had to be untreated at the time of sampling. As a consequence, RRMS patients were enrolled shortly after diagnosis, before commencing a disease-modifying treatments, and enrolled PP/SP patients had not shown "disease activity" (ie, clinical relapses, contrast-enhancing lesions on MRI T1-weighted images, or new lesions on T2-weighted images) in the previous years (at least three). In the absence of long-standing disease stability, they would, otherwise, as per clinical practice, have been candidates for currently licensed immunomodulatory/immunosuppressant treatments. Exclusion criteria were steroid treatment in the previous month and immunomodulatory/immunosuppressive treatment in the preceding 6 months.

Stored plasma samples were available for 70 progressive (27 PP and 43 SP) and 21 RRMS untreated patients, and for 10 healthy controls (HC), who were age-matched to progressive MS patients. Repeated pNFL measurements over time were available for nine PP/SP patients. Approval from the Modena Ethics Committee (Italy) was obtained for the study (protocol number 2843, July 25, 2017).

2.2 | Clinical data

We retrospectively retrieved clinical information from our center's electronic database, which is regularly updated by Neurostatus-trained neurologists (DF, FV, PS): age, sex, disease duration, Expanded Disability Status Scale (EDSS) at the time of sampling, the previous year, the following year and at last follow-up, Multiple Sclerosis Severity Score (MSSS)[16] at the time of sampling and at last follow-up, occurrence of relapses in the previous year and during follow-up. We then calculated whether there had been a disability progression in the preceding year or during follow-up. Disability progression was defined as either (a) an EDSS increase of at least one point for EDSS scores up to 5, (b) an EDSS increase of at least 0.5 points for EDSS scores of at least 5.5.

2.3 | Laboratory procedures

Blood was collected in EDTA-treated tubes and centrifuged for 15 minutes at 800 rpm at room temperature. Plasma was transferred into a clean cryogenic vials using a Pasteur pipette, apportioned into 0.5 mL aliquots, stored at −80°C, and shipped in dry ice. Concentrations of NFL in plasma were analyzed using a High Definition-1 (HD-1) Immunoassay Analyzer, SimoaTM, which runs ultra-sensitive paramagnetic bead-based enzyme-linked immunosorbent assays (ELISAs).[17] NFL concentrations were measured in duplicate from each sample with the Simoa HD-1 Analyzer (Quanterix) using a commercial NFL kit (102258) at the Quanterix laboratories by Quanterix staff who were blinded to clinical data and group membership.

2.4 | Statistical analysis

We used the Mann-Whitney test for comparison of continuous variables, the chi-square test for comparison of categorical variables, the Kruskal-Wallis test for comparison of multiple groups (followed by post hoc tests), and Spearman's rank coefficient for assessing correlations between variables. Data were analyzed using STATA 11 (StataCorp).

3 | RESULTS

3.1 | Subject characteristics and pNFL values

Enrolled subject characteristics and pNFL values are shown in Table 1. Briefly, median pNFL levels were 11.3 pg/mL in the whole patient population, 12.8 pg/mL in PP/SP patients, 9.7 pg/mL in RR patients, and 9.5 pg/mL in HC (Figure 1), with significant differences (post hoc analysis of Kruskal-Wallis test) of PP/SP versus RR patients ($P = .007$).

