Supplemental Online Content

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This supplemental material has been provided by the authors to give readers additional information about their work.

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eAppendix. Eligibility Criteria and Samples Management in the Validation Cohort, Exclusion Criteria, Sensitivity Analyses

All consecutive patients who underwent their first demyelinating event suggestive of multiple sclerosis (MS) with available blood samples within 12 months from disease onset were recruited. Patient were prospectively followed at least two years with clinical assessments at least every 6 months. The exclusion criteria were the same applied to the development cohort: (1) history of possible previous relapses before the first demyelinating event evaluated at first visit, (2) incomplete follow-up, (3) final diagnosis different than clinically isolated syndrome or MS, and (4) baseline magnetic resonance imaging (MRI) performed more than six months after sample collection. After thoroughly revising all patients, from 272 patients initially selected, we excluded 21 based on different criteria (eFigure). The inclusion periods and last follow-up from all centers were the following:

-Hospital Universitario Virgen Macarena: From October 2009 to July 2015, with last follow-up on August 12, 2022.
-Hospital Clínico San Carlos: From November 2006 to August 2016, and last follow-up was on January 13, 2022.
-Hospital Clínic de Barcelona: From August 1997 to September 2018 and followed until July 19, 2022.
-Hospital Universitari i Politécnic La Fe: From February 2010 to August 2020 and last follow-up was on July 01, 2022.
-Hospital Universitari Dr. Josep Trueta: From May 2011 to August 2014 and followed until August 03, 2022.
-Hospital Universitari Vall d'Hebron: From October 1995 to May 2004, and last follow-up was on August 16, 2022.
-Hospital del Mar: From February 2016 to July 2019, with last follow-up on August 13, 2022.
-Hospital Universitari Arnau de Vilanova: From June 2008 to June 2014 and followed until June 22, 2022.

Serum samples were collected during routine clinical practice visits from all patients and stored at –80ºC before analysis. Samples were transported in dry ice to Hospital Universitario Ramón y Cajal during July 2022, where they were processed and analyzed in duplicate using the single molecule array (SIMOA) technique in a SR-X instrument (Quanterix, MA, USA).

Blood samples were obtained from non-treated patients and stored at –80 ºC until assayed in all centers. Serum samples from the validation cohort were sent in dry ice for sNfL study at HRYC during July 2022. sNfL values were quantified in duplicate serum samples using the single molecule array (SIMOA) technique in a SR-X instrument (Quanterix, MA, USA) following the manufacturer's instructions. Intra- and inter-assay coefficients of variability were 5.9% and 6.9%, respectively.

Exclusion criteria: (1) history of possible previous relapses before the first demyelinating event evaluated at first visit, (2) incomplete follow-up, (3) final diagnosis different than clinically isolated syndrome (CIS) or MS, and (4) baseline magnetic resonance imaging (MRI) performed more than six months after sample collection. Serum NfL levels were

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retrospectively analyzed. Patients with blood samples collected more than 12 months after their first relapse were also excluded.

Sensitive analyses

We restricted the models to patients with relapse 30, 60, 90, and 120 days before sampling (eTable 3). A higher risk of both outcomes was observed when sampling was obtained separately from a relapse. Contrast-enhancing lesions were included in an additional model (eTable 4), and results did not differ from the baseline. Besides, the prognostic values of sNfL levels were studied to evaluate the association with sustained (irreversible) disability worsening (eTable 5), and similar results were observed.

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eTable 1. Study Design. Selection of Cohorts.	
Development cohort (n=327)	**Validation cohort (n=251)**
Hospital Universitario Ramón y Cajal (Madrid, Spain)	Hospital Universitario Virgen Macarena (Sevilla, Spain)
	Hospital Clínico San Carlos (Madrid, Spain)
	Hospital Clínic de Barcelona (Barcelona, Spain)
	Hospital Universitari i Politècnic La Fe (Valencia, Spain)
	Hospital Universitari Dr. Josep Trueta (Girona, Spain)
	Hospital Universitari Vall d'Hebron (Barcelona, Spain)
	Hospital del Mar (Barcelona, Spain)
	Hospital Universitari Arnau de Vilanova (Lleida, Spain)

