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Immune cell subpopulations and serum neurofilament light chain are associated with increased risk of disease worsening in multiple sclerosis

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ABSTRACT

Changes is lymphocyte subpopulations in peripheral blood have been proposed as biomarkers for evaluation of disease activity in multiple sclerosis (MS). Serum neurofilament light chain (sNfL) is a biomarker reflecting neuro-axonal injury in MS that could be used to monitor disease activity, response to drugs and to prognosticate disease course. Here we show a moderate correlation between sNfL and lymphocyte cell subpopulations, and our data furthermore suggest that sNfL and specific immune cell subpopulations together could predict future disease worsening in MS.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), leading to demyelination and neurodegeneration, visible as lesions and atrophy by magnetic resonance imaging (MRI) of the brain and spinal cord. MS is classified into relapsing-remitting MS (RRMS), which is the most common form of MS, and primary and secondary progressive MS, PPMS and SPMS, respectively. The clinical course of RRMS is characterized by recurrent attacks with new or aggravated neurological dysfunction followed by recovery periods, whereas in the progressive form of MS, the disease progresses without recoveries (Dobson and Giovannoni, 2019). Imbalanced

Abbreviations: MS, Multiple sclerosis; CNS, central nervous system; MRI, magnetic resonance imaging; RRMS, relapsing-remitting MS; PPMS, primary progressive MS; SPMS, secondary progressive MS; PMS, progressive MS; PBMCs, peripheral blood mononuclear cells; sNfL, serum neurofilament light chain; EDSS, expanded Disability Status Score; HC, healthy controls.

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interactions between lymphocytes, such as T cells and B cells, and myeloid cells, as well as their effector and regulatory subpopulations, and also CNS resident cells, are important drivers of the disease. In the relapsing stage of MS, abnormal immune responses are triggered in the periphery leading to trafficking of activated immune cells into the CNS. In contrast, the biology in the progressive stage of MS involves inflammation and degenerative disease mechanisms within the CNS behind a closed blood-brain barrier (Attfield et al., 2022; Bar-Or and Li, 2021; Rodríguez Murúa et al., 2022).

The clinical course of MS is heterogeneous, and biomarkers predicting clinical outcomes are highly warranted. Changes in the frequency and function of lymphocytes have been reported in MS patients, and studies have indicated that disease activity and disease progression are associated with systemic inflammation (Babaloo et al., 2015; D'ambrosio et al., 2015; Durelli et al., 2009; Hedegaard et al., 2008; Kalra et al., 2020; Kebir et al., 2009; Li et al., 2011; Moser et al., 2020; Teniente-Serra et al., 2016). Immune cell subset changes in peripheral blood mononuclear cells (PBMCs) have been proposed as surrogate biomarkers of disease activity, progression and response to treatment (Cellerino et al., 2020; Jaye et al., 2012; Mimpen et al., 2021; Pardo and Jones, 2017; Teniente-Serra et al., 2016). Neurofilament light chain (NfL) is one of the major components of the neuronal cytoskeleton and is released into the extracellular fluid upon axonal damage (Khalil et al., 2018). Thus, the extent of neuronal damage in CNS can be evaluated by measuring the NfL concentration of body fluids, and NfL as biomarker for MS as well as for other neurodegenerative disorders has gained increased attention over the last two decades (Bittner et al., 2021; Khalil et al., 2018). In MS, serum NfL (sNfL) concentrations are associated with disease activity, disability progression and treatment response (Barro et al., 2018; Barro et al., 2023b; Bittner et al., 2020; Chitnis et al., 2018; Disanto et al., 2017; Khalil et al., 2018; Kuhle et al., 2019; Leppert and Kuhle, 2019; Loonstra et al., 2023; Meier et al., 2023; Novakova et al., 2017; Rival et al., 2023). Furthermore, we and others have also shown that sNfL could act as a predictor for disease worsening (Barro et al., 2018; Benkert et al., 2022; Brune et al., 2022; Buchmann et al., 2023; Disanto et al., 2017; Kuhle et al., 2017; Steffen et al., 2023).

Hence, immune cell subset characteristics and sNfL measurements may each provide valuable information for elucidating subsequent MS disease activity. The combination of sNfL with other biomarkers of disease activity (optical coherence tomography and MRI) may be superior for the prediction of disease worsening than each biomarker alone (Kuhle et al., 2020; Lin et al., 2021). However, the combined association of immune cell subsets and sNfL as a predictor for future disease activity in a real-world MS population has not yet been explored. In this exploratory study, we use data from a well-characterized cohort of MS patients with baseline flow cytometry and sNfL measures and two-year MRI and clinical follow-up, aiming at investigating the association between the frequencies of different lymphocyte cell subpopulations and sNfL concentration and to explore their possible use as prognostic factors for MS disease worsening.

2. Materials and methods

2.1. Study population

A total of 328 patients with MS were prospectively enrolled between July 2016 and December 2017 at four European MS centers (Hospital Clinic of Barcelona, Spain; Oslo University Hospital, Norway; Charité-Universitaetsmedizin Berlin, Germany; Ospedale Policlinico San Martino, Genoa, Italy) through the ERACOSYSMED ERA-Net program (Sys4MS project, id:43). The inclusion and exclusion criteria are described elsewhere (Brune et al., 2022; Cellerino et al., 2020). Complete data sets with sNfL, flow cytometry, clinical and imaging measures were obtained from 222 MS patients at baseline. A full data set, also including clinical and imaging measures after two-years, were available for 140 of the patients (Fig. 1). The clinical (EDSS) and paraclinical

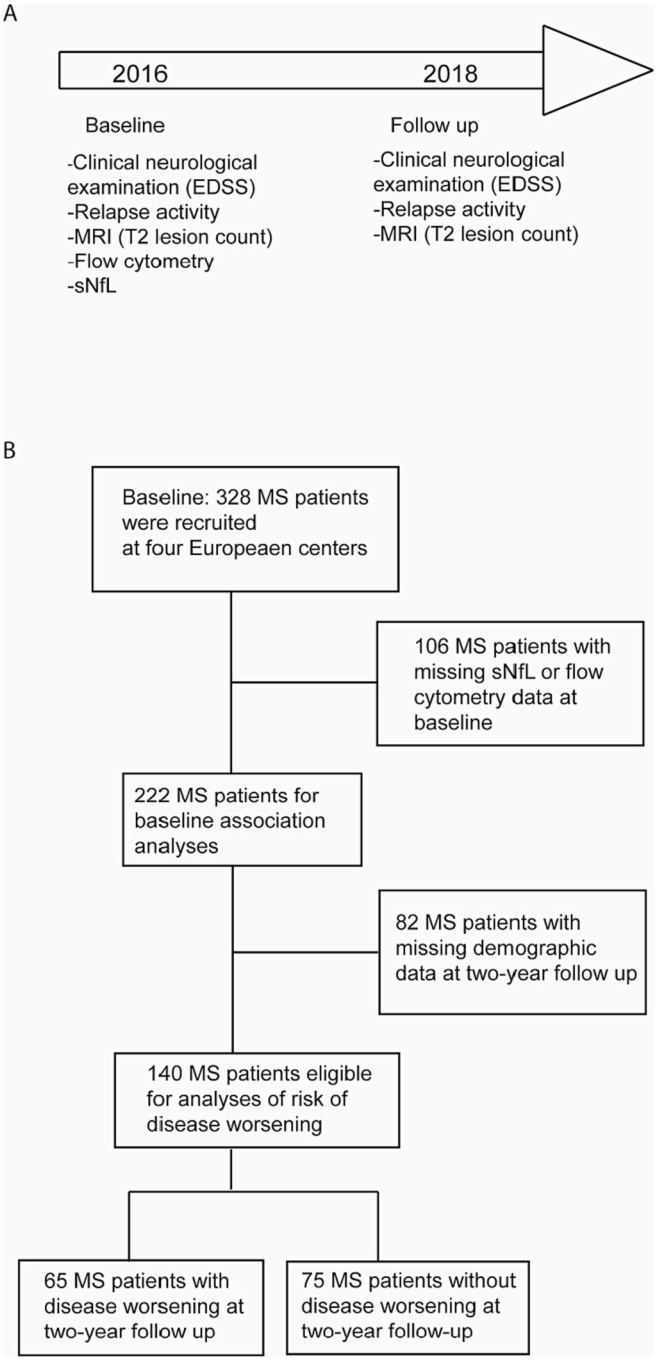


Fig. 1. An overview of the collected samples (A) and of the patient selection (B). EDSS = Expanded Disability Status Scale; sNfL = serum neurofilament light chain; disease worsening = ≥ 3 new cerebral MRI lesions and/or EDSS progression and/or evidence of a new clinical relapse.

