Multiple Sclerosis and Related Disorders 77 (2023) 104893

Contents lists available at ScienceDirect

Multiple Sclerosis and Related Disorders

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

Original article

Serum Neurofilaments are a reliable biomarker to early detect PML in Multiple Sclerosis patients

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ARTICLE INFO

Keywords:
Progressive multifocal leukoencephalopathy
Neurofilament light chain
Multiple Sclerosis
Natalizumab

ABSTRACT

Background: The earliest detection of progressive multifocal leukoencephalopathy (PML) is crucial in Natalizumab (NTZ)-treated Multiple Sclerosis (MS) patients. This study aims to assess serum Neurofilaments (sNFL) ability to early detect PML in longitudinal patients' follow-up.

Methods: NFL were retrospectively measured in four PML cases occurred at the Regional Referring Center for MS (CRESM, Italy), in samples collected since one year before PML diagnosis, at PML diagnosis, during PML and in post-PML follow-up. sNFL levels were interpreted according to previously defined reference values. Clinical examination and EDSS were performed at each NTZ infusion. Routinary MRI was undertaken every six months; after PML diagnosis, MRI was performed according to clinical evaluation. sNFL were also measured in 45 NTZ-treated patients experiencing NEDA-3 status for at least 12 months.

Results: Patients showed different PML onsets and manifestations: in 3 patients routinary brain MRI revealed radiological signs of PML preceding different clinical manifestations, while in one patient brain MRI was performed after the clinical onset. PML diagnosis was defined at the time of the first detection of JCV DNA in cerebrospinal fluid. The following different PML phases were considered: *1. Basal* (up to 4 months before PML diagnosis): sNFL values were in the normal range in all patients' samples, except for one (median 9.1 pg/ml, range 6.2–15.1 pg/ml) *2. Pre-PML* (within 3 months before PML diagnosis): sNFL were elevated in all available samples (median 19.50 pg/ml, range 15.50–33.80 pg/ml). *3. PML diagnosis*: sNFL were elevated in all patients (median 59.20 pg/ml, range 11.1–101.50 pg/ml). *4. PML/IRIS*: during this phase, sNFL levels reached their peak (median 96.35 pg/ml, range 20.5–272.9) in all patients. *5. Post-PML* (recovery phase, starting from the first MRI without enhancement, up to the end of follow-up): sNFL levels showed a decrease (median 12.80 pg/ml, range 9.30–30.60); however, based on reference values, sNFL were still elevated in 2 out of 4 patients at the end of their follow-up (622 and 887 days after PML diagnosis). sNFL were always elevated when MRI scan suggested a suspicious of PML. In NEDA-3 patients, sNFL levels were in the normal range in all patients' samples (median 4.7 pg/ml, range 1.4–8.6 pg/ml).

Conclusion: Elevated sNFL were observed not only at PML diagnosis, but also in pre-PML phase. At PML recovery, sNFL weren't normalized in all patients' samples, suggesting ongoing neuronal degeneration. sNFL represent a reliable biomarker and should be introduced in clinical practice as an additional/alternative parameter to MRI to early detect and monitor PML.

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https://doi.org/10.1016/j.msard.2023.104893
Received 16 May 2023; Accepted 13 July 2023
Available online 16 July 2023

1. Introduction

Natalizumab (NTZ) is one of the most effective treatments for highly active relapsing remitting multiple sclerosis (RRMS) patients (Morrow et al., 2022).

However, its use is associated to several adverse events, the most serious of which is the development of progressive multifocal leukoencephalopathy (PML), caused by John Cunningham virus (JCV). The occurrence of PML in MS entails serious prognostic implications, as it is responsible for the death of about 20% of patients or for serious disability to 40% of survivors. The clinical presentation of NTZ-associated PML consists of cognitive impairments, experienced in over half of the patients, together with motor symptoms, ataxia, neurovisual disturbances, dysphasia or agnosia in more than 40% of cases (European Medicines Agency, 2016).

Over the years, since NTZ approval for MS treatment, the indications for monitoring the risk of developing PML have evolved and three risk factors have been identified, which include the presence/levels of specific anti-JCV antibodies, the increasing duration of NTZ treatment and the previous use of immunosuppressive drugs. The current risk algorithm based on the combination of these factors aims to minimize the risk of PML in NTZ treated patients (Schwab et al., 2017).

Further, the monitoring of NTZ-treated patients is crucial to early recognize PML signs and symptoms. Since 2016, guidelines indicate that during treatment with NTZ, patients should be monitored at regular intervals for signs and symptoms of new neurological dysfunction, and a full brain MRI should be performed at least once a year for the duration of the treatment. For patients at higher risk of PML, more frequent MRIs (e.g. every 3–6 months) should be considered (European Medicines Agency, 2016).

In this context, additional sensitive biomarkers reflecting clinical signs and brain lesions are needed for the early detection of PML onset and to aid personalized treatment decisions in clinical practice.

Neurofilament-light chain (NFL) are structural axonal proteins, which are released in cerebrospinal fluid and in blood following and reflecting axonal damage (Barro et al., 2020; Bittner et al., 2021; Lambertsen et al., 2020; Thebault et al., 2020; Varhaug et al., 2019).