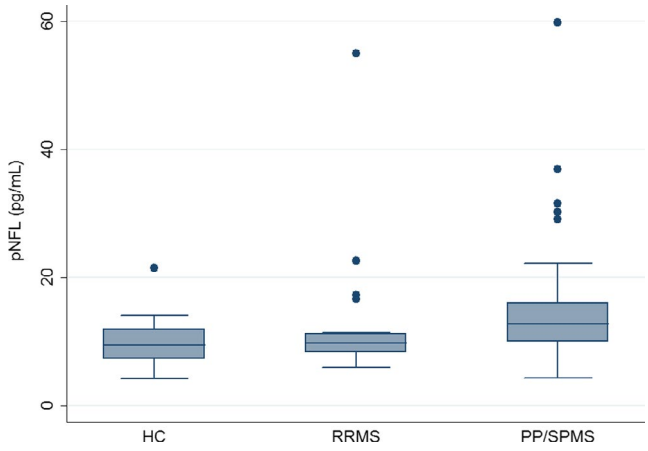

FIGURE 1 Plasma NFL values across different groups

3.2 | Correlations between pNFL values and clinical variables

In all MS patients taken together, pNFL levels correlate with EDSS and MSSS values at the time of sampling, although, after correcting for age, only EDSS at the time of sampling maintained a statistical significance (Table 2). In PP/SP patients, they correlate with disease duration and EDSS at the time of sampling, even after correcting for age. Figure 2 shows the correlation between EDSS at the time of sampling and pNFL levels in PP/SP patients.

3.3 | Plasma NFL levels and disease progression/activity

Out of the progressive patients, 10/70 (14%) had shown EDSS progression in the year preceding the pNFL sampling, and 30 (41%) showed progression between sampling and last follow-up visit, with no significant differences in pNFL levels between those who progressed and those who did not. Only one progressive patient had a relapse during the follow-up period. Among RR patients, 33% of patients (n = 7) presented with a relapse during follow-up and these had significantly higher pNFL levels (20.2 pg/mL) compared to those who did not relapse (8.7 pg/mL; *P* = .014).

3.4 | Repeated pNFL sampling

In nine progressive patients, pNFL levels were tested at least twice, with a median interval between testing of 25 months (range: 7-39). PNFL values increased in all patients from a median of 10.8 pg/mL (IQR: 9.8-13.9) to a median of 13.9 (IQR: 11.4-15.4) at final sampling, (Figure 3) while EDSS either remained stable (n = 7/9) or increased (n = 2/9).

4 | DISCUSSION

There is increasing research on sNFL levels as candidate biomarkers for monitoring disease progression, neurodegeneration, and treatment efficacy in different neurodegenerative diseases, including Alzheimer's disease, frontotemporal dementia and amyotrophic lateral sclerosis.[18-20] Patients with MS experience a higher rate of brain volume loss (BVL) than do healthy individuals and BVL in MS correlates with disease activity and predicts long-term disability status.[21,22] Patients with higher sNFL levels are at higher risk of experiencing accelerated brain and spinal cord volume loss at 2 and 5 years,[23,24] and a recent study confirmed the association of sNFL with spinal cord volume loss in a subgroup of progressive MS patients without detectable focal inflammatory MRI activity.[24] The authors argue that sNFL can represent a more accurate indicator of ongoing neuro-axonal loss and a better predictor of brain atrophy than MRI measures of acute and chronic lesional activity. As

TABLE 1 Subject characteristics and pNFL values

Variable	All subjects n = 101	Controls n = 10	RRMS n = 21	PRMS n = 70	*P*
pNFL (pg/mL)[a]	11.3 [9.1-15.7]	9.5 [7.3-11.9]	**9.7 [8.3-11.2]**	**12.8 [10-16]**	.007[b]
pNFL >11 pg/mL (yes/no)		3/7	7/14	45/25	.012
Sex (M/F)	31/70	6/4	15/6	49/21	ns
Age (y)[a]	57 [50-62]	59 [47-65]	**40 [37-51]**	**60 [54-63]**	<.001[b]
MS duration (y)[a]			11 [1-17]	20 [14-26]	<.001
Duration of follow-up (mo)[a]			56 [51-59]	27 [20-46]	<.001
EDSS[a]			1.5 [1-1.5]	6.5 [5.5-7]	<.001
EDSS last follow-up[a]			1.5 [0-2]	6.5 [6-7.5]	<.001
MSSS[a]			1 [0.7-2.3]	6.3 [4.6-7.5]	<.001
MSSS at last follow-up[a]			0.7 [0.3-1.6]	6.5 [5-7.8]	<.001

[a]Median [interquartile range].
[b]Result of significant post hoc test comparing data in bold letters.