examinations (MRI and blood samples) were obtained at the same timepoint for each patient, both at inclusion and follow-up. In addition, the history of a clinical relapse in the follow-up period was recorded at the two-year follow-up visit. The Sys4MS project followed the Declaration of Helsinki and was approved by the IRCCS Ospedale Policlinico San Martino, University of Oslo (REC ID: 2011/1846 A), Charité-Universitaetsmedizin, and Hospital Clinic of Barcelona. All participants provided written informed consent prior to their inclusion in the study.

2.2. Primary study outcome

The primary clinical study outcome was disease worsening two-year after inclusion to the study (baseline), characterized by either i) ≥ 3 new cerebral MRI T2 lesions, ii) Expanded Disability Status Score (EDSS) progression, or iii) evidence of a new clinical relapse. EDSS is a disability measure from 0 to 10, scoring increasing neurological disability, where progression was defined as an increase of (i) 1.5 or more if the EDSS baseline score was 0, (ii) 1.0 if the baseline EDSS score was >0 and less than 5.5 and (iii) 0.5 if the baseline EDSS score was ≥5.5.

2.3. Serum neurofilament light chain measurements

Serum NfL concentrations were measured using the single molecule array immunoassay (Simoa Technology; QUANTERIX Corporation) as described previously (Brune et al., 2022). Age-normative NfL percentile cut-offs were calculated from data from healthy controls (HC) ($n = 309$), dichotomizing patients based on their sNfL concentrations into high (≥75th age-corrected percentile) or normal (<75th age-corrected percentile) as described (Brune et al., 2022).

2.4. Flow cytometry

The multicentric study was set up to ensure the highest standardization of PBMC purification and flow cytometry acquisition and analyses of T, B and NK cell subsets to reduce possible biases as described previously (Cellerino et al., 2020).

2.5. Statistical analysis

Statistical analyses were performed using IBM SPSS version 29 for Mac (IBM Corp., Armonk, NY, USA). Descriptive statistics are presented as either mean with standard deviation (SD), median with range or proportions. For comparison between groups, student *t*-test, Mann-Whitney *U* test or analysis of covariance were used for continuous variables and $\chi 2$ or Fisher's exact test for categorical variables. Partial correlation (r_p) analyses were applied to test for associations between the frequency of different immune cell subpopulations and $\log_e$-transformed sNfL concentrations at baseline adjusted for age, gender and treatment level (no treatment, active treatment and highly active treatment). Concentrations of sNfL were logarithmically transformed in order to create normally distributed data. For the correlation coefficient *r*, small effects were defined as 0.1, medium effects as 0.3, and large effects were defined as 0.5 (Pd, 2010). To test if sNfL concentrations and immune cell subpopulation frequencies could predict disease worsening at two-year follow-up, univariable and multivariable logistic regression analyses were applied including sNfL concentrations above the age-corrected 75th percentile, age, gender, treatment level and the frequency of different immune cell subpopulations as covariates. Before performing the multivariable regression analysis, possible multicollinearities of the covariates were explored using a correlation coefficient ≥ 0.7 as a limit for multicollinearity. All tests were two-sided and a 1% significance level was used due to the number of tests performed.

3. Results

3.1. Patient characteristics

The study population consisted of 222 MS patients where both baseline flow cytometry and sNfL measures were available (Brune et al., 2022; Cellerino et al., 2020). Demographic and clinical characteristics of this study population are presented in Tables 1 and 2. The majority of the patients (80.2%) were diagnosed with RRMS, whereas 9.5 and 10.4% were diagnosed with SPMS and PPMS, respectively (Table 1). Among the RRMS patients, 23 (12.9%) were untreated, 100 (56.2%) on active treatment (interferon, glatiramer acetate, teriflunomide, dimethyl fumarate) and 55 (30.9%) on highly active treatment (fingolimod, natalizumab, alemtuzumab, rituximab, ocrelizumab). Among the progressive MS (PMS) patients, 29 (65.9%) were untreated and the remaining 15 (34.1%) were on active or highly active treatment (Table 2).

Table 1
Demographic and clinical features of the study population at baseline.

	MS ($n = 222$)
Age, years, mean (SD)	43.2 (±10.3)
Female, n (%)	149 (67.1)
MS classification, n (%)	
RRMS	178 (80.2)
SPMS	21 (9.5)
PPMS	23 (10.4)
Age at MS onset, years, mean (SD)	31.3 (±9.3)
Disease duration, median, years (range)	8.9 (0.6–43.5)
Follow-up time, mean, years (SD)	1.9 (±0.3)
Disease-modifying treatment	
None, n (%)	52 (23.4)
Active treatment, n (%)	104 (46.8)
Interferon	30 (13.5)
Glatiramer acetate	28 (12.6)
Teriflunomide	14 (6.3)
Dimethyl fumarate	32 (14.4)
Highly active treatment, n (%)	66 (29.8)
Fingolimod	28 (12.6)
Natalizumab	22 (9.9)
Alemtuzumab	7 (3.2)
Rituximab	6 (2.7)
Ocrelizumab	1 (0.5)
Daclizumab	2 (0.9)
EDSS, median (range)	2.0 (0.0–8.0)
sNfL (pg/mL), median (range)	7.4 (2.5–93.2)

Abbreviations: RRMS = relapsing remitting MS; SPMS = secondary progressive MS; PPMS = primary progressive MS; EDSS = expanded disability status scale; sNfL = serum neurofilament light chain. Descriptive statistics are presented as either mean with standard deviation (SD), median with range or proportions.

Flow cytometry and sNfL analyses were performed on samples obtained at inclusion (i.e., baseline), and the patients were clinically evaluated after 2 years (mean 2.0 ±0.3 years). A full data set including all clinical and imaging measures was available for 140 of the patients after two years (Fig. 1). Of these, 65 patients (46.4%) experienced disease worsening, whereas 75 patients (53.6%) were stable (Fig. 1 and Table 3). Of the 140 patients with a complete dataset at follow-up, 12 RRMS patients were untreated at baseline, and three of them started on active or highly active treatment during the follow-up period. Further, 62 RRMS patients were on active treatment at baseline, and 14 of them switched to highly active treatment. Of the 39 RRMS patients on highly active treatment at baseline, three discontinued the medication and one switched to active treatment. In the PMS group, 16 patients were untreated at baseline and four of them started on highly active treatment, whereas of the 11 PMS patients on active or highly active treatment at baseline, one of them discontinued the medication during the follow-up period.

The sNfL concentrations and the frequencies of immune cell subpopulations of the entire cohort ($n = 222$) as well as in the patient subgroups – all at baseline - are listed in Tables 4–6. The definition of each immune cell subset is explained in the table caption in Table 4.

