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K-INDEX AS A POTENTIAL PROGNOSTIC BIOMARKER OF DISEASE ACTIVITY IN MULTIPLE SCLEROSIS

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SUMMARY

ABSTRACT

Background:

The κ-index, a quantitative marker of intrathecal κ free light chain synthesis, shows high diagnostic accuracy in multiple sclerosis (MS), but its prognostic value remains insufficiently defined. Biomarkers capable of predicting NEDA-3 loss are needed to support individualized risk stratification.

Objectives:

To evaluate whether the κ-index predicts NEDA-3 loss in MS, to characterize its association with baseline clinical, radiological and CSF features and to explore the complementary prognostic contribution of serum neurofilament light chain (sNfL) z-scores.

Methods:

A longitudinal observational cohort of 242 MS patients was analyzed with standardized CSF and serum sampling and a median follow-up of 28 months. Cox regression models tested associations between baseline variables and time to NEDA-3 loss. κ-index and sNfL z-scores were studied as continuous and dichotomized predictors, including cohort-specific thresholds identified via maximally selected rank statistics. Flexible Cox models with cubic B-splines assessed potential non-linear associations, and a bivariate spline-based model evaluated the joint contribution of κ-index and sNfL.

Results:

Higher κ-index values correlated with larger T2 lesion burden and CSF inflammatory markers. κ-index >6.4 independently predicted NEDA-3 loss, whereas as a continuous variable it showed a non-linear risk profile, with increasing hazard at low and very high values and a plateau at intermediate values. sNfL z-scores were linearly associated with earlier NEDA-3 loss. In a bivariate spline-based model, κ-index and sNfL provided complementary prognostic information, although discrimination remained modest (~55%). Correlation analyses across DMT classes showed no differential association between κ-index and treatment response in anti-CD20–treated patients.

Conclusions:

The κ-index is a biologically meaningful marker of intrathecal immune activation associated with subsequent loss of disease control. Its non-linear prognostic profile and partial complementarity with sNfL support multi-biomarker approaches and flexible survival modeling. These findings provide a methodological rationale for prospective validation and developing individualized prognostic tools in MS.

INTRODUCTION

Multiple sclerosis (MS) is a common immune-mediated inflammatory demyelinating disease of the central nervous system (CNS), known for its heterogenous spectrum of symptoms, severity and response to therapy[1–3]. Despite the cause of MS is still unknown[4], several studies have demonstrated that both innate and adaptive immune systems and their effectors (e.g., microglia, activated macrophages, B and T lymphocytes) play a role in the inflammatory and neurodegenerative processes of the disease[3,5]. Therefore, several disease modifying treatments (DMTs) targeting different components of the immune system have been developed and licensed for the treatment of MS in the last decades[6].

Prognostic factors in Multiple Sclerosis

MS is among the leading causes of neurological disability in the young and middle aged[7]. Considering its highly variable course which can cause severe disability over time, identifying prognostic factors for long-term outcomes could be useful in order to identify patients at high risk of disability progression and help determine appropriate long-term treatment.[1] Despite several elements (demographic and clinical features, magnetic resonance imaging (MRI) measures and biomarkers) have been associated with poorer prognosis[2,8] (Figure 1), early reliable predictors are still lacking[1,9] and, as

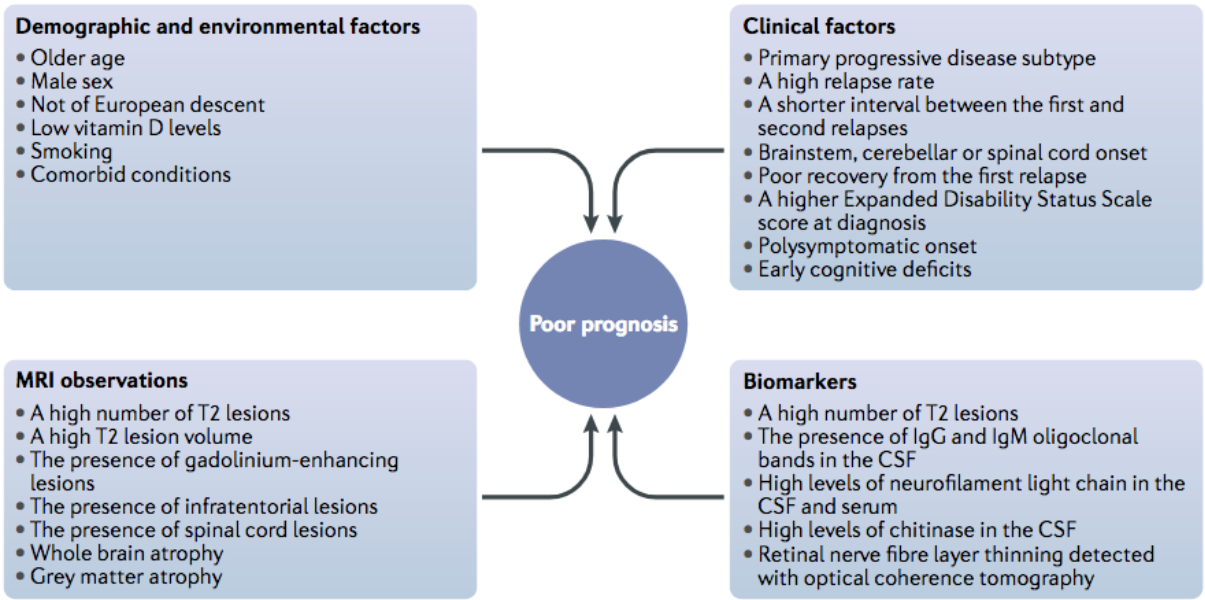

*Fig. 1 - **Predictors of a poor prognosis in MS**. CSF, cerebrospinal fluid; IgG, immunoglobulin G; IgM immunoglobulin M. From Rotstein and Montalban. 2019[2]*

a consequence, none of the prognostic predictive models proposed so far have been validated[10].

Thus, much effort has been put into the search for new prognostic biomarkers to be implemented in clinical practice.

Among fluid biomarkers, the presence and the number of CSF-restricted oligoclonal bands (OCBs), which represent a validated and clinically applied biomarker with diagnostic relevance[11], have been associated to worse prognosis and higher number of cortical lesions[2,12–18]. Although many studies have suggested a clear predictive power for CSF OCBs in MS, several others have not confirmed this hypothesis[19].

Several pieces of evidence concerning the potential prognostic role of neurofilaments light chain (NfL) have accumulated over the years. NfL are neuroaxonal cytoskeletal proteins released into the CSF and blood as a result of neuronal injury and their levels can be elevated in various neurological diseases[20]. In persons with MS (pwMS), higher CSF NfL levels have been associated with increased risk of reaching sustained disability worsening[21], earlier conversion to secondary progressive MS[22] and brain atrophy[23]. With the advent of highly sensitive single molecule array technology (Simoa), NfL can now be quantified in blood. The less invasively measured serum NfL (sNfL) levels have been found to be highly correlated with CSF NfL levels[24–26]. Although not always replicated, current evidence supports baseline serum neurofilament light chain as a prognostic biomarker for multiple sclerosis, correlating with disease activity, neurodegeneration, disability progression, cognitive decline and response to therapy[24,27–31]. The absence of representative reference values to correct for physiological age-dependent increases in sNfL has limited the diagnostic use of this biomarker at an individual level[32]; however, a statistically robust database to derive reference values corrected for age and body-mass index (BMI) has been recently established[33], laying the foundations for further studies. A recent international consensus[34] recommended interpreting sNfL through age- and BMI-adjusted z-scores or percentiles rather than absolute concentrations. Higher z-scores have been associated with an increased risk of future disease activity, although no formal cut-off has yet been validated for clinical use. Values above a z-score of 1.5 or the 90th percentile of healthy controls appear to identify patients at greater risk of subclinical or clinical progression. Recent systematic evidence further confirmed the prognostic

value of sNfL and highlighted the superior predictive accuracy of z-scores over absolute concentrations, while emphasizing the need for standardized thresholds before clinical implementation[35]. Prospective studies are necessary to assess the assay's utility for decision-making in individual patients and to explore the association of sNfL levels and disease outcomes in the long-term[23].