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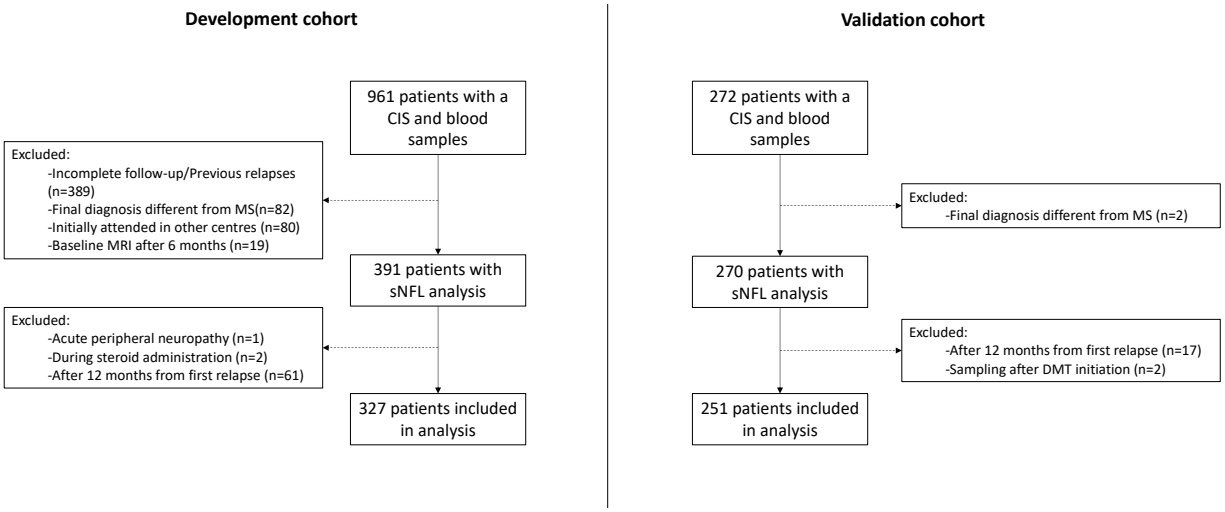
eFigure. Flowchart of Patients Included



Abbreviatures: CIS: clinically isolated syndrome; DMT: disease-modifying treatments; MRI: magnetic resonance imaging; MS: multiple sclerosis; sNfL: serum neurofilament light chain.

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eTable 2. Multivariable Cox Regression Models to Test Associations Between High sNfL Levels and the Risk of RAW and PIRA (n = 578)

Variables	RAW	PIRA
	HR (95% CI)	**HR (95% CI)**
	AIC 1099 / c statistics 0.69	**AIC 1292 / c statistics 0.63**
sNfL > 10 pg/ml	**1.87 (1.22 – 2.87)[d]**	**1.68 (1.13 – 2.49)[c]**
Age at first relapse	0.99 (0.97 – 1.01)	**1.04 (1.02 – 1.05)[e]**
Sex (male)	1.27 (0.82 – 1.98)	**1.61 (1.11 – 2.35)[c]**
Baseline EDSS	1.17 (0.96 – 1.43)	1.08 (0.91 – 1.28)
T2 lesion load	**1.36 (1.05 – 1.76)[c]**	1.05 (0.83 – 1.33)
Time from first relapse to sampling	0.99 (0.94 – 1.05)	1.04 (0.99 – 1.08)
Proportion of time on DMTs (excluding HE-DMTs)[a]	**0.35 (0.20 – 0.59)[e]**	1.47 (0.83 – 2.60)
Proportion of time on HE-DMTs[b]	**0.10 (0.03 – 0.36)[e]**	1.78 (0.82 – 3.87)
	AIC 1099 / c statistics 0.70	**AIC 1294 / c statistics 0.62**
sNfL Z-score > 1.5	**1.88 (1.22 – 2.89)[d]**	**1.51 (1.00 – 2.25)[c]**
Age at first relapse	1.00 (0.98 – 1.02)	**1.04 (1.02 – 1.06)[e]**
Sex (male)	1.26 (0.81 – 1.96)	**1.61 (1.10 – 2.34)[c]**
Baseline EDSS	1.17 (0.96 – 1.43)	1.09 (0.92 – 1.29)
T2 lesion load	**1.38 (1.07 – 1.79)[c]**	1.08 (0.85 – 1.37)
Time from first relapse to sampling	0.99 (0.94 – 1.05)	1.04 (0.99 – 1.08)
Proportion of time on DMTs (excluding HE-DMTs)[a]	**0.33 (0.19 – 0.57)[e]**	1.45 (0.82 – 2.55)
Proportion of time on HE-DMTs[b]	**0.10 (0.03 – 0.36)[e]**	1.80 (0.83 – 3.89)

Abbreviation: AIC: Akaike information criterion; CI: confidence interval; HE-DMT: high efficacy disease-modifying treatments; HR: hazard ratio; IQR: interquartile range; sNfL: serum neurofilament light chain.