The availability of ultrasensitive methods for the detection of NFL in serum (sNFL) has enabled the extensive demonstration of the strong correlation between NFL levels and clinical/radiological parameters in MS, supporting the role of sNFL as the most promising biomarker for disease activity and treatment response in MS (Barro et al., 2018; Benkert et al., 2022; Bittner et al., 2021; Delcoigne et al., 2020; Novakova et al., 2017; Thebault et al., 2020; Uher et al., 2020; Varhaug et al., 2019).

The possible applications of this biomarker in different clinical contexts are enormous.

In particular, the utility of sNFL in the monitoring of PML onset is of great interest in MS. In this context, a routinary use of sNFL in clinical practice requires analyses to be performed at individual level, based on shared and reliable normative values (Benkert et al., 2022; Hviid et al., 2020; Leppert and Kuhle, 2019; Valentino et al., 2021). Further, it is fundamental to establish if this measure is more sensitive than radiological or clinical evaluation.

Some few studies have previously demonstrated that sNFL levels are strongly elevated at PML diagnosis (Dalla Costa et al., 2019; Fissolo et al., 2021; Loonstra et al., 2019) and correlate with MRI lesions' volume; still, data analysis at individual level was lacking, limiting a simple and correct interpretation of results in single patients.

In the present study, we retrospectively measured longitudinal sNFL in the four PML cases diagnosed in our MS center between 2009 and 2015, which were monitored by MRI and clinical parameters.

Further, sNFL were tested in a group of NTZ-treated patients experiencing NEDA-3 status for at least 12 months, as controls. sNFL levels were interpreted according to previously defined reference values (Valentino et al., 2021).

2. Material and methods

2.1. Study participants

This retrospective study includes the four MS patients who developed PML at the Regional Referring Center for MS (CRESM, Orbassano, Turin, Italy) between years 2009 and 2015.

Including criteria were:

1) diagnosis of RRMS according to revised Mc Donald criteria (Thompson et al., 2018)
2) NTZ treatment
3) Confirmed PML diagnosis (Berger et al., 2013)
4) availability of follow-up samples collected during their longitudinal follow-up and stored in CRESM Biobank (University Hospital San Luigi Gonzaga, deliberation n° 56/2020) (Marnetto et al., 2020).

Clinical examination and EDSS were performed at each NTZ infusion during follow-up. Routinary MRI was undertaken every six months, while, after PML diagnosis, MRI was performed according to clinical evaluation. All data were collected in medical reports.

Cerebrospinal (CSF) analysis including JCV-DNA detection was performed to confirm and define PML diagnosis: CSF samples were analyzed for JCV–DNA detection in the local laboratory and in the centralized specialized lab of the National Institute of Health (NIH, Bethesda, USA).

Patients' follow-up was subdivided in five different phases:

1 *Basal phase*, up to 4 months before PML diagnosis
2 *Pre-PML*, within 3 months before PML diagnosis
3 *PML diagnosis,* corresponding to the time of first detection of JCV-DNA in CSF
4 *PML/IRIS*, during the evolution of PML disease and immune reconstitution inflammatory syndrome (IRIS)
5 *Post-PML*, recovery phase, starting from the first MRI without enhancement, up to the end of follow-up.

A group of 45 NTZ-treated patients in NEDA-3 status (no evidence of clinical/radiological activity, nor progression) throughout the last 12 months was included as control group. They were matched for sex (32 female, 13 male), age (median 35 years, range 21–49 years) and treatment duration (median 24 months, range 12–60 months) with patients developing PML.

2.2. Serum NFL measurement

Blood samples were collected in serum tubes (BD Vacutainer, Becton, Dickinson and Company) during follow-up routine visits and at lumbar puncture. Samples were processed within two hours from collection according to international guidelines (Teunissen et al., 2009). Blood tubes were centrifuged at 3000x*g* 10 min, and serum supernatant stored in at least two aliquots: one aliquot was used to perform anti-NTZ antibody test, while the leftover serum sample was stored at −80 °C. sNFL levels were measured by single molecule array (Simoa™) on SR-X instrument (Lambert et al., 2018) using NF-light assays (Quanterix). In each assay session, samples were run in duplicate together with a titration curve and two controls provided in the kit. In addition, two quality internal controls were run in each session to monitor inter-assay and inter-lot variability. sNFL levels were interpreted according previously defined reference values (Valentino et al., 2021).

2.3. Ethical committee approval

The study was approved by the Ethical Committee of San Luigi Gonzaga University Hospital (approvals number 7262/2019 and 18,390/2019). All participants provided written informed consent.

2.4. Statistical analysis

Statistical analysis was performed using R version 4.1.1. Normality of distribution and homogeneity of variances were evaluated by Shapiro-Wilk test and Levene's test.

To test differences in sNFL levels between groups, the Kruskal-Walis test with Dunn Post-hoc test was performed. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg method. p-values < 0.05 were considered statistically significant.

3. Results

Four NTZ-treated patients developed PML during their follow-up since 2007 at CRESM.

PML diagnosis was confirmed in each patient by detection of JCV DNA in CSF: the time of first JCV-DNA detection was defined as T0. sNFL levels were measured in all available serum samples ($n = 38$) collected since one year before PML diagnosis, during PML and after recovery (median follow-up 43 months, range 31–45 months).

3.1. Individual longitudinal sNFL levels in patients developing PML

Patients showed different PML onsets and manifestations: in 3 patients routinary brain MRI revealed radiological signs of PML preceding different clinical manifestations, while in one patient brain MRI was performed after the clinical onset.