Other potential prognostic biomarkers have been studied over time[36,37], among which one of the most promising is chitinase 3-Like 1 (CHI3L1)[38–41], a glycoprotein secreted by activated glia in the CNS. CHI3L1 is still subject of research and has not reached clinical application.

Kappa free light chains in Multiple Sclerosis

Above normal, intrathecal immunoglobulin (Ig) production is found in the vast majority of pwMS and has long been considered a hallmark of the disease. Excess intrathecal Ig can be demonstrated in several ways, with qualitative and quantitative tests. So far, the most widely used method of detection is isoelectrofocusing (IEF), showing CSF restricted OCBs, a qualitative (seldom quantitative) test. A more recently standardized quantitative method is the determination of kappa free light chain (κ-FLC) CSF levels. The rationale for this investigation lies in the fact that, in addition to intact Ig that consist of light chains and heavy chains bound together, B cells also produce light chains in excess over heavy chains and secrete them as free forms[42] (Fig. 2). κ-FLC, considered for years a waste product of Ig synthesis, are currently known to be very active molecules, helping to develop specific antibody affinity[43]. In order to determine the excess intrathecal κ-FLC fraction (independent from the blood-derived fraction), the concept of κ-FLC index (κ-index) has been introduced. To calculate the κ-index the CSF/serum albumin quotient (Qalb)[44] and the CSF/serum κ-FLC quotient (QκFLC) are considered. The κ-index is determined by the following formula[45,46]:

$$\kappa - \text{index} = \frac{\text{Q}\kappa\text{FLC}}{\text{Qalb}}$$

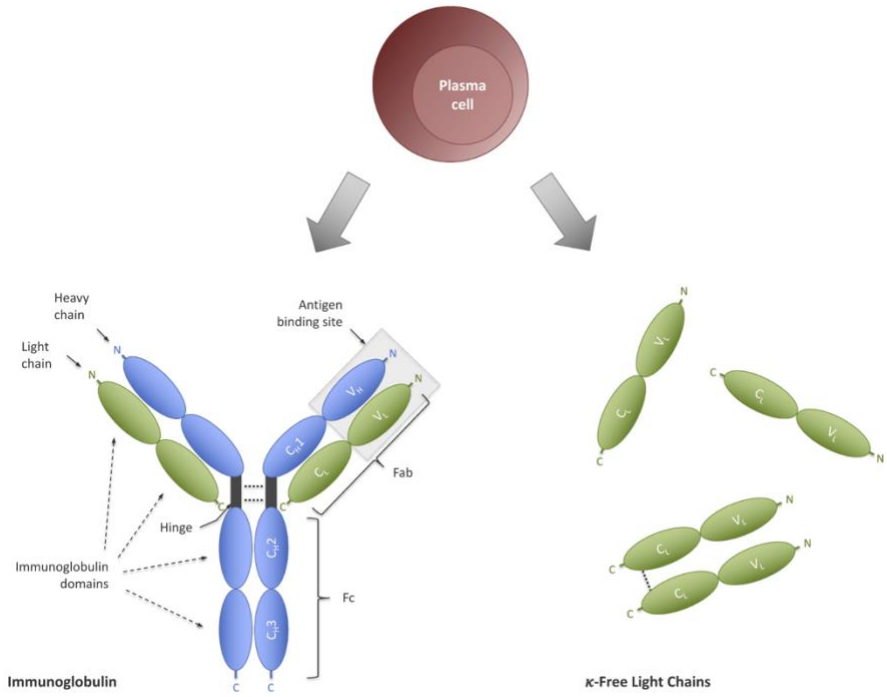

Fig. 2 - ***Schematic representation of intrathecal plasma cell activity and κ-free light chain production.*** *During immunoglobulin synthesis, κ-light chains are secreted in excess relative to heavy chains, regardless of immunoglobulin subtype. Adapted from*[47].

By correcting the κ-FLC ratio for albumin, the k-index offers greater sensitivity than isolated κ-FLC measurements in CSF if concentrations are low[47].

Over the last few years, a multitude of studies have highlighted the value of κ-FLC in CSF and κ-index as alternative biomarkers of intrathecal B cell activity in pwMS[48], and the significant advantages compared to OCBs, in terms of intra- and inter-observer variability, considering IEF is relatively complex to run and time consuming.

From a diagnostic point of view, it has been consistently demonstrated that the κ-index shows a high accuracy similar to that of OCBs in identifying patients with MS (Fig. 3) and it is a promising, easily accessible and easy-to-measure biomarker which might replace OCB detection[47–49] not only in relapsing-remitting MS (RRMS) but also in primary progressive MS (PPMS)[50].

In view of the growing evidence supporting the concordance between OCBs and this index, based on the opinion of an international expert panel[47], the k-index has been included as an equivalent tool to oligoclonal bands in the recent 2024 revision of the McDonald criteria for the diagnosis of multiple sclerosis[51]. In this regard, a recent systematic review and meta-analysis[52], subsequently confirmed and incorporated in

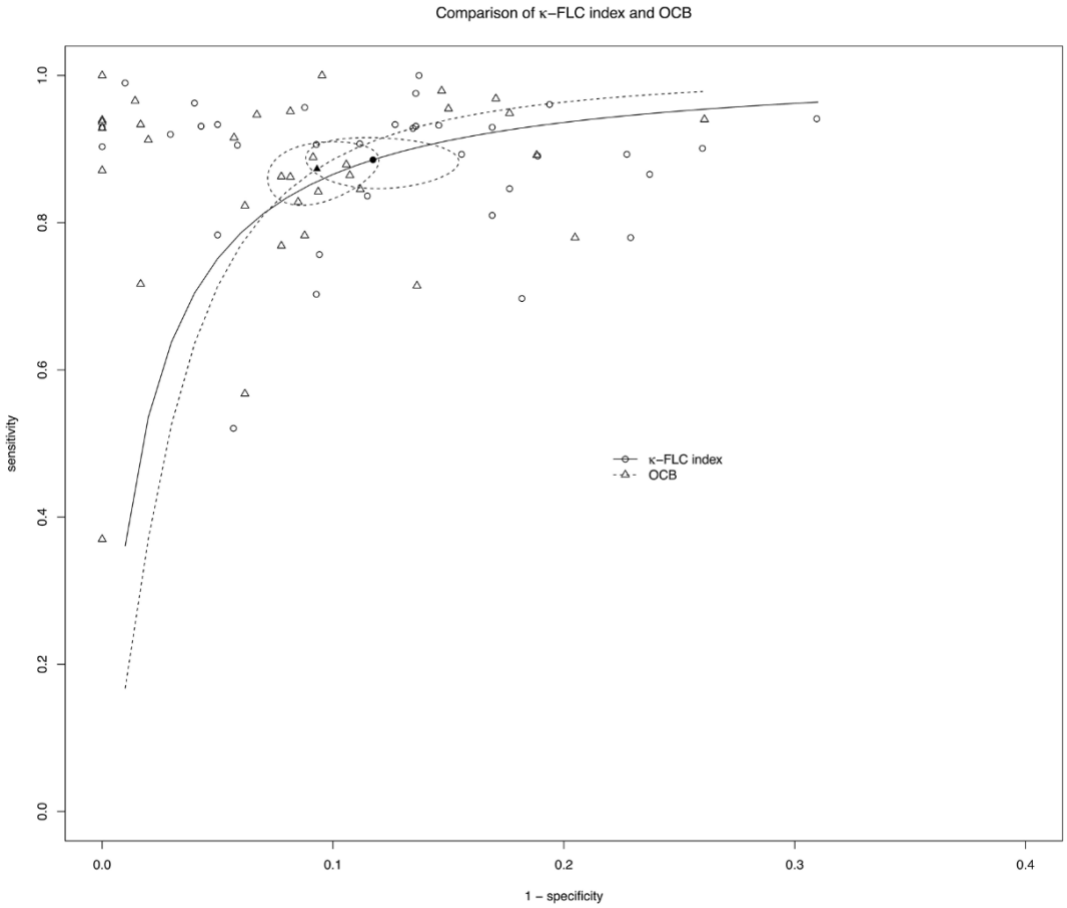

Fig. 3 – ***Comparison of the diagnostic accuracy of κ-index with OCBs to identify MS patients****. κ-FLC index, κ-index free light chains index; OCBs, oligoclonal bands. From Harald et al. 2012*[49]

the 2024 revision of the McDonald criteria and related commentary[53], proposed a diagnostic cut-off of 6.1 for the κ-index, showing sensitivity and specificity around 90%. However, as highlighted by Deisenhammer et al.[53], this threshold should be locally validated to account for methodological variability, and alternative laboratory-specific cut-offs, such as the value of 6.4 derived from the Italian multicenter study by Bernardi et al.[54], may be applied accordingly. Despite this important update on the use of the k-index in the diagnosis of multiple sclerosis, it should be emphasized that, as with OCBs, this value reflects the intrathecal production of antibodies also present in other diseases (including NMOSD and MOGAD[55,56]). The k-index value must therefore be interpreted in the clinical, laboratory and neuroradiological context of each patient.