3.2. Association between sNfL and immune cell subpopulations at baseline

To explore the relationship between sNfL and circulating immune cell subpopulations, we first performed partial correlation calculations between baseline sNfL concentrations and immune cell subpopulation frequencies in samples from all MS patients (n = 222), followed by analyses in subgroups of patients. There was no correlation between sNfL concentrations and immune cell subpopulation frequencies when analysing the entire MS cohort together (eTable 1). When subgrouping

Table 2
Demographic and clinical features of the study population in subgroups of patients at baseline.

	RRMS no treatment ($n = 23$)	RRMS active treatment ($n = 100$)	RRMS highly active treatment ($n = 55$)	PMS no treatment ($n = 29$)	PMS active or highly active treatment ($n = 15$)
Age, years, mean (SD)	41.6 (± 8.5)	41.2 (± 9.1)	38.5 (± 8.3)	56.3 (± 5.8)	50.5 (±9.7)
Female; n (%)	16 (69.6)	67 (67.0)	41 (74.5)	17 (58.6)	8 (53.3)
Age at MS onset, years, mean (SD)	31.4 (±8.6)	31.4 (±8.6)	28.3 (±8.2)	35.5 (±9.7)	33.5 (±14.7)
Disease duration, median, years (range)	8.9 (1.0–30.9)	8.3 (0.8–29.6)	7.5 (0.6–33.5)	21.5 (1.4–43.5)	11.0 (3.1–42.1)
Follow-up time, mean, years (SD)	1.92 (±0.3)	1.95 (±0.4)	1.96 (±0.3)	1.9 (±0.2)	2.0 (±0.2)
Disease-modifying treatment					
None, n (%)	23 (100)	0 (0)	0 (0)	29 (100)	0 (0)
Active treatment, n (%)	0 (0)	100 (100)	0 (0)	0 (0)	4 (26.7)
Interferon	0 (0)	29 (29.0)	0 (0)	0 (0)	1 (6.7)
Glatiramer acetate	0 (0)	26 (26.0)	0 (0)	0 (0)	2 (13.3)
Teriflunomide	0 (0)	14 (14.0)	0 (0)	0 (0)	0 (0)
Dimethyl fumarate	0 (0)	31 (31.0)	0 (0)	0 (0)	1 (6.7)
Highly active treatment, n (%)	0 (0)	0 (0)	55 (100)	0 (0)	11 (73.3)
Fingolimod	0 (0)	0 (0)	26 (47.3)	0 (0)	2 (13.2)
Natalizumab	0 (0)	0 (0)	21 (38.2)	0 (0)	1 (6.7)
Alemtuzumab	0 (0)	0 (0)	6 (10.9)	0 (0)	1 (6.7)
Rituximab	0 (0)	0 (0)	0 (0)	0 (0)	6 (40.0)
Ocrelizumab	0 (0)	0 (0)	0 (0)	0 (0)	1 (6.7)
Daclizumab	0 (0)	0 (0)	2 (3.6)	0 (0)	0 (0)
sNfL (pg/mL), median (range)	7.1 (4.0–20.6)	6.6 (2.4–93.2)	6.9 (3.9–17.0)	11.4 (4.8–28.4)	9.7 (4.2–14.1)
EDSS, median (range)	2.0 (0.0–4.0)	1.5 (0.0–6.5)	2.0 (0.0–6.5)	5.5 (2.0–8.0)	4.5 (2.5–6.5)

Abbreviations: RRMS = relapsing-remitting MS; PMS = progressive MS; EDSS = expanded disability status scale; sNfL = serum neurofilament light chain. Descriptive statistics are presented as either mean with standard deviation (SD), median with range or proportions.

Table 3
Baseline demographic and clinical features of patients with or without disease worsening at two-year follow-up.

	Patients with disease worsening ($n = 65$)	Patients without disease worsening ($n = 75$)	p
Age, years, mean (SD)	43.2 (± 10.5)	41.7 (± 10.5)	0.408
Female; n (%)	38 (58.5)	56 (74.7)	
Multiple sclerosis classification, n (%)			0.053
RRMS no treatment	5 (7.7)	7 (9.3)	
RRMS active treatment	30 (46.2)	32 (42.7)	
RRMS highly active treatment	12 (18.5)	27 (36.0)	
PMS no treatment	12 (18.5)	4 (5.3)	
PMS active or highly active treatment	6 (9.1)	5 (6.7)	
Age at MS onset, years, mean (SD)	31.3 (± 9.5)	29.5 (± 8.6)	0.264
Disease duration, median, years (range)	8.5 (1.0–43.5)	9.2 (1.6–34.7)	0.856
Follow-up time, mean, years (SD)	2.0 (± 0.4)	2.0 (± 0.3)	0.361
sNfL (pg/mL), median (range)	8.4 (2.9–93.2)	7.0 (2.7–20.2)	**0.034**
EDSS, median (range)	2.0 (0.0–6.5)	2.0 (0.0–6.5)	0.134

Abbreviations: RRMS = relapsing-remitting MS; SPMS = secondary progressive MS; PPMS = primary progressive MS; disease worsening = ≥ 3 new cerebral MRI lesions and/or confirmed Expanded Disability Status Score (EDSS) progression and/or evidence of a new clinical relapse. Descriptive statistics are presented as either mean with standard deviation (SD), or median with range or proportions. p = p value for the comparison between patients with disease worsening and without disease worsening, using the independent samples t-test, chi-squared test, Fisher exact test and analysis of covariance, adjusted for age, gender and treatment level. Significant differences between the groups are reported in bold.

the patients based on treatment level (eTable 2 and Fig. 2), we found a moderate positive correlation between sNfL concentration and the frequency of $CD4^+$ T cells ($r_p = 0.201$, $p = 0.047$) in the RRMS group on active treatment and a moderate positive correlation between sNfL concentration and the frequency of Th1 cells ($CD3^+CD4^+CCR6^-CD161^-CXCR3^+$) ($r_p = 0.276$, $p = 0.045$) in the RRMS group on highly active treatment. In the untreated PMS group, there was a moderate negative correlation between sNfL concentration and the frequency of B regulatory cells ($CD19^+CD24^{high}CD38^{high}$) ($r_p = -0.431$, $p = 0.025$), and in addition, there was a strong negative correlation between sNfL concentration and the frequency of plasma B cells ($CD19^+CD24^-CD38^{high}$) ($r_p = -0.643$, $p = 0.018$) in the PMS group on treatment.

We have previously shown that the greatest deviation in immunophenotype in MS patients was based on treatment and not disease course, with the strongest impact in fingolimod-treated patients (Cellerino et al., 2020). Therefore, sensitivity analyses excluding patients treated with fingolimod from the highly active treatment group, and analyses stratifying patients according to their specific medication and disease phenotype (RRMS or PMS) were performed (eTable 3 and eTable 4). The significant correlation between the frequency of Th1 cells ($CD3^+CD4^+CCR6^-CD161^-CXCR3^+$) and sNfL concentration was not present in the cross-sectional analyses excluding the fingolimod treated patients from the highly active treatment group. However, analyses in subgroups of patients, revealed a moderate positive correlation between sNfL concentration and the frequency of Th1 cells ($CD3^+CD4^+CCR6^-CD161^-CXCR3^+$) ($r_p$ 0.419, $p = 0.041$) in RRMS patients treated with fingolimod, suggesting that fingolimod is the driving force of this association also in the analyses of RRMS patients on highly active treatment including fingolimod. Further, there was a moderate negative correlation between sNfL concentration and the frequency of regulatory $CD8^+$ T cells ($CD3^+CD8^+CD28^-CD127^-$) ($r_p = -0.418$, $p = 0.030$) and between sNfL concentration and the frequency of regulatory NK cells ($CD56^{dim}$) ($r_p = -0.431$, $p = 0.025$) in RRMS patients treated with interferon and a strong positive correlation between sNfL concentration and the frequency of regulatory $CD4^+$ T cells ($CD3^+CD4^+CD25^+CD127^-$) ($r_p = 0.611$, $p = 0.035$) in RRMS patients treated with teriflunomide. None of the p values survived multiple testing corrections.