Briefly, *patient 1* (Fig. 1A) was a Caucasian woman, aged 32 at the time of PML diagnosis. She started NTZ therapy in June 2008; she was previously treated with Interferon beta 1a i.m.

In October 2009 she had her 18th NTZ infusion; routinary brain MRI performed after 18 infusions was reported as stable, without evidence of new lesions. In November 19th she came to our Center complaining of occasional left labial hemispasm; she underwent NTZ infusion in November and in December, as the symptom was not attributed to a possible PML manifestation. As hemispasm persisted and worsened, she underwent brain MRI, that showed a hyperintense lesion of right lenticular nucleus. A re-examination of the previous MRI revealed that a very small lesion was already present, despite not being considered suggestive of PML at the time.

NTZ was interrupted; in December 21st the patient underwent lumbar puncture (LP) that was negative for JCV PCR in local lab, but demonstrated positive (31 copies) when tested in the NIH Centralized lab, confirming PML diagnosis (T0); high dose intravenous steroids were started.

From November 2009 to May 2010 clinical and radiological worsening occurred, as patient progressively developed speech impairment up to anarthria and dysphagia needing percutaneous radiologic gastrostomy (PRG). Brain MRIs were performed 31, 66 and 112 days after T0 showing further progressive enlargement of lenticular nucleus lesion and development of cortical frontal atrophy. LP was repeated 36 and 66 days after T0 and JCV PCR analysis tested positive (39 and 70 copies respectively).

The patient underwent intravenous immunoglobulins (T0 + 66 days) and high dose steroid treatment. LP performed 254 days after T0 was negative for JCV-DNA; the patient was considered clinically stable in December 2010 and showed absence of Gadolinium (Gd) enhancing lesions at MRI 619 days after T0.

sNFL levels were in the normal range until 140 days before PML diagnosis. Interestingly, sNFL increased above reference levels (15.1 pg/ul) already 115 days before definite PML diagnosis, 83 days before the first clinical sign and 60 days before the MRI considered stable at that time. sNFL showed a continuous increase in the following months, during pre-PML phase (15.5 pg/ml, 19.5 pg/ml, 33.8 pg/ml) and reached their peak (229.3 pg/ul) during PML evolution, at day 81 after

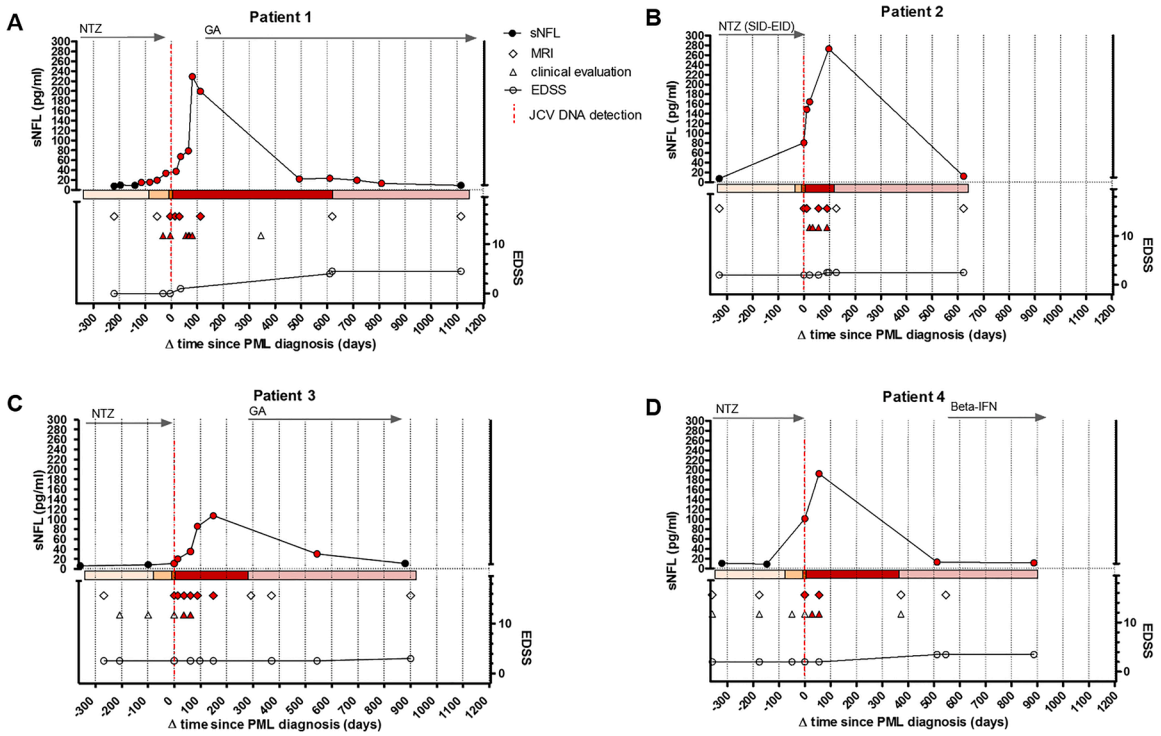


Fig. 1. Longitudinal clinical, radiological and biological data in four PML cases at CRESM. sNFL levels (pg/ml, *black circles*= normal levels, *red circle*= elevated/pathological levels, according to previously defined cut-off values, Valentino et al., 2021), MRI (*white rhombuses*= stable/inactive MRI, *red rhombuses*= active MRI), clinical signs (*white triangles*= clinical stability, *red triangles*= PML signs/symptoms), EDSS scores (*white circles*) and treatments are represented in panels A-D during the longitudinal follow-up of each patient. The different PML phases are represented with different bar sections under each sNFL graph. The *red dotted vertical line* shows the first detection of JCV DNA, considered as the PML diagnostic confirmation (T0 time point).