TABLE 2 Correlations between clinical variables and pNFL values

Variable	All patients (n = 90) ρ	All patients (n = 90) P	RRMS patients (n = 21) ρ	RRMS patients (n = 21) P	PP/SPMS patients (n = 70) ρ	PP/SPMS patients (n = 70) P
Age	.39	<.001	−.032	ns	.43	<.001
Disease duration	.19	ns	−.35	ns	.25	.04
EDSS at the time of sampling	.34	.001	.09	ns	.26	.027
MSSS at the time of sampling	.27	.01	.21	ns	.11	ns

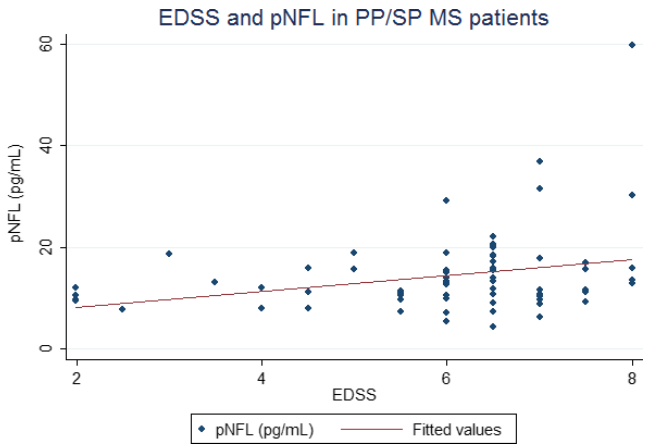

FIGURE 2 Correlation between EDSS and pNFL levels in PP/SP patients

opposed to BVL measurement, sNFL dosage could represent a more feasible and low-cost option for repetitively quantifying the rate of neuronal loss within the CNS at the time of sampling.

Similar to other studies, we found correlations between blood NFL levels and EDSS scores and a correlation between blood NFL levels and age.[11,12,24] Data on the higher blood NFL levels in progressive patients as opposed to RRMS patients are not conclusive since progressive patients were older than RRMS patients. With regard to the prognostic relevance of a single measurement, we could confirm higher pNFL levels in RR patients who presented with a relapse during follow-up,[11] but we could not discriminate PP/SP with disease progression from those without progression (neither in the preceding year nor during follow-up) based on a single pNFL concentration measurement. A single measurement may not be sufficiently informative in this context, while a slope showing an increasing (as opposed to a stable or decreasing) trend in repeated measurements may offer more information on the presence of active neurodegeneration within the CNS. The effect of aging—an increase in sNFL of approximately 2.2% yearly has been described[11,24]—and the correlation between CSF/sNFL and age must be kept in mind, but increases over time in this study were markedly higher with a mean increase of 23% between the first and second sample.

Patients enrolled in the present study were untreated; this means that they were judged by treating neurologists (based on absence of relapses and of focal MRI activity) as being "not active" and, thus, not candidates for immunosuppressive treatment. This has given us the opportunity to assess pNFL levels supposedly released as a result of chronic inflammatory and/or intrinsic neurodegenerative processes, in the absence of overt inflammation, although a recent study on progressive MS patients[25] argues that relapses and/or focal MRI activity alone probably do not capture the presence of active, ongoing inflammation, since in a substantial proportion of not-active progressive MS patients, there were detectable levels of CSF MMP-9 and of CXCL13, which is considered a marker of active intrathecal inflammation,[26-28] and these patients had increased CSF-NFL levels.