Table 4
Lymphocyte frequencies in peripheral blood from patients with MS at baseline.

	MS (n = 222)
Cell subpopulations, cell frequencies, % (SD)	
Total $CD3^+$	63.0 (±16.7)
$CD3^+CD4^+$	39.6 (±16.1)
$CD3^+CD8^+$	19.0 (±8.5)
$CD3^+CD4^+CD25^+CD127^-$ ($CD4^+$T-reg)	6.3 (±3.5)
$CD3^+CD8^+CD28^-CD127^-$ ($CD8^+$T-reg)	30.3 (±21.3)
$CD3^+CD4^+CCR6^-CD161^-CXCR3^+$ (Th1)	10.1 (±6.9)
$CD3^+CD4^+CCR6^+CD161^+CXCR3^-CCR4^+$ (Th17)	0.9 (±0.8)
$CD3^+CD4^+CCR6^+CD161^+CxCR3^{high}CCR4^{low}$ (Th1/Th17)	1.9 (±1.9)
Total $CD19^+$	7.6 (±5.6)
$CD19^+CD24^{high}CD38^{low}$ (B-memory)	13.3 (±8.8)
$CD19^+CD24^{low}CD38^{low}$ (B-mature)	46.4 (±17.9)
$CD19^+CD24^{high}CD38^{high}$ (B-reg)	12.7 (±12.5)
$CD19^+CD24^-CD38^{high}$ (plasma B-cells)	3.4 (±6.7)
$CD16^+CD56^{low}$ ($CD56^{dim}$ NK cells)	58.7 (±17.9)
$CD16^+CD56^{high}$ ($CD56^{bright}$ NK cells)	3.6 (±3.3)

Total $CD3^+$ = % $CD3^+$ T cells among the PBMCs; $CD3^+CD4^+$ = % $CD3^+CD4^+$ among the PBMCs; $CD3^+CD8^+$ = % $CD3^+$CD8 T cells among the PBMCs; $CD3^+CD4^+CD25^+CD127^-$ ($CD4^+$T-reg) = % $CD3^+CD4^+CD25^+CD127^-$ ($CD4^+$ T-reg) cells among the $CD3^+CD4^+$ T cell population; $CD3^+CD8^+CD28^-CD127^-$ ($CD8^+$T-reg) = % $CD3^+CD8^+CD28^-CD127^-$ ($CD8^+$T-reg) cells among the $CD3^+$ $CD8^+$ population; $CD3^+CD4^+CCR6^-CD161^-CXCR3^+$ (Th1) = % $CD3^+CD4^+$ $CCR6^-CD161^-CXCR3^+$ (Th1) among the $CD3^+CD4^+$ T cells; $CD3^+CD4^+CCR6^+$ $CD161^+CXCR3^-CCR4^+$ (Th17) = % $CD3^+CD4^+CCR6^+CD161^+CXCR3^-CCR4^+$ (Th17) among the$CD3^+CD4^+$Tcells;$CD3^+CD4^+CCR6^+CD161^+CXCR3^{high}CCR4$-^{low}(Th1/Th17)=% $CD3^+CD4^+CCR6^+CD161^+CXCR3^{high}CCR4^{low}$ (Th1/Th17) among the $CD3^+CD4^+$ T cells; Total $CD19^+$ = % $CD19^+$ B cells among the PBM Cs; $CD19^+CD24^{high}CD38^{low}$ (B-memory) = % $CD19^+CD24^{high}CD38^{low}$ (B-me mory) among $CD19^+$ B cells; $CD19^+CD24^{low}CD38^{low}$ (B-mature) = % $CD19^+$CD $24^{low}CD38^{low}$ (B-mature) among the $CD19^+$ B cells; $CD19^+CD24^{high}CD38^{high}$ (B-reg) = regulatory B cells; % $CD19^+CD24^{high}CD38^{high}$ (B-reg) among the $CD19^+$ B cells; $CD19^+CD24^-CD38^{high}$ (plasma B-cells) = % $CD19^+CD24^-CD38^{high}$ (plasma B-cells) among the $CD19^+$ B cells; $CD16^+CD56^{low}$ ($CD56^{dim}$ NK cells) = % $CD16^+CD56^{low}$ ($CD56^{dim}$ NK cells) of the $CD3^-CD19^-$ PBMC population; $CD16^+CD56^{high}$ ($CD56^{bright}$ NK cells) = % $CD16^+CD56^{high}$ ($CD56^{bright}$ NK cells) of the $CD3^-CD19^-$ PBMC population; Descriptive statistics are presented as mean with standard deviation (SD).

3.3. Association of sNfL and immune cell subpopulations with MS disease worsening

Two years after inclusion, the MS patients were classified as clinically stable or experiencing disease worsening as defined in the Materials and Methods (section 2.2). Serum NfL concentrations at baseline were significantly higher in patients with disease worsening compared with stable patients ($p = 0.034$ corrected for age, gender and treatment level; Table 3 and Fig. 3 A), although the p value did not survive multiple testing corrections. There were however no significant differences in distribution of any of the immune cell subpopulations between patients with and without disease worsening, except for the Th1 cell subset were patients experiencing disease worsening had higher frequencies of Th1 cells compared with stable patients ($p = 0.050$ corrected for age, gender and treatment level; Table 6 and Fig. 3 B). When performing univariable logistic regression analyses to evaluate whether specific immune cell subpopulation frequencies or sNfL concentrations were associated with elevated risk of disease worsening in the entire MS population, higher frequency of Th1 cells ($CD3^+CD4^+CCR6^-CD161^-$ $CXCR3^+$) was associated with a slightly increased risk of disease worsening (OR [95% CI] =1.05, [1.00–1.11], $p = 0.048$) (supplementary eTable 5). Further, a trend towards a positive association between high sNfL concentrations at baseline (≥75th age-corrected percentile) and the risk of disease worsening after two years was observed, although this did not reach statistical significance (OR [95% CI] = 1.96, [0.97–3.95], $p = 0.060$).

When stratifying by MS subgroups, RRMS patients on active treatment with high sNfL concentrations at baseline (≥75th age-corrected percentile) had a 2.9-fold increased risk of disease worsening at two-year follow up (OR [95% CI] =2.90, [1.02–8.21], $p = 0.046$) (supplementary eTable 6). There was however, no association between sNfL concentration and the risk of disease worsening in the other subgroups of patients. In addition, high frequency of regulatory $CD4^+$ T cells ($CD3^+CD4^+CD25^+CD127^-$) was associated with increased risk (OR [95% CI] = 1.24, [1.01–1.52], $p = 0.045$) of disease worsening among RRMS patients on active treatment, and high frequency of regulatory NK cells ($CD56^{dim}$) was associated with decreased risk (OR [95% CI] = 0.94, [0.88–0.99], $p = 0.031$) of disease worsening among RRMS patients on highly active treatment, also in analyses excluding the fingolimod treated patients (OR [95% CI] = 0.87, [0.76–0.99], $p = 0.048$) (Supplementary eTable 7). Finally, in the univariable analyses stratifying patients according to their specific medication and disease phenotype (RRMS or PMS), neither sNfL concentration nor immune cell subpopulations were associated with elevated risk of disease worsening (Supplementary eTable 8).

To analyse the potential prognostic value of combining baseline immune cell subpopulation frequencies and sNfL concentrations, we used multivariable models both in the total MS population and further in

Table 5
Lymphocyte frequencies in subgroups of MS patients at baseline.