T0. sNFL decreased during PML recovery and normalized 1115 days after diagnosis.

Patient 2 (Fig. 1B) was a Caucasian woman, aged 39 at the time of PML diagnosis. She started NTZ therapy in June 2010, and she had been previously treated with mitoxantrone and interferon beta 1a 44mcg 3/week.

Routinary brain MRI performed in July 2014 revealed a new right temporo-occipital lesion suggestive of PML; brain MRI was repeated in August 2014, showing an increase of the new lesion, without contrast enhancement. The same day the patient underwent LP and resulted positive for JCV PCR (80 copies), confirming PML diagnosis (T0). Neurological examination didn't show any new signs until September 2014 (T0 + 22 days), when the patient started complaining of right lateral hemianopsia and dysmetamorphopsia; brain MRI showed progressive enlargement of the right temporo-occipital lesion with signs of IRIS and cortical involvement (T0 + 57 days); worsening of radiological signs persisted until November 2014 (T0 + 90 days). Patient was treated with plasma-exchange and high dose intravenous steroids. In January 2015, MRI didn't show any contrast enhancement (T0 + 126 days). sNFL levels were normal in the basal phase (7.5 pg/ul, 330 days before diagnosis). No serum samples were available in the pre-PML phase. sNFL were strongly increased at PML diagnosis (81 pg/ul) and reached their peak (272 pg/ul) during PML IRIS, at T0 + 97 days. sNFL levels decreased during PML recovery (12.5 pg/ul measured in the last available sample, collected at T0 + 622 days).

Patient 3 (Fig. 1C) was a Caucasian man, aged 40 at the time of PML diagnosis. He started NTZ therapy in May 2010; he had been previously treated with interferon beta 1b and glatiramer acetate.

Routinary brain MRI performed in May 2015 (after 63 infusions) showed a new right temporal lesion suggestive of PML; NTZ was interrupted. PML diagnosis (T0) was confirmed by detection of JCV DNA in CSF (11 copies). Neurological examination was stable but patient started having epileptic seizure in July 2015 (T0 + 37 days); brain MRI performed at T0 + 37 days showed enlargement of the temporal lesion with evidence of IRIS. From July to September 2015 (37 to 97 days after T0) brain MRI showed progressive enlargement of the temporal lesion with contrast enhancement. The patients was treated with high dose of steroids. On October 2015 (T0 + 149 days), brain MRI showed dimensional reduction of two lesions with persistent contrast enhancement. In March 2016, brain MRI didn't show any contrast enhancement (T0 + 293 days).

sNFL levels were in the normal range during basal phase. At PML diagnosis (T0), sNFL demonstrated increased (11.1 pg/ml) and they raised to their peak (107.1 pg/ml) at T0 + 149 days during PML evolution. sNFL decreased during PML recovery and normalized 880 days after diagnosis.

Patient 4 (Fig. 1D) was a Caucasian man, aged 47 at the time of PML diagnosis. He started NTZ therapy in November 2009, after having being treated with Interferon beta 1a 22mcg 3/week, Azathioprine and Methotrexate.

Routinary brain MRI performed in April 2012 showed a new left frontal lesion suggestive of PML. NTZ was interrupted and LP was performed showing positive for JCV PCR (15 copies) (T0). Patient underwent plasma exchange.

In May 2012 (T0 + 28 days), the patient started complaining of speech impairment up to motor aphasia (T0 + 55 days); brain MRIs showed progressive enlargement of left frontal lesion, the appearance of right frontal lesion with linear enhancement (28 to 55 days after T0). In the following months, the patients gradually developed severe cognitive impairment with aphasia.

Brain MRI performed in September 2012 (T0 + 140 days) showed the absence of Gd enhancing lesions.

sNFL levels were normal during basal phase. At PML diagnosis (T0), sNFL demonstrated strongly increased (101.5 pg/ml); they reached their peak (192.6 pg/ml) 55 days after T0. sNFL levels decreased during PML recovery to 11.7 pg/ul corresponding to the last available sample (T0 + 887 days).

3.2. sNFL levels during the different phases of PML and in NTZ-control group

sNFL levels were analyzed and compared during the different phases of PML disease (Fig. 2A and B):

- *Basal* (up to 4 months before PML diagnosis): sNFL values were in the normal range in all patients' samples (median 9.1 pg/ml, range 6.2–15.1 pg/ml; $n = 9$ samples), except for one sample from patient 1: notably, this is the first time point at which the increase in sNFL levels occurred in this patient (Fig 1A).
- *Pre-PML* (within 3 months before PML onset): sNFL were elevated in all available samples (median 19.50 pg/ml, range 15.50–33.80 pg/ml; $n = 3$ samples).
- *PML diagnosis*: sNFL were elevated in all patients (median 59.20 pg/ml, range 11.1–101.50 pg/ml; $n = 4$ samples).
- *PML/IRIS*: during this phase, sNFL levels reached their peak (median 96.35 pg/ml, range 20.5–272.9; $n = 14$ samples) in all patients.
- *Post-PML* (recovery phase, starting from the first MRI without enhancement, up to the end of follow-up): sNFL levels showed a decrease (median 12.80 pg/ml, range 9.30–30.60; $n = 8$ samples); however, based on reference values, sNFL were still elevated in 2 out of 4 patients at the end of their follow-up (622 and 887 days after PML diagnosis).