This study has also given us the means, in a small sample with longitudinal measurements, and who were still untreated at the time of

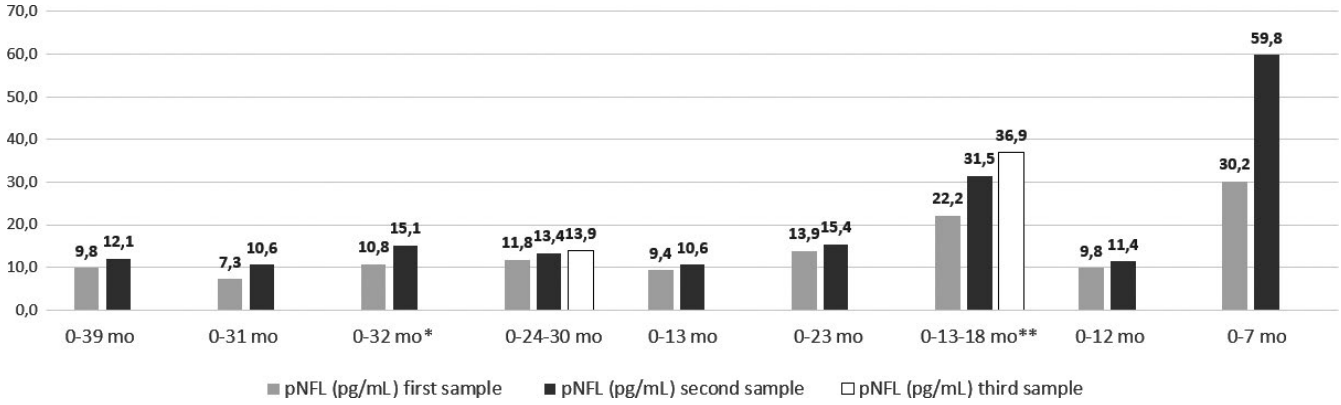

FIGURE 3 Repeated longitudinal pNFL measurements (patients n = 9). *EDSS increase from 5.5 to 6 between first and second sample. **EDSS increase from 6.5 to 7 between first and second sample

repeated sampling, to evaluate the trend across time. Interestingly, pNFL increased in all patients. Of these, only two had an EDSS increase between sampling. EDSS, however, is probably not the most appropriate clinical tool for assessing progression with its low sensitivity to change as well as its over-reliance on lower extremity function in its mid-ranges and its low sensitivity to measure cognition and upper limb function.[29-31]

The strength of the study is its focus on progressive MS patients, with a longitudinal analysis in a small subset. The study, however, was retrospective and disability was assessed using only EDSS, and this did not permit an in-depth evaluation of their disability.

With regard to the SIMOA-measured pNFL levels, reference ranges in different MS phenotypes and controls are still lacking and results throughout studies are not homogeneous. Disanto et al[11] report median sNFL values of 22.9 pg/mL in HC, 27.2 in RR/CIS patients and 41.4 pg/mL in PP/SP patients, which are higher than the ones reported both in the present study and in a recent study on 222 CIS patients[32] in which median baseline sNFL values (22 pg/mL) in patients were comparable to those found in HC (22.9 pg/mL) by Disanto et al The present results are similar to other large studies on the topic: Piehl et al[13] found a median value of 15.5 pg/mL in 241 MS patients switching to fingolimod and 8.2 pg/mL in a group of controls, while Novakova et al[12] found a median value of 16.9 pg/mL in 201 patients and 10.5 pg/mL in HC. Differences between studies could be due to the lack of standardization of laboratory methods and highlight the need for certified reference methods and materials. Finally, we did not find any significant differences between HC and MS subjects. This may be due to the small number of controls and to the fact that they were age-matched to progressive patients, (with an average age of 59 years) and NFL levels are known to correlate with age. Furthermore, studies have shown substantial overlap in sNFL levels between MS subjects and HC.[11,24]

5 | CONCLUSION

Plasma NFL levels were correlated with patient disability in PP/SP MS patients and increased over time in patients with repeated measurements. They were, however, not associated with prior or subsequent disability progression as measured by EDSS, which may have a too low sensitivity to change/disease progression in this context, or over a short time-period.