	RRMS no treatment (n = 23)	RRMS active treatment (n = 100)	RRMS highly active treatment (n = 55)	PMS no treatment (n = 29)	PMS active and highly active treatment (n = 15)
Cell subpopulations, cell frequencies, % (SD)					
Total $CD3^+$	71.9 (±12.8)	67.9 (±13.3)	50.7 (±18.7)	64.3 (±13.3)	58.9 (±15.2)
$CD3^+CD4^+$	46.9 (±9.3)	45.3 (±12.4)	24.7 (±17.0)	43.2 (±11.0)	37.8 (±15.9)
$CD3^+CD8^+$	21.6 (±9.4)	18.7 (±7.6)	19.7 (±8.7)	17.7 (±9.7)	17.2 (±9.7)
$CD3^+CD4^+CD25^+CD127^-$ ($CD4^+$T-reg)	4.8 (±2.0)	6.1 (±3.2)	7.2 (±4.9)	6.8 (±2.3)	5.9 (±1.8)
$CD3^+CD8^+CD28^-CD127^-$ ($CD8^+$T-reg)	28.0 (±17.3)	24.7 (±17.5)	39.3 (±25.7)	31.1(±19.8)	36.2 (±24.6)
$CD3^+CD4^+CCR6^-CD161^-CXCR3^+$ (Th1)	7.6 (±5.2)	8.9 (±6.2)	10.8 (±8.6)	13.3 (±6.4)	12.7 (±4.9)
$CD3^+CD4^+CCR6^+CD161^+CXCR3^-CCR4^+$ (Th17)	1.0 (±1.2)	0.9 (±0.8)	0.9 (±0.8)	0.7 (±0.5)	0.9 (±0.8)
$CD3^+CD4^+CCR6^+CD161^+CxCR3^{high}CCR4^{low}$ (Th1/Th17)	2.1 (±1.8)	1.9 (±2.1)	1.9 (±1.9)	1.7 (±1.1)	1.9 (±1.5)
Total $CD19^+$	8.2 (±5.7)	7.9 (±4.6)	8.5 (±7.9)	6.3 (±3.3)	4.5 (±4.1)
$CD19^+CD24^{high}CD38^{low}$ (B-memory)	21.3 (±10.1)	13.6 (±8.5)	12.6 (±7.8)	10.2 (±6.6)	8.2 (±9.1)
$CD19^+CD24^{low}CD38^{low}$ (B-mature)	41.4 (±14.9)	50.9 (±16.1)	37.1 (±16.5)	56.5 (±17.4)	37.6 (±20.3)
$CD19^+CD24^{high}CD38^{high}$ (B-reg)	6.2 (±4.4)	12.1 (±8.1)	17.5 (±16.4)	4.9 (±4.0)	23.3 (±22.4)
$CD19^+CD24^-CD38^{high}$ (plasma B-cells)	3.6 (±3.8)	2.5 (±2.7)	4.3 (±9.3)	1.7 (±1.5)	8.3 (±15.9)
$CD16^+CD56^{low}$ ($CD56^{dim}$ NK cells)	63.1 (±12.8)	54.5 (±20.1)	61.9 (±17.3)	62.1 (±14.4)	61.0 (±12.7)
$CD16^+CD56^{high}$ ($CD56^{bright}$ NK cells)	3.5 (±2.8)	3.9 (±3.0)	3.8 (±4.4)	2.7 (±2.0)	2.7 (±2.1)

Abbreviations: RRMS = relapsing-remitting MS; PMS = progressive MS. Active treatment = interferon, glatiramer acetate, teriflunomide and dimethyl fumarate. Highly active treatment = fingolimod, natalizumab, alemtuzumab, rituximab and ocrelizumab. Descriptive statistics are presented as mean with standard deviation (SD).

Table 6
Baseline lymphocyte frequencies in MS patients with and without disease worsening at two-year follow-up.

	Patients with disease worsening (n = 65)	Patients without disease worsening (n = 75)	p
Cell subpopulations, cell frequencies, % (SD)			
Total $CD3^+$	63.4 (± 15.2)	59.8 (± 18.8)	0.589
$CD3^+CD4^+$	41.2 (± 13.3)	36.9 (± 18.2)	0.450
$CD3^+CD8^+$	18.4 (± 8.3)	18.4 (± 8.6)	0.930
$CD3^+$ $CD4^+CD25^+CD127^-$ ($CD4^+$T-reg)	6.7 (± 3.4)	5.6 (± 3.5)	0.062
$CD3^+CD8^+CD28^-CD127^-$ ($CD8^+$T-reg)	26.8 (± 18.3)	32.5 (± 22.7)	0.159
$CD3^+CD4^+CCR6^-CD161^-CXCR3^+$ (Th1)	11.2 (± 7.1)	9.1 (± 7.2)	**0.050**
$CD3^+CD4^+CCR6^+CD161^+CXCR3^-CCR4^+$ (Th17)	0.9 (± 0.9)	0.8 (± 0.7)	0.363
$CD3^+CD4^+CCR6^+CD161^+CxCR3^{high}CCR4^{low}$(Th1/Th17)	1.8 (± 1.6)	1.5 (± 1.5)	0.272
Total $CD19^+$	7.4 (± 5.6)	8.6 (± 6.4)	0.454
$CD19^+CD24^{high}CD38^{low}$ (B-memory)	12.9 (± 8.9)	13.7 (± 9.0)	0.277
$CD19^+CD24^{low}CD38^{low}$ (B-mature)	47.5 (± 18.6)	45.7 (± 17.2)	0.865
$CD19^+CD24^{high}CD38^{high}$ (B-reg)	13.2 (± 14.5)	13.5 (± 11.8)	0.307
$CD19^+CD24^-CD38^{high}$ (plasma B-cells)	3.9 (± 9.7)	2.5 (± 3.9)	0.131
$CD16^+CD56^{low}$ ($CD56^{dim}$ NK cells)	55.2 (± 18.8)	57.7 (± 17.1)	0.184
$CD16^+CD56^{high}$ ($CD56^{bright}$ NK cells)	3.7 (± 3.2)	4.1 (± 3.9)	0.838

Abbreviations: disease worsening = ≥ 3 new cerebral MRI lesions and/or confirmed Expanded Disability Status Score (EDSS) progression and/or evidence of a new clinical relapse. Descriptive statistics are presented as mean with standard deviation (SD). p = p value for the comparison between patients with and without disease worsening, using analysis of covariance, adjusted for age, gender and treatment level.

RRMS patients on active treatment (eTable 9 and Fig. 4 A) the only subgroup of patients with sufficient sample size for these analyses. When analysing data from all MS patients together, the risk of disease worsening was slightly and significantly increased when combining sNfL concentration and regulatory B cell levels ($CD19^+CD24^{high}CD38^{high}$) (OR [95% CI] =2.13, [1.04–4.38], $p = 0.040$), and when combing sNfL concentration and plasma B cell levels ($CD19^+CD24^-CD38^{high}$) (OR [95% CI] =2.14, [1.05–4.40], $p = 0.037$), whereas the risk of disease worsening was not affected when combining sNfL with subpopulation frequencies of other immune cells. In RRMS patients on active treatment, the significant association between sNfL and disease worsening was more pronounced when combining sNfL with $CD4^+$ T cells (OR [95% CI] =3.22, [1.02–10.2], $p = 0.047$), $CD8^+$ T cells (OR [95% CI] =3.03, [1.02–9.0], $p = 0.046$), Th17 cells ($CD3^+CD4^+CCR6^+CD161^+CXCR3^-CCR4^+$;OR [95% CI] =3.28, [1.08–9.92], $p = 0.036$), memory B cells ($CD19^+CD24^{high}CD38^{low}$; OR [95% CI] =2.98, [1.01–8.83], $p = 0.049$) or regulatory B cells ($CD19^+CD24^-CD38^{high}$; OR [95% CI] = 3.23, [1.04–10.03], $p = 0.042$) (Fig. 4 A) as compared with each parameter in separate univariable models (Fig. 4 B). None of the *p* values survived multiple testing corrections.