κ-index prognostic value has been studied to a much lesser extent. In general, the role of immunoglobulins produced in the CNS in MS pathogenesis and progression is

largely unknown, although some studies have shown that they may have a pathogenic role and contribute to disease worsening[57–59]. While prognostic studies on OCBs have shown inconsistent results as predictors of MS progression[60,61], evidence of a possible prognostic role for the k-index has been accumulating over the last few years. These studies can broadly be divided into two main categories based on their objectives. The first group includes investigations aimed at determining whether the κ-FLC index can predict the occurrence and timing of a second demyelinating event, that is, the conversion from clinically isolated syndrome to clinically definite multiple sclerosis. The second group has focused on the association between κ-FLC index values and markers of inflammatory activity in the early years beyond disease onset, as well as on cumulative disability accrual over this period.

Specifically, higher κ-FLC index values have been observed in individuals carrying the HLA-DRB1*15:01 allele and were found to correlate positively with the number of CSF mononuclear cells, IgG index, and CSF concentrations of CXCL13 and CSF NfL[62]. In addition, a higher κ-index has been associated with a greater brain T2-lesion burden on baseline MRI[63]. Elevated κ-index values have further been linked to an increased risk of clinical relapses[64], progression independent of relapse activity (PIRA) [65] and a greater likelihood of treatment escalation during follow-up[66]. In particular, one longitudinal study showed that a κ-index determined at disease onset predicted both time to relapse and time to disability accrual independently of other well-established prognostic factors, using a cut-off value of 100[67]. Similarly, patients who developed new infratentorial or spinal cord lesions during follow-up exhibited higher baseline κ-FLC index values, and an index ≥100 was significantly associated with the subsequent development of such lesions[68]. However, not all studies have confirmed the prognostic value of the κ-FLC index. A small case–control study with approximately two decades of follow-up found no difference in κ-FLC index between patients with benign and aggressive MS phenotypes. Nevertheless, this analysis included only 20 benign and 15 aggressive cases and was likely affected by survivor bias, limiting the strength of its conclusions[69].

Despite this growing evidence, the results currently available are difficult to compare due to differences in study design, inclusion criteria and κ-index cut-off values used. Furthermore, it is not known whether the predictive value of the κ-index is also valid

in progressive forms of MS and whether it can predict the response to the different types of DMTs currently available.

In view of this and the fact that data on the prognostic value of the κ-index remain limited, further studies are needed in order to translate these preliminary data into clinical practice.

Personalized medicine in Multiple Sclerosis

The identification of blood and CSF biomarkers is necessary not only for a better prediction of disability outcomes, but also to help choose, among the many DMTs currently available, the most appropriate therapy for an individual pwMS. A better understanding of the individual pwMS disease profile, prognosis, treatment response, and adverse events is crucial to develop the concept of personalized medicine in MS, looking at individual patients instead of patient populations. Indeed, MS is well suited to personalized treatment because of the heterogeneity of its clinical features[2]. Furthermore, a recent approach to treatment using early high efficacy treatments versus the more traditional approach in which treatment is scaled up progressively to prevent the accumulation of disability in patients with aggressive forms has generated the need for prognostic markers[70,71]. Validation and incorporation of biomarkers in prognostication, including also the prediction of response to therapy, would allow the development of models to optimize initial treatment choice while reducing the risk of adverse events, of disability accumulation and the consequent costs related to disease progression[72].

Currently, there are no data on the value of the κ-index in predicting response to therapy and, therefore, on its usefulness in the decision-making process when choosing the initial DMT, close to diagnosis. Reflecting ongoing intrathecal B-cell activity, κ-index value is potentially informative on the extent of B-cell participation in the MS pathogenetic process on an individual level in pwMS. According to this hypothesis, the best candidates for anti-B DMTs (e.g., anti-CD20) could be identified a priori, based on the κ-index value. As a result, neurologists could rely on this biomarker for a strategic approach for therapeutic decisions and individual disease management.

OBJECTIVES

The main objective of the study is to verify the prognostic role of the κ-index obtained at diagnostic lumbar puncture (LP) by evaluating its relationship with disease activity during follow-up in pwMS.

The secondary objectives are:

- to study the association between κ-index value and clinical, laboratory and radiological features at MS onset.
- to evaluate the association between disease activity and clinical, radiological and laboratory characteristics (including κ-index), collected during follow up in clinical practice.
- to assess whether baseline κ-index is differentially associated with the risk of NEDA-3 loss across RRMS patients treated with anti-CD20, other moderate-high efficacy DMTs or first line therapies.
- To explore the combined prognostic value of baseline κ-index and sNfL levels in predicting disease activity in pwMS.

MATERIALS AND METHODS

Based on prospectively acquired data, this was a retrospective and prospective observational longitudinal study conducted on patients referred to the UOSI Riabilitazione Sclerosi Multipla, IRCCS Istituto delle Scienze Neurologiche, Bologna, Italy.

Study population

From June 1st, 2017, to July 31st, 2025, all consecutive patients older than 18 years undergoing a diagnostic workup for MS, including lumbar puncture (LP) were recruited. Patients were included in the study according to the following criteria:

- Inclusion criteria:
    - patients suspected to have MS referred to our center undergoing diagnostic LP during the diagnostic work-up with subsequent diagnostic confirmation according to the 2017 McDonald criteria[11]
    - minimum follow up of one year after LP
    - ability to understand and sign the informed consent
- Exclusion criteria:
    - neurological and/or psychiatric comorbidities
    - comorbidities severely impacting on the EDSS score[73]
    - severe systemic diseases (e.g., cardiac, renal, hepatic etc.)
    - high-dose corticosteroid therapy within the 4 weeks preceding the diagnostic LP, considering the documented transient increase in κ-index values following corticosteroid administration[74]

The time from onset of the disease to LP was not consider as an exclusion criterion.

Study procedures

Patients who fulfilled the selection criteria of the study were divided into a retrospective and a prospective population based on the timing of recruitment. Patients of the first group were enrolled retrospectively at a follow up visit, while those of the second group were included at the time of MS diagnosis.

By reviewing medical records, clinical, treatment, laboratory (including κ-index and sNfL, if available) and MRI data (see "*Collected data*") were collected at each visit performed according to clinical practice every 6-12 months. For patients of the retrospective group who underwent LP before 2019 (the year in which κ-index determination was implemented in our center's clinical practice) and of whom paired CSF and serum samples were retained, the κ-index at the time of LP was determined retrospectively.

Based on these data, at each follow-up, the possible maintenance of so-called "No Evidence of Disease Activity" (NEDA-3)[75] was assessed for both retrospective and prospective population, as required by clinical practice. No evidence of disease activity is defined as:

- Absence of clinical relapse, defined as neurological signs and symptoms lasting at least 24 h that could not be explained by another cause[11]
- Absence of confirmed disability worsening (CDW) sustained for at least 6 months, defined as a significant EDSS increase:
    - < 1.5, if baseline EDSS = 0
    - < 1, if baseline EDSS ≥ 1
    - < 0.5, if baseline EDSS ≥ 5.5
- Absence of new or enlarged lesions in T2-weighted sequences and/or gadolinium-enhancing lesions in T1-weighted sequences in follow up MRI[75]

Exclusively for prospectively enrolled patients, a blood sample was collected at each follow up visit to determine sNfL levels. In this way, for this subgroup of patients, it was possible to assess the maintenance of NEDA+, defined specifically for this study as follows: no relapses, no progression, no MRI activity, sNFL z-score ≤ 1.5 (obtained adjusting sNfL levels for age and BMI[33]).