In addition, a group of matched NTZ-treated MS patients experiencing NEDA-3 status for at least 12 months was included as control group and sNFL levels were measured in one sample per patient: sNFL levels were in the normal range in all patients' samples (median 4.7 pg/ml, range 1.4–8.6 pg/ml). sNFL levels measured in PML patients at any disease phase (since basal to post-PML) demonstrate statistically higher compared to values measured in NTZ-control patients (Kruskal Wallis test with Dunn post hoc test, basal $p = 0.0130$, pre-PML $p = 0.0118$, PML diagnosis $p = 0.0014$, PML/IRIS $P<0.0001$ and post-PML $p = 0.0009$).

3. Discussion

The early detection of PML in the management of NTZ-treated patients is crucial: MRI is currently the main monitoring tool for early PML detection. Frequent MRI are not always applicable and are highly expensive. sNFL in MS has been established as marker of inflammatory disease activity and treatment response. sNFL increase reflects central nervous system (CNS) axonal damage associated with clinical/subclinical disease activity, MRI new lesions, neurodegeneration in relapse-free phases (Akgün et al., 2019; Barro et al., 2020; Bittner et al., 2021; Thebault et al., 2020; Varhaug et al., 2019), and PML (Dalla Costa et al., 2019; Fissolo et al., 2021; Loonstra et al., 2019).

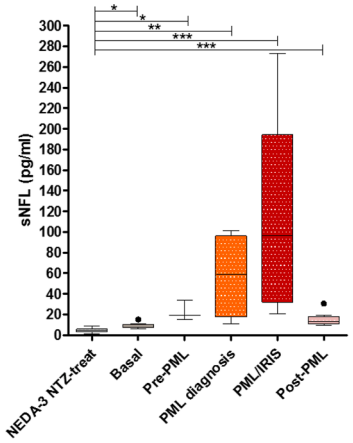
The availability of reliable reference values is of utmost importance to detect an increase of sNFL at individual level and to enable their routine use in clinical practice.

Few previous studies demonstrated that sNFL levels are increased at PML diagnosis and correlate with volume of MRI lesions (Dalla Costa et al., 2019; Fissolo et al., 2021; Loonstra et al., 2019). In particular, Loonstra et al. suggests that an increase of sNFL could precede PML diagnosis in some patients; nevertheless, their data were not analyzed at an individual level based on reference values. Moreover, data about sNFL levels during PML recovery are scarcely assessed and discussed.

A detailed longitudinal characterization of PML disease course, including clinical/radiological and biological parameters, could improve earlier PML detection and monitoring at individual level. Different specific phases can be identified in the follow-up of patients developing PML: a basal phase, an asymptomatic pre-PML phase, the PML diagnosis, the PML/IRIS and a recovery post-PML phase.

The present study retrospectively assessed sNFL levels during the

A



B

	NEDA-3 NTZ-treat	Basal	Pre-PML	PML diagnosis	PML/IRIS	Post-PML
PATIENTS	45	4	4	4	4	4
n° samples	45	9	3	4	14	8
median sNFL (range) pg/ml	4.7 (1.4-8.6)	9.1 (6.2-15.1)	19.50 (15.50-33.8)	59.2 (11.10-101.5)	96.35 (20.5-272.9)	12.80 (9.3-30.6)
sNFL *(cut-off based interpretation)*	↓45/45	8 ↓, 1 ↑	3 ↑	4 ↑	14 ↑	6 ↑, 2 ↓
PT 1		3 ↓, 1 ↑	3 ↑	1 ↑	6 ↑	2 ↑, 1 ↓
PT 2		1 ↓	-	1 ↑	3 ↑	1 ↑
PT 3		2 ↓	-	1 ↑	4 ↑	1 ↑, 1 ↓
PT 4		2 ↓	-	1 ↑	1 ↑	2 ↑

Fig. 2. Comparison between sNFL levels in patients developing PML and in NEDA-3 patients as control group.
A) sNFL levels are shown in 4 PML patients during the different phases of PML disease (basal, pre-PML, PML diagnosis and post-PML, as defined in the text) and in a control group of NEDA-3 patients ($n = 45$). sNFL are significantly increased in PML patients at each disease phase relative to NEDA-3 patients Kruskal wallis test with Dunn post hoc test, $p = 0.0130$, $p = 0.0118$, $p = 0.0014$, $p<0.0001$ and $p = 0.0009$, respectively). B) The table shows data about patients samples in each group, including sNFL levels (pg/ml) and interpretation according to cut-off values specific for age decade (↑=elevated sNFL value, ↓= normal sNFL value).

longitudinal follow-up of four NTZ-treated patients developing PML in our MS center. sNFL levels were measured in 38 samples collected before and during the different PML phases (median time 43 months, range 31–45 months), and interpreted according to the previously defined reference values (Valentino et al., 2021).