4. Discussion

The prognostic value of combined measures of immune cell subpopulation frequencies and sNfL concentration was investigated for disease worsening in a longitudinal multicentre real-world MS cohort. High sNfL concentration has already been shown to be associated with disease worsening in this heterogenous MS cohort (Brune et al., 2022). Additional analyses in subgroups of patients now revealed that sNfL predicted disease worsening in the RRMS patient group using active treatment, but not in the other subgroups of patients. In the RRMS patient group on active treatment, a higher proportion of the patients, about 50%, experienced disease worsening after two years, compared to about 30% among the RRMS patients on highly active treatment, indicating suboptimal effect of therapy among the patients using active treatment (Table 3), and that switching therapy to highly active treatment might improve their outcome (Simonsen et al., 2022; Spelman et al., 2021). A high proportion, 75%, of the untreated PMS patient group also experienced disease worsening after two years. However, there was now association between high sNfL at baseline and the risk of disease worsening two years after in this patient population. Recent studies have demonstrated that the association between sNfL and disease progression is less pronounced in patients with progressive MS, and that serum glial fibrillary acidic protein, a biomarker of astrocytic activation, is more associated with disease progression in progressive MS patients (Barro et al., 2023a; Meier et al., 2023).

To evaluate the potential of immune cell subpopulation frequencies as a prognostic biomarker in this patient cohort, the association between immune cell subpopulations at baseline and the risk of disease worsening after two years was investigated. Interestingly, higher frequency of Th1 cells, a pro inflammatory cell subtype involved in the development of several autoimmune diseases (Chatzileontiadou et al., 2020), including MS, was associated with slightly increased risk of disease worsening. Surprisingly, higher frequencies of regulatory $CD4^+$ T cells ($CD3^+CD4^+CD25^+CD127^-$) were associated with increased risk of disease worsening among RRMS patients using active treatment. Regulatory T cells prevent autoimmunity by regulating the immune homeostasis and maintain tolerance. Of note, there are discrepancies in the literature concerning level of regulatory T cells and MS, as reduced (Venken et al., 2008), unaltered (Feger et al., 2007; Noori-Zadeh et al., 2016; Putheti et al., 2004) and increased (Khosravi et al., 2019; Kumar et al., 2006) frequencies of regulatory T cells have been reported. However, other studies have shown impairment of regulatory T cell function in MS patients, and that their effector T cells are more resistant towards regulation by regulatory T cells (Mexhitaj et al., 2019; Schneider et al., 2013). This suggests that the increased frequency of regulatory T cells, which was associated with disease worsening, does not necessarily involve tighter immune regulation, but may be a compensatory mechanism for the possible impact of their impaired immune cell function. Further, high frequency of $CD56^{dim}$ NK cells, an immune-regulatory NK cell subtype, which may be important in controlling MS because of its antiviral function (Laroni and Uccelli, 2020), was associated with decreased risk of disease worsening among RRMS patients treated with highly active treatment. However, these results need to be interpreted with caution given the heterogenous MS population treated with medication with different mechanisms of action.

By combining sNfL concentrations and immune cell subpopulation frequencies in multivariable models, we then explored whether baseline sNfL concentrations and immune cell subset frequencies together could be a stronger predictor of disease worsening two years after than each parameter alone. Therapy influences the immune cell composition of the patients, however, due to the availability of efficient DMTs, the untreated patient group was too small to perform multivariable calculations. Of the MS subgroups, only the group with RRMS patients on active treatment did reach a sufficient sample size for such analyses. Notably, the multivariable analyses revealed an increased risk of disease worsening in this patient group when combining sNfL with Th17 frequencies (OR [95% CI] =3.28, [1.08–9.92], $p = 0.036$). The level of Th17 cells is elevated in RRMS patients, both in blood, cerebrospinal fluid and in

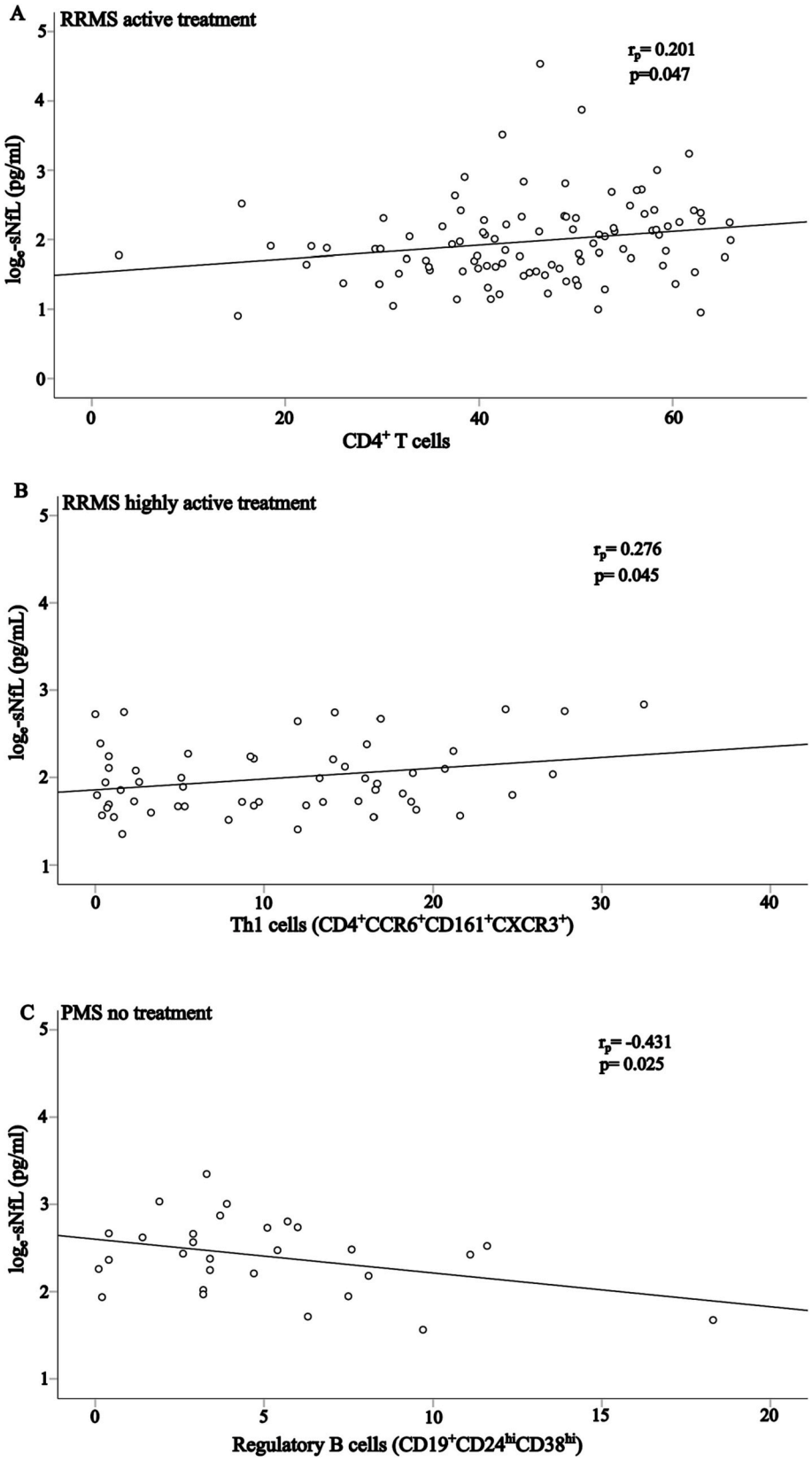


Fig. 2. Scatter plots depicting the associations (partial correlations) between baseline $\log_e$-transformed sNfL concentrations and baseline (A) $CD4^+$ T cells in the RRMS group on active treatment (r_p 0.201, p = 0.047), (B) Th1 cells ($CD3^+CD4^+CCR6^-CD161^-CXCR3^+$) in the RRMS group on highly active treatment (r_p 0.276, p = 0.045) and (C) regulatory B cells ($CD19^+CD24^{high}CD38^{high}$) in the untreated PMS group (r_p = −431, p = 0.025). sNfL = serum neurofilament light chain; RRMS = relapsing remitting multiple sclerosis; PMS = progressive multiple sclerosis; r_p = partial correlation coefficient (corrected for age and gender). None of the *p* values survived multiple testing corrections.