The primary outcome of the study was disease activity, which can be defined as a change in NEDA-3 or NEDA+ status from "no activity" to "disease activity".

Collected data

All data were pseudonymized and collected using an ad-hoc database on REDCap platform, ensuring standardized and secure data collection.

For all included patients, the following data were recorded at baseline:

- Demographic data: age, sex.
- Clinical data: date and type of symptoms of MS onset, date of MS diagnosis, MS clinical phenotype, comorbidities.
- Treatment data: steroid use before the diagnostic workup, type of DMT and start date.
- Laboratory data: time from MS onset to blood and CSF sampling, CSF protein, CSF glucose, CSF white blood cell counts, IgG index, albumin quotient, OCBs, κ-index, sNfL.
- MRI data: number and location of T2 lesions on the index brain and spinal cord MRI, presence of gadolinium-enhancing T1-weighted lesion on index MRI. The baseline MRI was defined as the scan performed closest to the onset of clinical symptoms.

At every follow-up visit, the following data were recorded: date, EDSS, clinical attack occurrence suggestive of MS, presence of MRI activity (new or enlarged T2 lesions, presence of gadolinium-enhancing lesions), and the initiation of disease-modifying treatment. In addition to these, sNfL levels were also recorded for prospective patients.

Analyses of intrathecal immunoglobulin synthesis

Following lumbar puncture, serum and CSF samples were centrifuged at 3000g and 2000g, respectively, for 10 minutes and stored at -80°C until analysis.

Serum and CSF levels of κ-FLC and albumin and, thus, the calculation of κ-index were determined using the Optilite® turbidimetric analyzer (The Binding Site Ltd, Birmingham, UK). The κ-FLC was measured with the Freelite Mx™ kit according to manufacturer's instructions. The κ-index was calculated using the equation [(CSF κ-FLC/serum κ-FLC)/(CSF albumin/serum albumin)].

All analyses were performed at the Laboratorio Unico Metropolitano in Bologna, Italy by board-certified laboratory technicians.

Analyses of serum neurofilament light chains levels

sNfL concentrations, in the entire sample cohort, were determined with the Single molecule array (Simoa) technology on a Simoa SR-X instrument (Quanterix, Billerica, MA, United States) using the commercially available NF-light advantage kit

(Quanterix). The mean intra- and inter-assay coefficients of variation (CVs) were below 15%.

Statistical analysis

Quantitative variables were reported as mean and standard deviation or as median and interquartile range, as appropriate. Categorical variables were expressed as absolute frequencies and percentages. The normality of distributions was assessed using the Shapiro-Wilk test, and homoscedasticity of normally distributed variables was evaluated with Bartlett's test. Correlations among continuous variables were examined using Pearson's or Spearman's coefficients, as appropriate. Associations between categorical and continuous variables were tested using Student's *t*-test, Mann-Whitney *U* test, one-way ANOVA, or Kruskal-Wallis test. When the assumption of homoscedasticity was violated, the Welch correction was applied to parametric tests.

The primary endpoint was time to loss of NEDA-3 status, calculated from either lumbar puncture or treatment initiation, depending on the analysis. Survival curves were estimated using the Kaplan-Meier method, and hazard ratios (HR) with 95% confidence intervals (95% CI) were obtained through Cox proportional hazards regression models. Patients were censored at the last available follow-up, transfer to another center, or death from unrelated causes. When multiple observations per individual were available, standard errors were corrected using a cluster-robust sandwich estimator. Univariate Cox regression analyses were first performed to explore the association between baseline variables and the risk of NEDA-3 loss, separately for RRMS and PPMS patients. Variables with a p-value ≤ 0.15 were subsequently entered into multivariate models, with a mild L2 penalization (penalizer = 0.1) applied to improve numerical stability and manage potential collinearity. To ensure a complete dataset for regression analyses, missing values were handled by simple imputation (mode for some categorical variables, mean for continuous variables); in specific cases, categorical missing values were imputed based on domain knowledge - for example, missing MRI lesions data were interpreted as absence of lesions.

Serum neurofilament light chain (sNfL) values were normalized for age and body mass index according to Benkert et al. (https://shiny.dkfbasel.ch/baselnflreference/) and

expressed as z-scores. Both κ-index and sNfL were analyzed as continuous and dichotomized variables. For the κ-index, dichotomization was performed according to the 6.4 cut-off derived from a multicenter Italian diagnostic study, previously proposed prognostic thresholds (100[64,65], 106[63], 130[66]), and data-driven thresholds identified through maximally selected rank statistics (MSRS). For sNfL, a threshold of 1.5 z-score derived from the literature[35] and an exploratory cut-off of 2.0 z-score were tested, along with the data-driven MSRS threshold.

A univariate Cox regression analysis was conducted to explore the non-linear association between the κ-index and the risk of NEDA-3 loss by modeling the regressor as cubic B-splines[76]. This flexible approach was adopted in line with recent methodological recommendations, which emphasize that overly restrictive linear assumptions may reduce predictive performance in survival analysis[77]. To assess the joint association between baseline κ-index and sNfL z-score and the risk of NEDA-3 loss over time from lumbar puncture, proportional hazards models with flexible spline terms were also implemented. The κ-index was modeled as a B-spline continuous variable, while the sNfL z-score was included as a linear covariate based on its previously observed associations with the outcome. The proportional hazards assumption was verified using Schoenfeld residuals. Competing spline complexities (degrees of freedom 3-6) were compared using partial Akaike Information Criterion (AIC), likelihood ratio tests, and concordance index (C-index) to identify the optimal balance between model fit and parsimony. The overall non-linear effect of the κ-index was evaluated by comparing the B-spline model with its linear counterpart via likelihood ratio testing.

Additional Cox regression analyses were conducted within the RRMS subgroup to assess baseline predictors of the individual NEDA-3 components (clinical relapses, MRI activity, and progression independent of relapse activity, PIRA) and separately for the pre-treatment and post-treatment periods. In post-DMT analyses, patients were stratified by therapy type: first-line DMTs (interferons, glatiramer acetate, dimethyl fumarate, teriflunomide, or no treatment) and moderate-to-high efficacy DMTs (sphingosine-1-phosphate receptor modulators, cladribine, natalizumab, anti-CD20 therapies, and alemtuzumab). Analyses were repeated within treatment subgroups to evaluate whether predictors of NEDA-3 loss differed according to therapeutic efficacy.

ROC curve analysis and a bivariate grid search based on the Youden index were used to identify optimal cutoff values of k-index and sNFL z-score associated with the absolute risk of NEDA-3 loss. The association between κ-index and NEDA-3 status was evaluated using point-biserial correlation, comparing anti-CD20 with other groups of therapies through Fisher's z transformation.

All analyses were performed using Python (packages *lifelines*, *scipy*, *numpy*, *matplotlib*) and R (for maximally selected rank statistics). A two-sided p-value < 0.05 was considered statistically significant.

Ethical consideration

The study was conducted following good clinical practice (GCP) guidelines, incorporating the Declaration of Helsinki and it has been approved by the local Ethics Committee (Comitato Etico Area Vasta Emilia Centro, CE-AVEC, code CE23043; protocol number 0052580, SIRER ID 5903). Written informed consent was obtained from all patients.