Our data suggest that sNFL are a reliable tool to early detect and monitor PML: briefly, we observed that:

1) sNFL were already elevated in all available samples in pre-PML phase
2) sNFL levels were elevated in every patient at PML diagnosis
3) sNFL were always elevated when MRI scan suggested a suspicious of PML
4) sNFL levels were not normalized in all patients' samples despite radiological stability during PML recovery (622 and 887 days after PML diagnosis).

Patient 1 is the most informative case of the present study: the systematic collection of serum samples in pre-PML phase allowed to clearly show that sNFL levels had been increasing before the first clinical and radiological signs, and before PML diagnostic confirmation by JCV-DNA detection in CSF.

In addition, the very high sNFL values observed at PML diagnosis in patients 2 and 4 (81.0 and 101.5 pg/ml respectively, about 10-fold higher than the respective basal values) are suggestive of an increase in the preceding months, although samples collected during pre-PML phase were unfortunately lacking.

Elevated sNFL levels, based on specific reference values, reflect CNS lesions that could be associated with disease activity, progression or PML, to be urgently investigated by MRI.

Importantly, we found sNFL levels in the normal range in all NTZ-treated patients of the control group ($n = 45$) consistently with their NEDA-3 status, as well shown also in previously published data (Johnsson et al., 2022; Valentino et al., 2022).

These evidences suggest that:

1) sNFL are as sensitive as MRI in the detection of PML onset
2) sNFL can early detect PML: in particular, a quarterly blood sampling (every three months) seems to be optimal for an earlier detection of sNFL increase
3) sNFL can be used to monitor PML and its recovery: the increase of NFL levels and their subsequent reduction reflect disease evolution; in particular during PML recovery, persisting elevated sNFL levels (over the reference values) could suggest an ongoing neuronal degeneration.

Further, sNFL dosage could be implemented as monitoring tool in patients treated with any other immunosuppressive drug, at high PML risk (Oshima et al., 2019), for which the same strict MRI monitoring is not always applicable.

The main limitation of the present study is represented by the few PML patients included. However, considering the rarity and severity of this disease, the described four PML cases, including detailed pre- and post-PML follow-up data, add precious information and suggest the utility of sNFL dosing for a better routinary management of MS patients at risk of PML development.

4. Conclusions

Brain MRI monitoring is crucial in patients at high PML risk. sNFL quantification represents an easy accessible, sustainable and sensitive marker of CNS lesions, therefore useful to early detect and monitor PML .

The measurement of sNFL should be introduced in clinical practice as an additional/alternative parameter to MRI for patients treated with any immunosuppressive drug.

Role of funding source

This work was supported by Roche, by the Italian Ministry of Health (grant number RF-2013-02357497), by FISM - Fondazione Italiana Sclerosi Multipla (grant number 2020/S/5) and financed or co-financed with the '5 per mille' public funding.

Declaration of Competing Interest

Valentino Paola received speaker honoraria from Roche, research support from Merck and grant support from Quanterix.

Malucchi Simona received speaker honoraria from Biogen and Roche.

Bava Cecilia Irene: nothing to discose

Martire Serena: nothing to discose

Capobianco Marco served on the scientific advisory board of Biogen, Sanofi Genzyme, Novartis,

Roche, Becton-Dickinson, Alexion and Horizon and received speaker

honoraria from Almirall, Biogen, Novartis, Sanofi Genzyme.

Malentacchi Maria: nothing to discose

Sperli Francesca: nothing to discose

Oggero Alessandra: nothing to discose

Di Sapio Alessia received personal compensation for speaking and consulting by Biogen, Novartis, Roche, Sanofi and Alexion and has been reimbursed by Merck, Biogen, Genzyme and Roche for attending several conferences.

Bertolotto Antonio served on the scientific advisory board of Almirall, Bayer, Biogen, and Genzyme; received speaker honoraria from Biogen, Novartis and Sanofi and grant support from Almiral, Biogen, Associazione San Luigi Gonzaga ONLUS, Fondazione per la Ricerca Biomedica ONLUS, Mylan, Novartis and the Italian Multiple sclerosis Society.

Acknowledgement

This work was supported by Roche, by the Italian Ministry of Health (grant number RF-2013-02357497), by FISM - Fondazione Italiana Sclerosi Multipla (cod. 2020/S/5) and financed or co-financed with the '5 per mille' public funding.

The authors thank CRESM Biobank for sample and data collection, Da Col family and Associazione San Luigi Gonzaga ONLUS for the donation that allowed SR-X system purchase.

References

Akgün, K., Kretschmann, N., Haase, R., Proschmann, U., Kitzler, H.H., Reichmann, H., Ziemssen, T., 2019. Profiling individual clinical responses by high-frequency serum neurofilament assessment in MS. Neurol. Neuroimmunol. NeuroInflammation 6. https://doi.org/10.1212/NXI.0000000000000555.

Barro, C., Benkert, P., Disanto, G., Tsagkas, C., Amann, M., Naegelin, Y., Leppert, D., Gobbi, C., Granziera, C., Yaldizli, Ö., Michalak, Z., Wuerfel, J., Kappos, L., Parmar, K., Kuhle, J., 2018. Serum neurofilament as a predictor of disease worsening and brain and spinal cord atrophy in multiple sclerosis. Brain 141, 2382–2391. https://doi.org/10.1093/brain/awy154.

Barro, C., Chitnis, T., Weiner, H.L., 2020. Blood neurofilament light: a critical review of its application to neurologic disease. Ann. Clin. Transl. Neurol. 7, 2508–2523. https://doi.org/10.1002/acn3.51234.