active MS plaques. The Th17 cells promote pathogenesis by producing IL-17, an interleukin with ability to impair the blood-brain-barrier (Moser et al., 2020), thereby making the CNS more prone to inflammation leading to neuroaxonal damage and release of NfL.

In comparison to NfL alone (OR 2.90), by adding either $CD4^+$ T cell, $CD8^+$ T cell, memory or regulatory B cell frequencies to the model, this led to an increase in the risk for disease worsening, although to a slightly less extent than Th17 cells (OR 2.98–3.23 compared with 3.28 for Th17

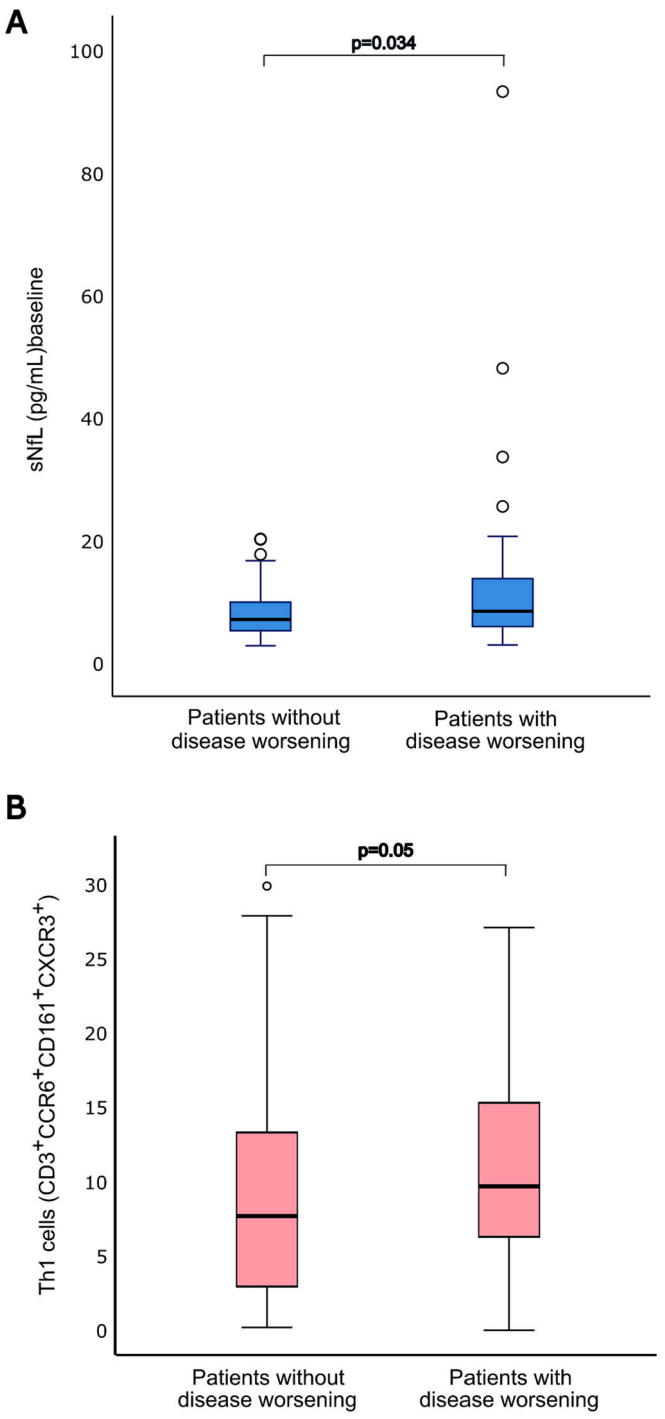


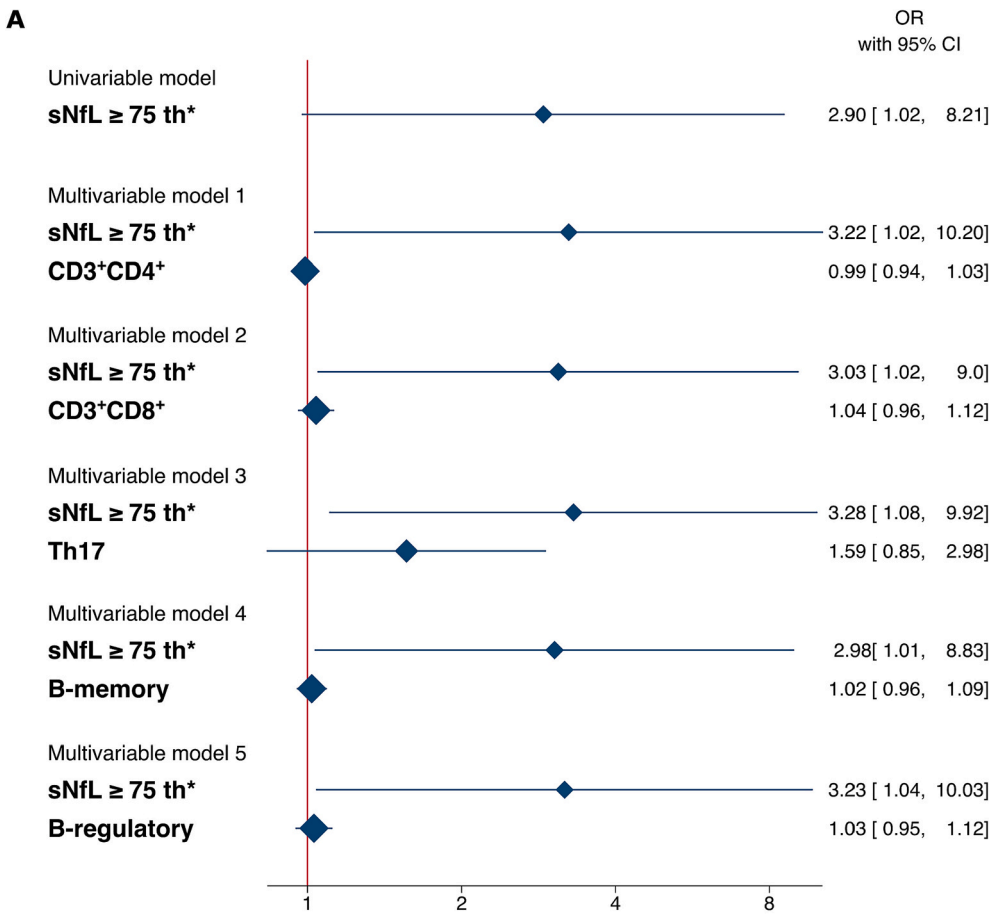
Fig. 3. Differences in sNfL concentration (A) and in the frequency of Th1 cells ($CD3^+CD4^+CCR6^-CD161^-CXCR3^+$) (B) at baseline between patients with and without disease worsening after two years of follow up. p = p value is calculated using analysis of covariance, adjusted for age, gender and treatment level. Bars represent median with interquartile range. Outliers are depicted with circles.

cells). Both $CD4^+$ and $CD8^+$ T cells are heterogeneous groups of cells, containing both proinflammatory cell populations as well as regulatory cells, making it difficult to interpret the mechanism for the observed effect on disease worsening risk. However, it is likely that the balance between pro- and anti-inflammatory subpopulations within these T cell populations is an important predictor for outcome. This has also been suggested in other studies, both in animal models and in patients (Bar-Or and Li, 2021; Mexhitaj et al., 2019). Careful immunophenotyping in bigger cohorts of patients grouped, based on medication subtypes, might give a clearer distinction as of whether additional cell subtypes have valuable prognostic value. DMTs such as dimethyl fumarate have major impact on $CD8^+$ T cells, memory and regulatory B cells (Cellerino et al., 2020; Fleischer et al., 2018; Lundy et al., 2016; Spencer et al., 2015; Staun-Ram et al., 2018), and thus these cell populations might be of prognostic value as patients responding properly to treatment will experience an effect in the frequencies of these immune cell subtypes.