[a]HE-DMTs: Natalizumab, Alemtuzumab, Ocrelizumab, Rituximab, Ofatumumab, Mitoxantrone.

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[b]Other DMTs: subcutaneous or intramuscular interferon-ß, glatiramer acetate, teriflunomide, dimethyl fumarate, fingolimod, oral cladribine, daclizumab, azathioprine, tacrolimus.
[c]p<0.05; [d]p<0.01; [e]p<0.001.
Multivariable models were adjusted by age at first relapse, sex, T2 lesion load, time from first relapse to sampling, proportion of time that the patient was treated with DMTs excluding HE-DMTs (obtained by dividing the time with DMTs by the time of disease evolution until outcome of interest), and proportion of time on HE-DMTs.
Bolt text indicates differences are significant (P < 0.05).

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eTable 3. Multivariable Cox Regression Models to Test Associations Between High sNfL Levels and the Risk of 6-Month CDW and EDSS Score of 3 Stratifying by the Time of Sampling With Respect to First Relapse

	6-month CDW			EDSS score of 3		
sNfL > 10 pg/ml	**HR (95% CI)**	**AIC**	**c statistics**	**HR (95% CI)**	**AIC**	**c statistics**
Total (n=578)	1.88 (1.37 – 2.60)[b]	1938	0.65	2.48 (1.69 – 3.64)[b]	1415	0.75
Relapse > 30 days before sampling (n=442)	2.10 (1.45 – 3.06)[b]	1424	0.67	3.31 (2.10 – 5.24)[b]	1039	0.79
Relapse > 60 days before sampling (n=361)	2.43 (1.56 – 3.80)[b]	1046	0.68	4.29 (2.47 – 7.45)[b]	759	0.80
Relapse > 90 days before sampling (n=306)	2.69 (1.67 – 4.34)[b]	889	0.69	4.95 (2.72 – 9.00)[b]	630	0.80
Relapse > 120 days before sampling (n=227)	2.62 (1.52 – 4.52)[b]	699	0.69	3.68 (1.95 – 6.93)[b]	548	0.77
sNfL Z-score >1.5						
Total (n=578)	1.80 (1.30 – 2.50)[b]	1941	0.65	2.13 (1.46 – 3.11)[b]	1422	0.75
Relapse > 30 days before sampling (n=442)	1.93 (1.32 – 2.82)[a]	1428	0.66	2.73 (1.75 – 4.25)[b]	1047	0.79
Relapse > 60 days before sampling (n=361)	2.29 (1.47 – 3.56)[b]	1049	0.68	3.31 (1.99 – 5.55)[b]	768	0.80
Relapse > 90 days before sampling (n=306)	2.41 (1.50 – 3.87)[b]	892	0.68	3.95 (2.26 – 6.90)[b]	637	0.80
Relapse > 120 days before sampling (n=227)	2.13 (1.24 – 3.65)[a]	704	0.69	2.79 (1.54 – 5.05)[a]	555	0.78

Abbreviation: AIC: Akaike information criterion; 6-month CDW: 6-month confirmed disability worsening; CI: confidence interval; EDSS: expanded disability status scale; HR: hazard ratio; sNfL: serum neurofilament light chain.

Multivariable models were adjusted by age at first relapse, sex, T2 lesion load, time from first relapse to sampling, proportion of time that the patient was treated with DMTs excluding HE-DMTs (obtained by dividing the time with DMTs by the time of disease evolution until outcome of interest), and proportion of time on HE-DMTs.

[a]$P < 0.01$; [b]$P < 0.001$.

Bolt text indicates differences are significant ($P < 0.05$).