Benkert, P., Meier, S., Schaedelin, S., Manouchehrinia, A., Yaldizli, Ö., Maceski, A., Oechtering, J., Achtnichts, L., Conen, D., Derfuss, T., Lalive, P.H., Mueller, C., Müller, S., Naegelin, Y., Oksenberg, J.R., Pot, C., Salmen, A., Willemse, E., Kockum, I., Blennow, K., Zetterberg, H., Gobbi, C., Kappos, L., Wiendl, H., Berger, K., Sormani, M.P., Granziera, C., Piehl, F., Leppert, D., Kuhle, J., Aeschbacher, S., Barakovic, M., Buser, A., Chan, A., Disanto, G., D'Souza, M., Du Pasquier, R., Findling, O., Galbusera, R., Hrusovsky, K., Khalil, M., Lorscheider, J., Mathias, A., Orleth, A., Radue, E.W., Rahmanzadeh, R., Sinnecker, T., Subramaniam, S., Vehoff, J., Wellmann, S., Wuerfel, J., Zecca, C., 2022. Serum neurofilament light chain for individual prognostication of disease activity in people with multiple sclerosis: a retrospective modelling and validation study. Lancet Neurol. 21, 246–257. https://doi.org/10.1016/S1474-4422(22)00009-6.

Berger, J.R., Aksamit, A.J., Clifford, D.B., Davis, L., Koralnik, I.J., Sejvar, J.J., Bartt, R., Major, E.O., Nath, A., 2013. PML diagnostic criteria: consensus statement from the AAN neuroinfectious disease section. Neurology 80, 1430–1438. https://doi.org/10.1212/WNL.0b013e31828c2fa1.

Bittner, S., Oh, J., Havrdová, E.K., Tintoré, M., Zipp, F., 2021. The potential of serum neurofilament as biomarker for multiple sclerosis. Brain 144, 2954–2963. https://doi.org/10.1093/brain/awab241.

Dalla Costa, G., Martinelli, V., Moiola, L., Sangalli, F., Colombo, B., Finardi, A., Cinque, P., Kolb, E.M., Haghikia, A., Gold, R., Furlan, R., Comi, G., 2019. Serum neurofilaments increase at progressive multifocal leukoencephalopathy onset in natalizumab-treated multiple sclerosis patients. Ann. Neurol. 85, 606–610. https://doi.org/10.1002/ana.25437.

Delcoigne, B., Manouchehrinia, A., Barro, C., Benkert, P., Michalak, Z., Kappos, L., Leppert, D., Tsai, J.A., Plavina, T., Kieseier, B.C., Lycke, J., Alfredsson, L., Kockum, I., Kuhle, J., Olsson, T., Piehl, F., 2020. Blood neurofilament light levels segregate treatment effects in multiple sclerosis. Neurology 94, e1201–e1212. https://doi.org/10.1212/WNL.0000000000009097.

European Medicines Agency, 2016. EMA confirms recommendations to minimise risk of brain infection PML with Tysabri [WWW Document]. Sci. Med. Heal. URL http://www.ema.europa.eu/docs/en_GB/document_library/Press_release/2016/02/WC500202389.pdf.

Fissolo, N., Pignolet, B., Rio, J., Vermersch, P., Ruet, A., deSèze, J., Labauge, P., Vukusic, S., Papeix, C., Martinez-Almoyna, L., Tourbah, A., Clavelou, P., Moreau, T., Pelletier, J., Lebrun-Frenay, C., Bourre, B., Defer, G., Montalban, X., Brassat, D., Comabella, M., 2021. Serum Neurofilament Levels and PML Risk in Patients With Multiple Sclerosis Treated With Natalizumab. Neurol. Neuroimmunol. neuroinflammation 8, 1–6. https://doi.org/10.1212/NXI.0000000000001003.

Hviid, C.V.B., Knudsen, C.S., Parkner, T., 2020. Reference interval and preanalytical properties of serum neurofilament light chain in Scandinavian adults. Scand. J. Clin. Lab. Invest. 80, 291–295. https://doi.org/10.1080/00365513.2020.1730434.

Johnsson, M., Farman, H.H., Blennow, K., Zetterberg, H., Malmeström, C., Axelsson, M., Lycke, J., 2022. No increase of serum neurofilament light in relapsing-remitting multiple sclerosis patients switching from standard to extended-interval dosing of natalizumab. Mult. Scler. J. 28, 2070–2080. https://doi.org/10.1177/13524585221108080.

Lambert, J., Chang, L., Song, L., Patel, P.P., Shan, D., Johnson, J., Rissin, D., 2018. P2-098: comparison of Two Platforms Quantitating Fg/Ml Neurological Biomarkers Using Single Molecule Arrays and Digital Elisa: the Benchtop Reader Sr-X™ and the Fully Automated Analyzer Hd-1™. Alzheimer's Dement 14, P706. https://doi.org/10.1016/j.jalz.2018.06.783. –P706.

Lambertsen, K.L., Soares, C.B., Gaist, D., Nielsen, H.H., 2020. Neurofilaments: the C-reactive protein of neurology. Brain Sci 10, 1–29. https://doi.org/10.3390/brainsci10010056.

Leppert, D., Kuhle, J., 2019. Blood neurofilament light chain at the doorstep of clinical application. Neurol. Neuroimmunol. NeuroInflammation 6, 4–5. https://doi.org/10.1212/NXI.0000000000000599.