RESULTS

Baseline characteristics of the study population

The study patient cohort consisted of 242 pwMS (155 female, 64%), including 194 RRMS, 23 PPMS, 16 CIS, 7 RIS and 3 SPMS (Fig. 4). 192 patients were enrolled retrospectively, while 50 patients were included in the prospective population. Overall, the median age at symptom onset was 34 years. Most of the patients presented with myelitis (51.2%), followed by optic neuritis (22.3%), brainstem (20.7%) and supratentorial symptoms (5%); 2 patients (0.8%) presented with multifocal onset. Median EDSS was 1.5 at the time of the lumbar puncture. Median disease duration prior to LP was 6.5 months [3;22]. Forty-one patients (17.1%) had been treated with intravenous pulse steroids before LP, but none in the previous 4 weeks. No patients were on immunomodulatory therapy at baseline. At baseline MRI, 106 patients (43.8%) had ≥9 lesions on brain MRI, with 73 patients (33.2%) with one or more gadolinium-enhancing lesions. Considering spinal cord MRI at baseline, 151 (77.1%) showed spinal cord involvement, of which 33 (18.6%) with active lesions. Regarding laboratory data, the median baseline sNfL z-score was 0.92, while median CSF white blood cells were 3/mm^3, with a median IgG index of 0.68. CSF-restricted oligoclonal bands (OCB) were present in 211 patients (87.2%), with a median κ-index of 31.98 [13.02;92.49]. Positive OCB and κ-index (binarized according to the 6.4 diagnostic cutoff[54]) were concordant in 196 patients (81%). Of the 46 remaining patients (19%), 13 of them (5.4%) had a negative κ-index with positive OCB, 9 (3.7%) had positive κ-index with negative OCB and 22 (9.1%) had negative κ-index and negative OCB.

Table 1 shows baseline data for the entire population and for the RRMS and PPMS subgroups. Patients diagnosed with CIS, RIS and SPMS were excluded from the analyses due to the small sample size.

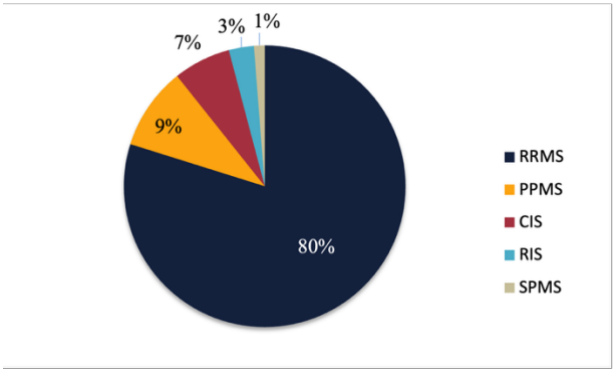

Fig. 4 - ***Distribution of phenotypes in the study population.***
RRMS: relapsing-remitting multiple sclerosis; PPMS: primary progressive multiple sclerosis; CIS: clinically isolated syndrome; RIS: radiologically isolated syndrome; SPMS: secondary progressive multiple sclerosis

Table 1 – Baseline demographic, clinical, laboratory and MRI data

Demographic and clinical data

	Total population (n=242)	RRMS (n=194)	PPMS (n=23)
Sex (female), N (%)	155 (64)	125 (64.4)	14 (60.9)
Retrospective group	192	157	18
Prospective group	50	37	5
Age (y) at MS onset, median [IQR]	34 [28;42]	34 [27;41.75]	44 [39;49.5]
Type of clinical disease onset (monofocal), N (%)			
Optic neuritis	54 (22.3)	49 (25.3)	0 (0)
Supratentorial symptoms	12 (5)	11 (5.7)	1 (4.3)
Brainstem symptoms	50 (20.7)	44 (22.7)	1 (4.3)
Myelitis	124 (51.2)	93 (47.9)	21 (91.3)
Steroid use before LP, N (%)	41 (17.1)	35 (18.2)	3 (13)
Time of disease duration at LP (m), median [IQR]	6.5 [3;22]	6 [3;15]	33 [22;43]
EDSS at the time of LP, median [IQR]	1.5 [1;2]	1.5 [1;2]	3.5 [2.75;4.75]

Baseline MRI data

	Total population (n=242)	RRMS (n=194)	PPMS (n=23)
Baseline MRI, N (%)			
Brain MRI without Gd enhancement	22 (9.1)	15 (7.7)	3 (13)
Cervical spinal cord MRI without Gd enhancement	19 (7.9)	12 (6.2)	2 (8.7)

Dorsal spinal cord MRI without Gd enhancement	12 (5.0)	7 (3.6)	1 (4.4)
Brain MRI with Gd enhancement	220 (90.9)	178 (91.8)	20 (87)
Cervical spinal cord MRI with Gd enhancement	177 (73.1)	140 (72.2)	20 (87)
Dorsal spinal cord MRI with Gd enhancement	124 (51.2)	90 (46.4)	16 (69.6)
No. of total T2 brain lesions, N (%)			
0	5 (2.1)	2 (1.0)	0 (0)
1-2	18 (7.4)	13 (6.7)	0 (0)
3-8	53 (21.9)	44 (22.7)	2 (8.7)
>=9	106 (43.8)	87 (44.8)	13 (56.5)
Unknown	60 (27.5)	48 (24.7)	8 (34.8)
No. of periventricular T2 lesions, N (%)[a]			
0	11 (6.0)	7 (4.8)	0 (0)
1-2	29 (15.9)	23 (15.8)	0 (0)
>=3	88 (48.4)	75 (51.4)	10 (66)
Unknown	54 (29.7)	41 (28.1)	5 (33)
No. of iuxta/cortical T2 lesions, N (%)[a]			
0	58 (31.9)	47 (32.2)	2 (13.3)
1	10 (5.5)	9 (6.2)	1 (6.7)
>1	65 (35.7)	54 (37.0)	8 (53.3)
Unknown	49 (26.9)	36 (24.6)	4 (26.7)
No. of infratentorial T2 lesions, N (%)[a]			
0	49 (26.9)	39 (26.7)	3 (20)
1	34 (18.7)	28 (19.2)	0 (0)
>1	81 (44.5)	66 (45.2)	12 (80)
Unknown	18 (9.9)	13 (8.9)	0 (0)
No. of patients with brain Gd-enhancing lesions, N (%)[b]	73 (33.2)	66 (37.1)	2 (10)
No. of brain Gd-enhancing lesions, median [IQR][b]	1 [1;3]	1 [1;3]	2 [1.5;2.5]
No. of total T2 spinal cord lesions, N (%)[c]			
0	43 (21.9)	31 (16)	4 (17.4)

1-2	85 (43.4)	71 (36.6)	5 (21.7)
>=3	66 (33.7)	48 (24.7)	13 (56.5)
Unknown	2 (1.0)	44 (22.7)	0 (0)
No. of cervical T2 spinal cord lesions, N (%)[d]			
0	14 (7.1)	10 (6.5)	2 (11.8)
1-2	82 (41.8)	71 (45.8)	4 (23.5)
>=3	49 (25.0)	34 (21.9)	10 (58.8)
Unknown	51 (26.0)	40 (25.9)	1 (5.9)
No. of dorsal T2 spinal cord lesions, N (%)[e]			
0	30 (15.5)	27 (25.7)	2 (11.8)
1-2	53 (27.3)	44 (41.9)	4 (23.5)
>=3	20 (10.3)	10 (9.5)	7 (41.2)
Unknown	91 (46.9)	24 (22.9)	4 (23.5)
No. of patients with spinal Gd-enhancing lesions, N (%)[f]	33 (18.6)	32 (22.4)	1 (5)
No. of spinal Gd-enhancing lesions, median [IQR]	1 [1;1]	1 [1;1]	1 [1;1]

Laboratory data

	Total population (n=242)	**RRMS (n=194)**	**PPMS (n=23)**
CSF white blood cell count (/mm^3), median [IQR]	3 [2;7]	3.5 [2;8]	2.5 [1.25;4]
CSF proteins (mg/dl), median [IQR]	39 [31;48.25]	39 [31;46.25]	43 [37;51]
Albumin quotient, median [IQR]	5.1 [3.7;628]	5.05 [3.62;6.2]	5.7 [4.35;7.15]
IgG index, median [IQR]	0.7 [0.6;1]	0.7 [0.59;0.97]	0.72 [0.58;0.95]
Intrathecal OCB, N (%)			
Pattern 1	27 (11.2)	13 (6.7)	0 (0)
Pattern 2	160 (66.1)	136 (70.1)	16 (69.6)
Pattern 3	51 (21.1)	42 (21.6)	7 (30.4)
Pattern 4	4 (1.7)	3 (1.5)	0 (0)

Pattern 5	0 (0)	0 (0)	0 (0)
κ-index, median [IQR]	31.98 [13.02;92.49]	34.86 [17.13;98.76]	28.22 [16.31;54.92]
OCB - κ-index concordance, N (%)			
Positive OCB, positive κ-index	196 (81%)	167 (86.1%)	77 (91.7%)
Positive OCB, negative κ-index	13 (5.4%)	9 (4.6%)	3 (3.6%)
Negative OCB, positive κ-index	9 (3.7%)	5 (2.6%)	2 (2.4%)
Negative OCV, negative κ-index	22 (9.1%)	11 (5.7%)	2 (2.4%)
sNfL z-score, median [IQR]	0.92 [-0.18;1.8]	0.96 [0.05;2.0]	0.06 [-1.02;1.08]

MS: multiple sclerosis; LP: lumbar puncture; Gd: gadolinium; EDSS: expanded disability status scale; CSF: cerebrospinal fluid; OCB: oligoclonal bands; sNfL: serum neurofilament light chain.