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eTable 4. Multivariable Cox Regression Models to Test Associations Between sNfL Levels and the Risk of 6-Month CDW and EDSS of 3 Using the Cut-Off Values of 10 pg/mL and *z* Score of 1.5 and Including Contrast-Enhancing Lesions

	6-month CDW			EDSS of 3		
Variables	**Development cohort (n=299)**	**Validation cohort (n=212)**	**Total (n=511)**	**Development cohort (n=299)**	**Validation cohort (n=212)**	**Total (n=511)**
	HR (95% CI)	**HR (95% CI)**	**HR (95% CI)**	**HR (95% CI)**	**HR (95% CI)**	**HR (95% CI)**
	AIC 603 / c statistics 0.71	**AIC 837 / c statistics 0.64**	**AIC 1658 / c statistics 0.65**	**AIC 508 / c statistics 0.82**	**AIC 575 / c statistics 0.71**	**AIC 1250 / c statistics 0.75**
sNfL > 10 pg/ml	**1.86 (1.04 – 3.34)[c]**	**1.58 (1.01 – 2.49)[c]**	**1.65 (1.17 – 2.34)[d]**	**2.99 (1.55 – 6.83)[d]**	**2.01 (1.16 – 3.46)[c]**	**2.11 (1.40 – 3.18)[e]**
Age at first relapse	1.00 (0.98 – 1.03)	1.01 (0.99 – 1.03)	1.01 (0.99 – 1.02)	1.01 (0.98 – 1.04)	1.01 (0.98 – 1.03)	1.01 (0.99 – 1.03)
Sex (male)	**2.01 (1.19 – 3.38)[d]**	1.17 (0.73 – 1.88)	**1.49 (1.06 – 2.08)[c]**	1.41 (0.76 – 2.60)	1.39 (0.79 – 2.44)	1.30 (0.87 – 1.94)
Baseline EDSS	**1.51 (1.24 – 1.85)[e]**	1.25 (0.95 – 1.65)	**1.26 (1.08 – 1.47)[d]**	**1.70 (1.39 – 2.09)[e]**	**2.07 (1.50 – 2.84)[e]**	**1.55 (1.33 – 1.80)[e]**
T2 lesion load	**1.42 (1.06 – 1.90)[c]**	1.03 (0.75 – 1.41)	**1.26 (1.03 – 1.55)[c]**	1.27 (0.94 – 1.72)	1.03 (0.71 – 1.50)	**1.27 (1.01 – 1.58)[c]**
T1 contrast-enhancing lesions	1.02 (0.95 – 1.09)	0.98 (0.89 – 1.08)	1.00 (0.94 – 1.06)	1.05 (0.98 – 1.12)	0.97 (0.87 – 1.07)	1.02 (0.96 – 1.08)
Time from first relapse to sampling	1.05 (0.98 – 1.13)	1.00 (0.94 – 1.05)	1.01 (0.97 – 1.05)	**1.09 (1.01 – 1.17)[c]**	1.00 (0.94 – 1.07)	1.03 (0.98 – 1.08)
Proportion of time on DMTs (excluding HE-DMTs)[a]	**0.44 (0.21 – 0.95)[c]**	**0.48 (0.27 – 0.85)[c]**	**0.53 (0.34 – 0.83)[d]**	**0.25 (0.11 – 0.59)[c]**	**0.24 (0.12 – 0.50)[e]**	**0.32 (0.19 – 0.54)[e]**
Proportion of time on HE-DMTs[b]	**0.21 (0.06 – 0.71)[c]**	**0.18 (0.04 – 0.91)[c]**	**0.23 (0.09 – 0.57)[d]**	**0.04 (0.01 – 0.19)[e]**	**0.22 (0.05 – 0.99)[c]**	**0.10 (0.03 – 0.28)[e]**
Variables	**Development cohort (n=299)**	**Validation cohort (n=212)**	**Total (n=511)**	**Development cohort (n=299)**	**Validation cohort (n=212)**	**Total (n=511)**
	HR (95% CI)	**HR (95% CI)**	**HR (95% CI)**	**HR (95% CI)**	**HR (95% CI)**	**HR (95% CI)**
	AIC 604 / c statistics 0.72	**AIC 836 / c statistics 0.64**	**AIC 1656 / c statistics 0.66**	**AIC 512 / c statistics 0.83**	**AIC 576 / c statistics 0.71**	**AIC 1253 / c statistics 0.75**
sNfL Z-score >1.5	**1.79 (1.01 – 3.16)[c]**	**1.70 (1.06 – 2.71)[c]**	**1.73 (1.22 – 2.46)[d]**	**2.39 (1.29 – 4.41)[d]**	**2.03 (1.15 – 3.60)[c]**	**1.90 (1.27 – 2.84)[d]**
Age at first relapse	1.01 (0.98 – 1.03)	1.01 (0.99 – 1.03)	1.01 (0.99 – 1.03)	1.02 (0.99 – 1.04)	1.01 (0.99 – 1.04)	1.02 (0.99 – 1.03)
Sex (male)	**1.89 (1.12 – 3.18)[c]**	1.22 (0.76 – 1.96)	**1.45 (1.04 – 2.04)[c]**	1.27 (0.69 – 2.34)	1.51 (0.86 – 2.66)	1.26 (0.85 – 1.88)