Loonstra, F.C., Verberk, I.M.W., Wijburg, M.T., Wattjes, M.P., Teunissen, C.E., van Oosten, B.W., Uitdehaag, B.M.J., Killestein, J., van Kempen, Z.L.E., 2019. Serum neurofilaments as candidate biomarkers of natalizumab associated progressive multifocal leukoencephalopathy. Ann. Neurol. 86, 322–324. https://doi.org/10.1002/ANA.25523.

Marnetto, F., Valentino, P., Caldano, M., Ficorilli, A., Paudice, A., Antonio, B., 2020. BB-cresm: a structured institutional biobank for quality research in multiple sclerosis_LB1245. Mult. Scler. J. 26, 43–117. https://doi.org/10.1177/1352458520974938.

Morrow, S.A., Clift, F., Devonshire, V., Lapointe, E., Schneider, R., Stefanelli, M., Vosoughi, R., 2022. Use of natalizumab in persons with multiple sclerosis: 2022 update. Mult. Scler. Relat. Disord. 65, 103995 https://doi.org/10.1016/j.msard.2022.103995.

Novakova, L., Zetterberg, H., Sundström, P., Axelsson, M., Khademi, M., Gunnarsson, M., Malmeström, C., Svenningsson, A., Olsson, T., Piehl, F., Blennow, K., Lycke, J., 2017. Monitoring disease activity in multiple sclerosis using serum neurofilament light protein. Neurology 89, 2230–2237. https://doi.org/10.1212/WNL.0000000000004683.

Oshima, Y., Tanimoto, T., Yuji, K., Tojo, A., 2019. Drug-associated progressive multifocal leukoencephalopathy in multiple sclerosis patients. Mult. Scler. J. 25, 1141–1149. https://doi.org/10.1177/1352458518786075.

Schwab, N., Schneider-, T., Melzer, N., Cutter, G., Wiendl, H., 2017. Natalizumab-associated PML.

Teunissen, C.E., Tumani, H.T., Bennett, J.L., Berven, F.S., Brundin, L., Comabella, M., Franciotta, D., Federiksen, J.L., Fleming, J.O., Furlan, R., Hintzen, R.Q., Hughes, S. G., Johnson, M.H., Krasulova, E., Kuhle, J., Magnone, M.C., Petzold, A., Rajda, C., Rejdak, K., Schmidt, H.K., Van Pesch, V., Waubant, E., Wolf, C., Hemmer, B., Deisenhammer, F., Giovannoni, G., 2009. Short commentary on ?a consensus protocol for the standardization of cerebrospinal fluid collection and biobanking? Mult. Scler. 16, 129–132. https://doi.org/10.1177/1352458509356368.

Thebault, S., Abdoli, M., Fereshtehnejad, S.M., Tessier, D., Tabard-Cossa, V., Freedman, M.S., 2020. Serum neurofilament light chain predicts long term clinical outcomes in multiple sclerosis. Sci. Rep. 10, 1–11. https://doi.org/10.1038/s41598-020-67504-6.

Thompson, A.J., Banwell, B.L., Barkhof, F., Carroll, W.M., Coetzee, T., Comi, G., Correale, J., Fazekas, F., Filippi, M., Freedman, M.S., Fujihara, K., Galetta, S.L., Hartung, H.P., Kappos, L., Lublin, F.D., Marrie, R.A., Miller, A.E., Miller, D.H., Montalban, X., Mowry, E.M., Sorensen, P.S., Tintoré, M., Traboulsee, A.L., Trojano, M., Uitdehaag, B.M.J., Vukusic, S., Waubant, E., Weinshenker, B.G., Reingold, S.C., Cohen, J.A., 2018. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. Lancet Neurol. 17, 162–173. https://doi.org/10.1016/S1474-4422(17)30470-2.

Uher, T., Schaedelin, S., Srpova, B., Barro, C., Bergsland, N., Dwyer, M., Tyblova, M., Vodehnalova, K., Benkert, P., Oechtering, J., Leppert, D., Naegelin, Y., Krasensky, J., Vaneckova, M., Kubala Havrdova, E., Kappos, L., Zivadinov, R., Horakova, D., Kuhle, J., Kalincik, T., 2020. Monitoring of radiologic disease activity by serum neurofilaments in MS. Neurol. Neuroimmunol. neuroinflammation 7. https://doi.org/10.1212/NXI.0000000000000714.

Valentino, P., Malucchi, S., Martire, S., Bava, C.I., Capobianco, M.A., Bertolotto, A., 2022. sNFL applicability as additional monitoring tool in natalizumab extended interval dosing regimen for RRMS patients. Mult. Scler. Relat. Disord. 67, 104176 https://doi.org/10.1016/j.msard.2022.104176.

Valentino, P., Marnetto, F., Martire, S., Malucchi, S., Bava, C.I., Popovic, M., Bertolotto, A., 2021. Serum neurofilament light chain levels in healthy individuals: a proposal of cut-off values for use in multiple sclerosis clinical practice. Mult. Scler. Relat. Disord. 54 https://doi.org/10.1016/j.msard.2021.103090.

Varhaug, K.N., Torkildsen, Ø., Myhr, K.M., Vedeler, C.A., 2019. Neurofilament light chain as a biomarker in multiple sclerosis. Front. Neurol. https://doi.org/10.3389/fneur.2019.00338.