[a]*Calculated on patients with known number of total T2 brain lesions (n =182 for total population, n =146 for RRMS group, n = 15 for PPMS group).*

[b]*Calculated on patients who underwent a brain MRI with Gd (n =220 for total population, n=178 for RRMS group, n = 20 for PPMS group).*

[c]*Calculated on patients who underwent a spinal cord MRI (n =196 for total population, n=155 for RRMS group, n = 22 for PPMS group).*

[d]*Calculated on patients who underwent a cervical spinal cord MRI (n=194 for total population, n=155 for RRMS group, n = 17 for PPMS group).*

[e]*Calculated on patients who underwent a dorsal spinal cord MRI (n=194 for total population, n=105 for RRMS group, n = 17 for PPMS group).*

[f]*Calculated on patients who underwent a spinal cord MRI with Gd (n=177 for total population, n=143 for RRMS group, n = 20 for PPMS group).*

Correlation between κ-index and clinical, laboratory and radiological baseline variables

With reference to the entire study population, analysis of the relationship between the κ-index and other baseline variables showed a weak negative correlation with age at MS onset ($r = -0.145$, $p = 0.025$).

Considering neuroradiological findings, patients with higher κ-index values showed a significantly higher number of total T2 lesions in the brain ($p = 0.027$), periventricular ($p < 0.001$), infratentorial ($p = 0.008$) and spinal lesions ($p = 0.033$) on MRI used for the diagnosis of MS; moreover, a moderate positive correlation emerged between the κ-index and the number of spinal cord lesions with Gd-enhancement ($r = 0.486$, $p = 0.005$), while the association with the number of gadolinium-enhancing brain lesions showed a trend toward statistical significance ($p = 0.06$).

With regard to CSF parameters, the κ-index correlated moderately and positively with CSF white blood cell count ($r = 0.367$, $p < 0.001$), strongly with IgG index ($r = 0.848$, $p < 0.001$), and was significantly associated with OCB positivity ($p < 0.001$). A weak, non-significant correlation was observed between κ-index and baseline sNfL z-score ($r = 0.114$, $p = 0.098$).

Within the RRMS subgroup, a statistically significant association was confirmed between the κ-index value and the number of periventricular ($p = 0.012$), infratentorial ($p = 0.046$) and medullary ($p = 0.017$) T2 lesions at baseline MRI, as well as a moderate positive correlation with the number of Gd-enhancing spinal lesions ($r = 0.482$, $p = 0.006$). However, no significant association was found with the total number of brain lesions. Regarding CSF findings, a moderate positive correlation was also found in this subgroup between the κ-index and CSF white blood cell count ($r = 0.35$, $p < 0.001$), a strong positive correlation with the IgG index ($r = 0.864$, $p < 0.001$) and a significant association with OCB positivity ($p=0.004$). In addition, a weak negative correlation was found between the κ-index and albumin quotient ($r = -0.144$, $p = 0.046$), with no evidence of a meaningful correlation with basal sNfL z-score ($r = 0.082$, $p = 0.289$).

In the subset of patients with PPMS, a strong negative correlation was found between κ-index and age at MS onset ($r = -0.606$, $p = 0.002$). No significant associations were detected with neuroradiological characteristics, although a trend towards statistical significance was observed between the κ-index and the number of iuxta/cortical T2

lesions. Moderate positive correlations with CSF white blood cell count ($r = 0.469$, $p = 0.028$) and strong correlations with IgG index ($r = 0.556$, $p = 0.006$) were confirmed. In this subgroup, it was not possible to perform some association analyses due to the small sample size.

The results of univariate analysis between κ-index and the different baseline variables are listed in Table 2.

Table 2 - Univariate associations between κ-index and baseline clinical, radiological and laboratory characteristics

	Total population			RRMS			PPMS		
Covariate	***Results***	***p-value****	***N***	***Results***	***p-value****	***N***	***Results***	***p-value****	***N***
Sex									
Male	32.11 [10.88;93.5]	0.964	87	34.64 [12.58;99.26]	0.91	69	28.22 [26.21;41.28]	0.449	9
Female	31.98[15.12;90.15]		155	35.25 [17.92;97.95]		125	27.56 [16.02;63.25]		14
Age at MS onset	-0.145	**0.025**	242	-0.122	0.092	194	-0.606	**0.002**	23
Disease duration prior LP	-0.017	0.795	234	-0.031	0.668	191	-0.175	0.424	23
Type of clinical disease onset									
Optic neuritis	30.66 [7.59;94.21]	0.315	54	34.64 [11.4;98.24]	0.251	48	NA	NA	0
Supratentorial symptoms	45.61 [17.37;164.9]		12	54.0 [24.43;183.86]		11	3.23 [3.23;3.23]		1
Brainstem symptoms	30.92 [17.51;65.56]		50	31.46 [18.07;65.56]		42	NA		0
Myelitis	32.92 [16.6;102.76]		124	36.75 [17.69;108.8]		93	29.64 [18.01;57.8]		22
No. of brain T2 lesions at baseline									
0	16.09 [8.84;26.31]	**0.027**	5	92.38 [54.24;130.5]	0.155	2	NA	NA	0
1-2	24.06 [9.79;53.37]		18	30.06 [21.36;88.86]		13	NA		0
3-8	22.76 [7.42;51.46]		53	23.79 [9.84;67.13]		44	43.66 [40.9;46.41]		2
>=9	37.22 [21.02;101.2]		106	41.66 [21.17;115.7]		87	26.21 [15.74;41.28]		13
No. of periventricular T2 lesions at baseline									
0	11.08 [0.0;19.36]	**<0.001**	11	17.35 [11.5;37.78]	**0.012**	7	NA	NA	0
1-2	26.75 [12.18;41.39]		29	29.09 [16.99;46.14]		23	NA		0
>=3	39.09 [20.33;118.8]		88	40.7 [19.98;126.41]		75	28.64 [18.01;39.16]		10
No. of iuxta/cortical T2 lesions at baseline									
0	23.66 [9.15;57.31]	0.513	58	30.06 [15.45;86.12]	0.538	47	9.48 [6.36;12.61]	0.067	2
1	40.24 [32.89;97.5]		10	41.39 [36.75;101.2]		9	21.39 [21.39;21.39]		1