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Baseline EDSS	**1.52 (1.24 – 1.86)[e]**	1.27 (0.96 – 1.68)	**1.25 (1.07 – 1.47)[d]**	**1.71 (1.40 – 2.10)[e]**	**2.11 (1.53 – 2.91)[e]**	**1.55 (1.33 – 1.80)[e]**
T2 lesion load	**1.44 (1.08 – 1.92)[c]**	1.03 (0.75 – 1.41)	**1.27 (1.04 – 1.56)[c]**	1.30 (0.97 – 1.75)	1.05 (0.72 – 1.52)	**1.30 (1.04 – 1.61)[c]**
T1 contrast-enhancing lesions	1.02 (0.95 – 1.10)	0.98 (0.89 – 1.08)	1.00 (0.95 – 1.06)	1.05 (0.98 – 1.12)	0.97 (0.87 – 1.07)	1.02 (0.96 – 1.08)
Time from first relapse to sampling	1.06 (0.98 – 1.13)	1.00 (0.94 – 1.06)	1.01 (0.97 – 1.05)	1.09 (1.01 – 1.17)	1.00 (0.94 – 1.08)	1.03 (0.98 – 1.08)
Proportion of time on DMTs (excluding HE-DMTs)[a]	**0.44 (0.21 – 0.94)[c]**	**0.44 (0.24 – 0.80)[d]**	**0.51 (0.32 – 0.80)[d]**	**0.26 (0.11 – 0.61)[d]**	**0.21 (0.10 – 0.44)[e]**	**0.31 (0.18 – 0.52)[e]**
Proportion of time on HE-DMTs[b]	**0.20 (0.06 – 0.69)[d]**	**0.19 (0.04 – 0.93)[c]**	**0.22 (0.09 – 0.55)[d]**	**0.05 (0.01 – 0.20)[e]**	0.22 (0.05 – 1.00)	**0.10 (0.04 – 0.28)[e]**

Abbreviation: AIC: Akaike information criterion; 6-month CDW: 6-month confirmed disability worsening; CI: confidence interval; EDSS: expanded disability status scale; HE-DMT: high efficacy disease-modifying treatments; HR: hazard ratio; IQR: interquartile range; sNfL: serum neurofilament light chain.

[a]HE-DMTs: Natalizumab, Alemtuzumab, Ocrelizumab, Rituximab, Ofatumumab, Mitoxantrone.

[b]Other DMTs: subcutaneous or intramuscular interferon-ß, glatiramer acetate, teriflunomide, dimethyl fumarate, fingolimod, oral cladribine, daclizumab, azathioprine, tacrolimus.

[c]$p<0.05$; [d]$p<0.01$; [e]$p<0.001$.

Multivariable models were adjusted by age at first relapse, sex, T2 lesion load, T1 contrast-enhancing lesions, time from first relapse to sampling, proportion of time that the patient was treated with DMTs excluding HE-DMTs (obtained by dividing the time with DMTs by the time of disease evolution until outcome of interest), and proportion of time on HE-DMTs.

Bolt text indicates differences are significant ($P < 0.05$).