Larger studies, with longitudinal measurements and in-depth clinical assessments, and standardized laboratory procedures are warranted in order to verify if SIMOA-measured sNFL concentrations and their modifications over time can be useful, at an individual level, as a paraclinical marker of disease progression in patients with progressive MS.

ACKNOWLEDGMENTS

This work was supported by Italian Multiple Sclerosis Foundation (FISM) project 2016/R/20 to MP. Sara De Biasi is a Marylou Ingram Scholar for the International Society for Advancement of Cytometry (ISAC). We thank Dr Rosalberta Pullano (University of Modena and Reggio Emilia) for recruiting healthy subjects included in the study.

CONFLICT OF INTEREST

Authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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REFERENCES

1. Teunissen CE, Iacobaeus E, Khademi M, et al. Combination of CSF N-acetylaspartate and neurofilaments in multiple sclerosis. *Neurology*. 2009;72(15):1322-1329.
2. Kuhle J, Leppert D, Petzold A, et al. Neurofilament heavy chain in CSF correlates with relapses and disability in multiple sclerosis. *Neurology*. 2011;76(14):1206-1213.
3. Lim ET, Sellebjerg F, Jensen CV, et al. Acute axonal damage predicts clinical outcome in patients with multiple sclerosis. *Mult Scler*. 2005;11(5):532-536.
4. Kuhle J, Disanto G, Lorscheider J, et al. Fingolimod and CSF neurofilament light chain levels in relapsing-remitting multiple sclerosis. *Neurology*. 2015;84(16):1639-1643.
5. Mellergård J, Tisell A, Blystad I, et al. Cerebrospinal fluid levels of neurofilament and tau correlate with brain atrophy in natalizumab-treated multiple sclerosis. *Eur J Neurol*. 2017;24(1):112-121.
6. Norgren N, Sundström P, Svenningsson A, Rosengren L, Stigbrand T, Gunnarsson M. Neurofilament and glial fibrillary acidic protein in multiple sclerosis. *Neurology*. 2004;63(9):1586-1590.
7. Gunnarsson M, Malmeström C, Axelsson M, et al. Axonal damage in relapsing multiple sclerosis is markedly reduced by natalizumab. *Ann Neurol*. 2011;69(1):83-89.
8. Axelsson M, Malmeström C, Gunnarsson M, et al. Immunosuppressive therapy reduces axonal damage in progressive multiple sclerosis. *Mult Scler*. 2014;20(1):43-50.
9. Fialová L, Bartos A, Svarcová J, Zimova D, Kotoucova J, Malbohan I. Serum and cerebrospinal fluid light neurofilaments and antibodies against them in clinically isolated syndrome and multiple sclerosis. *J Neuroimmunol*. 2013;262(1-2):113-120.
10. Kuhle J, Barro C, Andreasson U, et al. Comparison of three analytical platforms for quantification of the neurofilament light chain in blood samples: ELISA, electrochemiluminescence immunoassay and Simoa. *Clin Chem Lab Med*. 2016;54(10):1655-1661.
11. Disanto G, Barro C, Benkert P, et al. Serum neurofilament light: a biomarker of neuronal damage in multiple sclerosis. *Ann Neurol*. 2017;81(6):857-870.
12. Novakova L, Zetterberg H, Sundström P, et al. Monitoring disease activity in multiple sclerosis using serum neurofilament light protein. *Neurology*. 2017;89(22):2230-2237.
13. Piehl F, Kockum I, Khademi M, et al. Plasma neurofilament light chain levels in patients with MS switching from injectable therapies to fingolimod. *Mult Scler J*. 2017;24:1046–1054.