>1	32.73 [15.96;97.86]		65	29.27 [10.06;101.3]		53	35.47 [29.85;68.46]		8
No. of infratentorial T2 lesions at baseline									
0	23.79 [8.8;48.04]		49	30.06 [12.27;77.47]		39	16.88 [10.06;19.13]		3
1	26.76 [6.85;75.14]	**0.008**	34	32.92 [8.82;101.25]	**0.046**	28	NA	NA	0
>1	36.82 [21.36;101.2]		81	35.86 [21.1;109]		66	35.47 [23.6;52.9]		12
Brain Gd-enhancing lesions									
Yes	36.82 [18.42;105.4]	0.06	73	35.86 [18.64;100.6]	0.315	66	190.3 [108.5;272.0]	0.442	2
No	31.36 [13.01;89.23]		145	32.86 [16.86;94.42]		112	31.92 [14.56;57.8]		18
No. of brain Gd-enhancing lesions	-0.148	0.243	64	-0.201	0.134	57	0.006	0.976	27
No. of total T2 spinal cord lesions									
0	23.76 [5.03;53.7]		43	23.76 [11.63;53.7]		31	27.22 [20.46;37.2]		4
1-2	33.67 [17.6;92.49]	**0.033**	85	34.86 [18.99;93.5]	**0.017**	71	24.06 [14.16;41.28]	0.412	5
>=3	53.4 [19.5;122.02]		66	64.9 [20.51;130.22]		48	32.79 [21.39;60.67]		13
No. of cervical T2 spinal cord lesions									
0	29.22 [14.2;118.96]		14	29.22 [18.35;156.1]		10	66.56 [40.36;92.76]	0.948	2
1-2	35.25 [18.94;93.5]	0.568	82	36.75 [19.41;93]	0.302	71	32.67 [19.6;68.46]		4
>=3	40.48 [17.35;119.9]		49	64.9 [19.24;139.62]		34	28.89 [17.15;36.8]		10
No. of dorsal T2 spinal cord lesions									
0	36.06 [21.45;97.7]		30	34.9 [21.54;94.98]		27	25.66 [17.85;33.47]		2
1-2	26.52 [14.72;86.98]	0.302	53	29.22 [18.05;94.04]	0.273	44	20.44 [12.17;49.78]	0.427	4
>=3	271 [26.95;127.18]		20	101.28 [34.5;153.8]		10	32.79 [26.22;94.07]		7
Spinal Gd-enhancing lesions									
Yes	33.06 [16.09;108.1]	0.818	33	33.98 [15.32;108.5]	0.982	32	26.7 [26.72;26.72]	0.900	1
No	31.21 [15.93;80.46]		147	32.52 [17.81;85]		111	31.06 [14.95;56.64]		19
No. of spinal Gd-enhancing lesions	0.486	**0.005**	32	0.482	**0.006**	31	0.545	0.103	10
EDSS at the time of LP	0.019	0.771	240	0.055	0.45	192	0.105	0.344	84

CSF white blood cell count	0.367	**<0.001**	237	0.35	**<0.001**	190	0.469	**0.028**	22
CSF proteins	-0.037	0.57	238	-0.065	0.374	190	0.041	0.852	23
Albumin quotient	-0.1	0.122	240	-0.144	**0.046**	192	0.044	0.843	23
IgG index	0.848	**<0.001**	240	0.864	**<0.001**	192	0.556	**0.006**	23
Intrathecal OCB Pattern 1, 4, 5 Pattern 2 ,3	 0.01 [0.0;7.13] 37.22 [18.99;100.4]	**<0.001**	 31 211	 4.2 [0.0;7.41] 39.22 [19.13;101.9]	**0.004**	 16 178	 NA 28.22 [16.31;54.92]	NA	 0 23
sNfL z-score	0.114	0.098	211	0.082	0.289	170	-0.065	0.787	20

Results are shown as median [IQR] or Spearman rho depending on the type of variable. MS: multiple sclerosis; LP: lumbar puncture; Gd: gadolinium; EDSS: expanded disability status scale; CSF: cerebrospinal fluid; OCB: oligoclonal bands; sNfL: serum neurofilament light chain.
** Statistically significant at a $p < 0.05$ level. Bold text indicates $p < 0.05$.*

Follow up data

Following MS diagnosis, 224 patients (92.6%) were prescribed a DMT, of whom 112 (48.8%) a moderate-high efficacy DMT (Fig. 5).

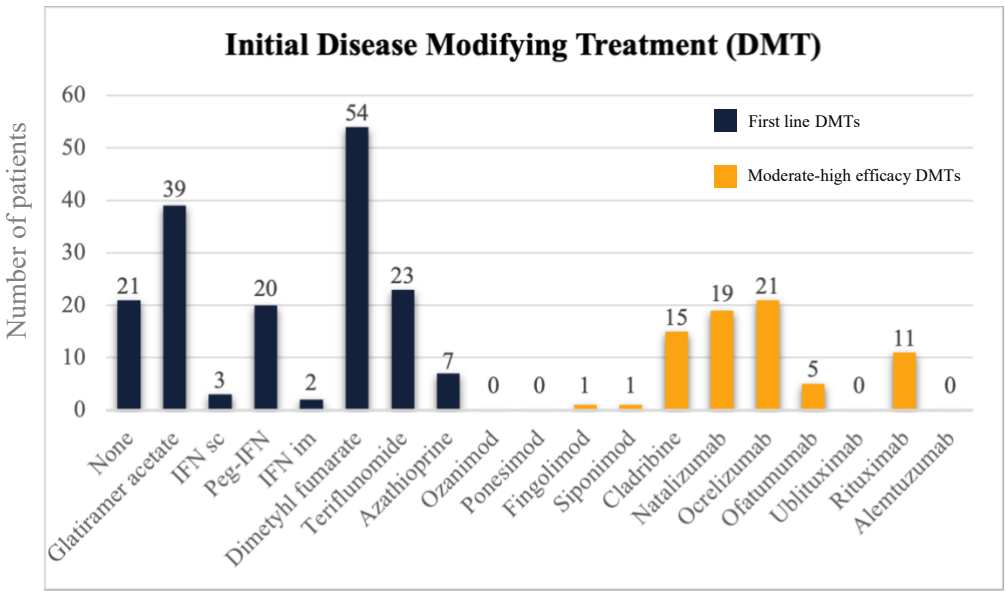

Fig. 5 - ***Type of DMTs initiated following MS diagnosis****.*

The median total follow-up time for the whole cohort was 28.0 [16.0;45.0] months, with longer follow up observed for retrospectively enrolled patients compared with the prospective cohort (median 30.0 vs 12.5 months, respectively).

Less than half of the individuals (102, 42.2%) maintained NEDA-3 status according to the definition above throughout the entire follow-up period, with a lower proportion in the PPMS subgroup (6, 26.1%). In the overall population, among those with evidence of disease activity, 36 (25.7%) experienced clinical relapses, 119 (85%) showed MRI activity (new or enlarging T2-lesions, lesions Gd-enhancing) and 32 (22.9%) progression independent of relapse activity (PIRA), defined as 6-month confirmed disability worsening. MRI activity was the main cause of NEDA-3 loss also within the RRMS (89%) and PPMS (47.1%) subgroups.

When considering only the treatment period with DMTs, thus excluding any disease activity occurring in the first 6 months after therapy initiation, 112 (46.3%) patients from the total cohort lost NEDA-3 status (48.9% and 56.5% in the RRMS and PPMS subgroups, respectively).

In the prospective cohort, given the relatively short follow-up period (median 12.5 months), 14 patients experienced NEDA+ loss, three of whom based on an sNfL z-score > 1.5. Specifically, one PPMS and two RRMS patients showed an increase in sNfL z-score > 1.5 at 6 and 12 months after DMT initiation, respectively, compared with baseline. (Fig. 6).

During follow up, 84 patients (34.7%) of the total population switched therapy, most frequently (39, 46.4%) due to persistent clinical and/or MRI disease activity.

Follow up data are shown in Table 3.