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eTable 5. Multivariable Cox Regression Models to Test Associations Between sNfL Levels and the Risk of Sustained Disability Worsening Using the Cut-Off Values of 10 pg/mL and *z* Score of 1.5

	Sustained disability worsening		
Variables	**Development cohort (n=327)**	**Validation cohort (n=251)**	**Total (n=578)**
	HR (95% CI)	**HR (95% CI)**	**HR (95% CI)**
	AIC 501 / c statistics 0.76	**AIC 873 / c statistics 0.66**	**AIC 1576 / c statistics 0.68**
sNfL > 10 pg/ml	**4.25 (2.12 – 8.53)[e]**	**1.58 (1.03 – 2.45)[c]**	**2.15 (1.50 – 3.08)[e]**
Age at first relapse	1.02 (0.99 – 1.05)	1.01 (0.99 – 1.03)	1.01 (0.99 – 1.03)
Sex (male)	**2.11 (1.19 – 3.76)[c]**	1.36 (0.86 – 2.17)	**1.52 (1.08 – 2.15)[c]**
Baseline EDSS	1.07 (0.85 – 1.36)	1.15 (0.88 – 1.49)	1.02 (0.87 – 1.20)
T2 lesion load	1.17 (0.85 – 1.62)	1.05 (0.77 – 1.43)	1.17 (0.95 – 1.44)
Time from first relapse to sampling	**1.11 (1.02 – 1.19)[c]**	1.00 (0.94 – 1.06)	1.03 (0.98 – 1.07)
Proportion of time on DMTs (excluding HE-DMTs)[a]	**0.25 (0.11 – 0.58)[d]**	**0.38 (0.22 – 0.65)[e]**	**0.36 (0.23 – 0.56)[e]**
Proportion of time on HE-DMTs[b]	0.45 (0.14 – 1.38)	0.61 (0.19 – 1.95)	0.51 (0.24 – 1.10)
Variables	**Development cohort (n=327)**	**Validation cohort (n=251)**	**Total (n=578)**
	HR (95% CI)	**HR (95% CI)**	**HR (95% CI)**
	AIC 504 / c statistics 0.76	**AIC 873 / c statistics 0.66**	**AIC 1576 / c statistics 0.69**
sNfL Z-score >1.5	**3.69 (1.92 – 7.07)[e]**	**1.63 (1.04 – 2.55)[c]**	**2.17 (1.51 – 3.12)[e]**
Age at first relapse	**1.04 (1.01 – 1.07)[c]**	1.01 (0.99 – 1.04)	1.02 (1.01 – 1.04)[d]
Sex (male)	**1.91 (1.08 – 3.39)[c]**	1.43 (0.89 – 2.29)	**1.51 (1.06 – 2.13)[c]**
Baseline EDSS	1.07 (0.84 – 1.37)	1.15 (0.89 – 1.50)	1.02 (0.87 – 1.20)
T2 lesion load	1.23 (0.89 – 1.69)	1.06 (0.78 – 1.45)	1.19 (0.97 – 1.47)
Time from first relapse to sampling	**1.11 (1.03 – 1.19)[d]**	1.00 (0.94 – 1.06)	1.03 (0.99 – 1.08)

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Proportion of time on DMTs (excluding HE-DMTs)[a]	**0.25 (0.11 – 0.56)[d]**	**0.36 (0.21 – 0.63)[e]**	**0.34 (0.22 – 0.54)[e]**
Proportion of time on HE-DMTs[b]	0.46 (0.15 – 1.37)	0.63 (0.20 – 2.01)	0.51 (0.24 – 1.07)

Abbreviation: AIC: Akaike information criterion; CI: confidence interval; HE-DMT: high efficacy disease-modifying treatments; HR: hazard ratio; IQR: interquartile range; sNfL: serum neurofilament light chain.

[a]HE-DMTs: Natalizumab, Alemtuzumab, Ocrelizumab, Rituximab, Ofatumumab, Mitoxantrone.

[b]Other DMTs: subcutaneous or intramuscular interferon-ß, glatiramer acetate, teriflunomide, dimethyl fumarate, fingolimod, oral cladribine, daclizumab, azathioprine, tacrolimus.

[c]p<0.05; [d]p<0.01; [e]p<0.001.

Multivariable models were adjusted by age at first relapse, sex, T2 lesion load, time from first relapse to sampling, proportion of time that the patient was treated with DMTs excluding HE-DMTs (obtained by dividing the time with DMTs by the time of disease evolution until outcome of interest), and proportion of time on HE-DMTs.

Bolt text indicates differences are significant (P < 0.05).

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