Serum concentration of NfL is a biomarker for MS disease activity (Buchmann et al., 2023, Disanto et al., 2017, Khalil et al., 2018, Kuhle et al., 2019, Loonstra et al., 2023), and in our cross-sectional analyses of baseline samples we showed positive correlation between sNfL concentration and cell subpopulations containing pro-inflammatory cells, such as $CD4^+$ T cells in RRMS patients on active treatment and Th1 ($CD3^+CD4^+CCR6^-CD161^-CXCR3^+$Th1classic) cells in RRMS patients on highly active treatment. (Bielekova et al., 2006; Jiang et al., 2011). Previous analyses of this cohort of untreated patients with PMS have demonstrated a deviation in the frequencies of B-cell subpopulations, with a decrease of B-regulatory cells and an increase of the B-mature arm (Cellerino et al., 2020). Interestingly, we found a trend, although not statistically significant, towards a moderate positive correlation between sNfL and the frequency of $CD19^+$ B cells and a significantly moderate negative correlation between sNfL and the frequency of B regulatory cells in untreated PMS patients. Together, these findings are of particular interest as the first DMT affecting disability progression in PMS is the B-cell-depleting agent, ocrelizumab (Montalban et al., 2017). Overall, these findings suggest that there might be a biological association between sNfL concentrations and immune cell subpopulations, both being associated with disease activity.

A strength of this study is the large multicentric analysis of the immunologic profile of T, B and NK cells and of sNfL in a cohort of 222 MS patients. PBMC purification and flow cytometry analyses were standardized across the four centers, and blood samples for NfL analyses were collected under standardized conditions (Brune et al., 2022; Cellerino et al., 2020). A limitation of the study is the heterogeneous patient population, including patients at different disease stages and treated with different DMTs influencing the cellular immune subpopulations in blood, making it difficult to interpret the results. Previous analyses, in this cohort of MS patients, have demonstrated a stronger deviation in the immunologic profile of patients with MS in relation to treatment rather than to disease phenotype, especially among the fingolimod-treated patients (Cellerino et al., 2020). Sensitivity analyses, stratifying patients according to their specific medication and disease phenotype, were performed to overcome this limitation, but unfortunately too few patients were included in each medication subgroup, making it difficult to draw conclusions from the data. Another limitation is that we have data from a lower number of patients at follow-up compared with baseline. Nevertheless, this was due to lack of information regarding disease worsening in these patients, and not due to a real drop out of patients from the study. Finally, functional changes of the immune cells instead of immune cell frequencies may be more informative as a potential biomarker of disease activity in MS. However, such functional data were not available from the cohorts in this multicentre study.

In conclusion, our exploratory analyses revealed moderate correlations between sNfL and immune cell subpopulations, confirms that sNfL is a biomarker of disease worsening, and suggests that combining circulating immune cell subset frequencies with sNfL concentrations could be a better predictor for disease worsening than one parameter alone. However, because of the heterogenous patient population, the data needs to be interpreted with caution and further studies using data from more homogenous patient populations are needed to explore the possible associations between different immune cell subsets and disease worsening, and to explore the validity and use of sNfL together with immune cell subpopulation frequencies as a potential prognostic marker in MS.



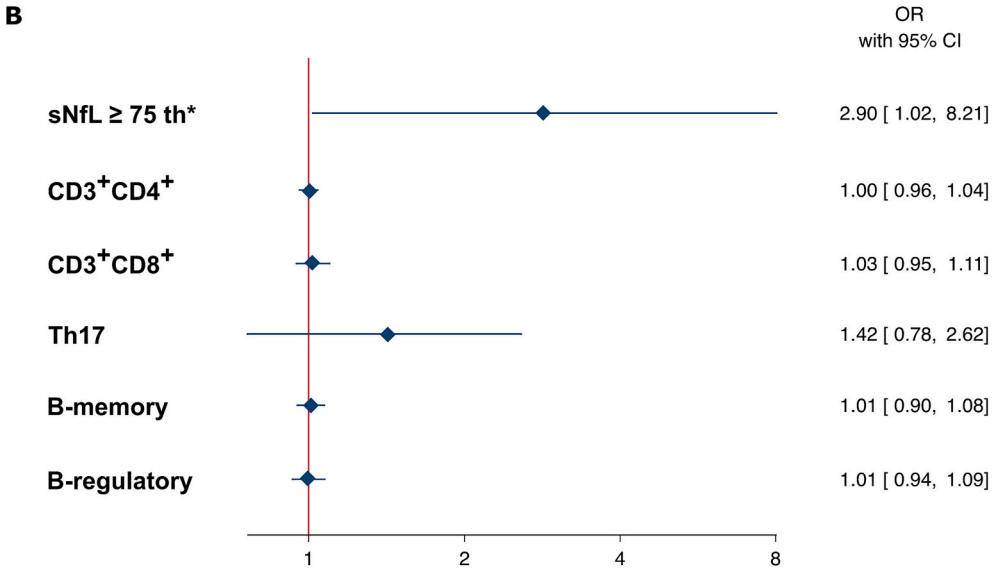


Fig. 4. A: A forest plot depicting the univariable and multivariable logistic regression models for disease worsening at two-year follow-up in RRMS patients using active treatment. The first line in the figure depicts the risk of disease worsening in patients with high baseline sNfL concentrations (univariable model). The multivariable models, 1–5, illustrates the risk of disease worsening when combining baseline sNfL concentrations and different immune cell subpopulation frequencies. In this forest plot, only significant results with an increase in OR in the multivariable analyses compared to the univariable analyses are shown. Results are presented with odds ratio (OR) and 95% confidence interval (CI). B: A forest plot depicting the univariable logistic regression model for disease worsening at two-year follow-up. Results are presented with odds ratio (OR) and 95% confidence interval (CI).

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Declaration of Competing Interest

Synne Brune-Ingebretsen has received honoraria for lecturing from Biogen and Novartis.

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Nicole Kerlero de Rosbo reports no disclosures.

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Cathrine Brunborg reports no disclosures.

Kaj Blennow has served as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon, Biogen, JOMDD/Shimadzu. Julius Clinical, Lilly, MagQu, Novartis, Roche Diagnostics, and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program.

Henrik Zetterberg has served at scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alector, Alzinova, ALZPath, Annexon, Apellis, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, Nervgen, Novo Nordisk, Optoceutics, Passage Bio, Pinteon Therapeutics, Prothena, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure, Biogen, and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work).

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Data availability statement

Anonymized data not published within this article will be made available by request from any qualified investigator. Raw data will be available at the MultipleMS database (www.multipleMS.eu).

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Appendix A. Supplementary data

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