14. Varhaug KN, Barro C, Bjørnevik K, et al. Neurofilament light chain predicts disease activity in relapsing-remitting MS. *Neurol Neuroimmunol NeuroInflammation*. 2018;5(1):1-7.
15. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol*. 2011;69(2):292-302.
16. Roxburgh RH, Seaman SR, Masterman T, et al. Multiple sclerosis severity score: using disability and disease duration to rate disease severity. *Neurology*. 2005;64(7):1144-1151.
17. Rissin DM, Kan CW, Campbell TG, et al. Single-Molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. *Nat Biotechnol*. 2010;28(6):595-599.
18. Rohrer JD, Woollacott I, Dick KM, et al. Serum neurofilament light chain protein is a measure of disease intensity in frontotemporal dementia. *Neurology*. 2016;87(13):1329-1336.
19. Mattsson N, Andreasson U, Zetterberg H, Blennow K. Association of plasma neurofilament light with neurodegeneration in patients with Alzheimer disease. *JAMA Neurol*. 2017;74(5):557-566.
20. Feneberg E, Oeckl P, Steinacker P, Verde F, Barro C. Multicenter evaluation of neuro fi laments in early symptom onset amyotrophic lateral sclerosis. *Neurology*. 2018;90(1):e22-e30.
21. Fisher E, Rudick RA, Simon JH, et al. Eight-year follow-up study of brain atrophy in patients with MS. *Neurology*. 2002;59(9):1412-1420.
22. Horakova D, Dwyer MG, Havrdova E, et al. Gray matter atrophy and disability progression in patients with early relapsing–remitting multiple sclerosis. *J Neurol Sci*. 2009;282(1-2):112-119.
23. Siller N, Kuhle J, Muthuraman M, et al. Serum neurofilament light chain is a biomarker of acute and chronic neuronal damage in early multiple sclerosis. *Mult Scler J*. 2018;25(5):678-686.
24. Barro C, Benkert P, Disanto G, et al. Serum neurofilament as a predictor of disease worsening and brain and spinal cord atrophy in multiple sclerosis. *Brain*. 2018;141(8):2382-2391.
25. Sellebjerg F, Börnsen L, Ammitzbøll C, et al. Defining active progressive multiple sclerosis. *Mult Scler J*. 2017;23(13):1727-1735.
26. Sellebjerg F, Bornsen L, Khademi M, et al. Increased cerebrospinal fluid concentrations of the chemokine CXCL13 in active MS. *Neurology*. 2009;73(23):2003-2010.
27. Ferraro D, Galli V, Vitetta F, et al. Cerebrospinal fluid CXCL13 in clinically isolated syndrome patients: association with oligoclonal IgM bands and prediction of multiple sclerosis diagnosis. *J Neuroimmunol*. 2015;283:64-69.
28. Khademi M, Kockum I, Andersson ML, et al. Cerebrospinal fluid CXCL13 in multiple sclerosis: a suggestive prognostic marker for the disease course. *Mult Scler*. 2011;17(3):335-343.
29. Cohen JA, Reingold SC, Polman CH, Wolinsky JS, International Advisory Committee on Clinical Trials in Multiple Sclerosis. Disability outcome measures in multiple sclerosis clinical trials: current status and future prospects. *Lancet Neurol*. 2012;11(5):467-476.
30. Brissart H, Sauvée M, Latarche C, Dillier C, Debouverie M. Integration of cognitive impairment in the expanded disability status scale of 215 patients with multiple sclerosis. *Eur Neurol*. 2010;64(6):345-350.
31. Ontaneda D, Cohen JA, Amato MP. Clinical outcome measures for progressive MS trials. *Mult Scler*. 2017;23(12):1627-1635.
32. Dalla Costa G, Martinelli V, Sangalli F, et al. Prognostic value of serum neurofilaments in patients with clinically isolated syndromes. *Neurology*. 2019;92(7):E733-E741.

How to cite this article: Ferraro D, Guicciardi C, De Biasi S, et al. Plasma neurofilaments correlate with disability in progressive multiple sclerosis patients. *Acta Neurol Scand*. 2019;00:1–6. https://doi.org/10.1111/ane.13152