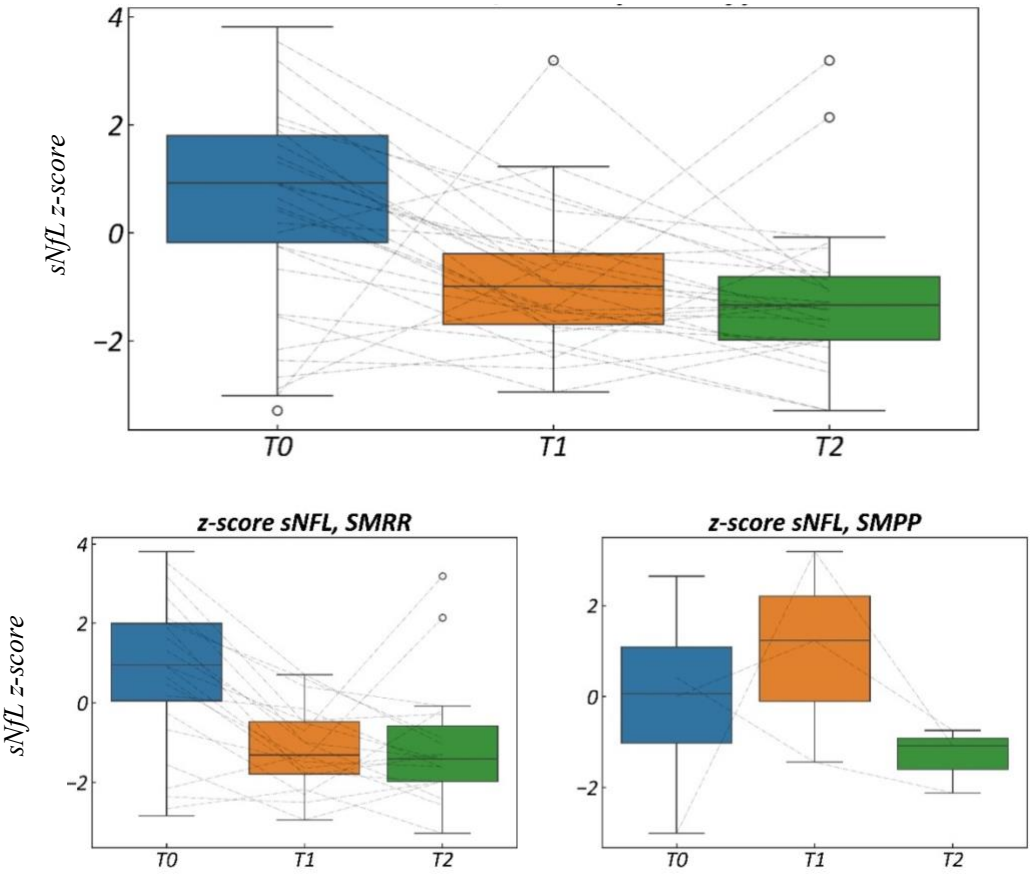

Fig. 6 ***- Longitudinal evolution of sNfL z-scores in the prospective population.*** *Top panel: overall cohort. Bottom panels: RRMS (left) and PPMS (right) subgroups. Measurements are shown at baseline (T0), 6 months (T1), and 12 months (T2) after DMT initiation. Individual trajectories are represented by grey lines.*

Table 3 - Follow up data

	Total population	RRMS	PPMS
Follow up duration (m), median [IQR]			
Total population	28.0 [16.0;45.0]	28.0 [16.0;44.0]	30.0 [17.0;46.0]
Retrospective population	30.0 [18.0;46.0]	30.0 [17.75;45.0]	33.0 [20.0;47.75]
Prospective population	12.5 [9.0;17.0]	13.0 [9.0;17.0]	13.0 [10.5;17.0]
Patients with NEDA-3 loss	25.0 [14.0;41.0]	26.0 [15.0;41.5]	17.0 [11.75;23.5]
Patients with NEDA-3 persistence	30.0 [17.0;46.0]	30.0 [17.0;45.0]	33.0 [20.0;47.0]
No. of patients prescribed a DMT, N (%)	224 (92.6)	191 (98.45)	21 (91.3)
Time from LP to DMT start (m), mean (SD)	5.92 (±8.71)	4.86 (±6.67)	9.48 (±15.5)
Initial treatment strategy, N (%)			
First line DMTs	153 (59.5)	136 (70.1)	2 (8.7)
Moderate-high efficacy DMTs	112 (48.76)	89 (46.9)	4 (17.4)
No DMTs	21 (8.68)	5 (2.58)	17 (73.9)
No. of patients with NEDA-3 loss during follow up, N (%)	140 (57.85)	109 (56.19)	17 (73.9)
Time to NEDA-3 loss after LP (m), median [IQR]	14.0 [9.0;26.5]	13.0 [9.0;28.0]	22.0 [13.0;34.0]
No. of patients with NEDA-3 loss after DMT start, N (%)	112 (46.28)	94 (48.45)	13 (56.52)
Time to NEDA-3 loss after DMT start (m), median [IQR]	8.5 [6.0;23.25]	8.0 [6.0;23.0]	19.0 [13.0;25.0]
Reason for NEDA-3 loss, N (%[a])			
Clinical relapse	36 (25.71)	31 (28.44)	2 (11.76)
MRI activity	119 (85.0)	97 (88.99)	8 (47.06)
PIRA	32 (22.86)	19 (17.43)	7 (30.43)
No. of patients with NEDA+ loss during follow up, N (%[b])	14 (28)	10 (20)	2 (4)
Clinical relapse	0 (0)	0 (0)	0 (0)
MRI activity	11 (22)	8 (16)	1 (2)
PIRA	0 (0)	0 (0)	0 (0)

sNfL z-score > 1.5	3 (6)	2 (4)	1 (2)
No. of patients who switched DMT during follow up, N (%[c])	84 (34.71)	75 (38.66)	7 (30.43)
Reason for DMT switch, N (%)			
Persistence of disease activity (clinical or MRI)	39 (46.43)	37 (49.33)	0 (0)
Progression	5 (5.95)	0 (0)	5 (71.43)
Side effects	18 (21.43)	17 (22.67)	1 (14.29)
Poor tolerability	15 (17.86)	14 (18.67)	1 (14.29)
Safety	6 (7.14)	6 (8.0)	0 (0)
Comorbidities	1 (1.19)	1 (1.33)	0 (0)

DMT = disease modifying therapies; LP = lumbar puncture; NEDA-3 = no evidence of disease activity; PIRA = progression independent of relapse activity.
a = Calculated on patients with NEDA-3 loss during follow up (n=140).
b = Calculated on prospective patients (n = 50)
c = Calculated on patients who switched DMT during follow up (n=84).

Correlation between baseline data and NEDA status during follow up

With regard to the main outcome of this study, correlations between baseline variables and NEDA-3 loss after MS diagnosis are reported in Table 4–6. Considering the differences in pathogenesis and disease course, these analyses were performed separately for RRMS and PPMS subgroups.

In RRMS patients, univariate Cox regression analysis showed that higher age at MS onset was associated with a lower risk of NEDA-3 loss during follow-up (HR = 0.98, 95% CI 0.97–0.99, p = 0.002), whereas the presence of brain Gd-enhancing lesions at diagnosis was significantly related to a higher risk (HR = 1.5, 95% CI 1.2–1.8, p < 0.001). Among laboratory variables, higher CSF white blood cell count (HR = 1.02, 95% CI 1.00–1.04, p = 0.032), κ-index > 6.4 (HR = 1.50, 95% CI 1.10–1.90, p = 0.008), and higher baseline sNfL z-score (per-unit increase; HR = 1.09, 95% CI 1.02–1.17, p = 0.014) were associated with increased hazard of NEDA-3 loss.

In an exploratory dichotomization, the literature-based threshold of 1.5 z-scores and an additional exploratory cut-off of 2.0 were tested. Only sNfL z-score > 2.0 was associated with a significantly higher risk (HR = 1.40, p = 0.002), whereas the other cut-offs were not significant. To complement this approach, maximally selected log-hazard ratio statistics (MSRS) were applied to κ-index and sNfL z-score, identifying data-driven thresholds of 6.51 (p = 0.060) and 1.98 (p = 0.006), respectively, both corresponding to higher risk of NEDA-3 loss. When the κ-index dichotomized at 6.51 was entered in the Cox regression model, its association with NEDA-3 loss remained significant (HR = 1.50, 95% CI 1.10–1.90, p = 0.008) and persisted in the multivariable model (HR = 1.29, 95% CI 1.01–1.64, p = 0.041), confirming the robustness of this threshold.

Variables with a significance level of 0.15 or lower were included in the multivariable Cox model. The presence of Gd-enhancing lesions was independently associated with a higher risk of losing NEDA-3 status (HR = 1.40, 95% CI 1.15–1.67, p = 0.001). Similarly, κ-index > 6.4 and κ-index > 6.51 both retained statistical significance in the multivariable model (HR = 1.20, 95% CI 1.00–1.38, p = 0.041 for both), confirming the robustness of this threshold. In addition, baseline sNfL z-score values above the

data-driven cut-off (1.98) and the literature-based cut-off (2.0) remained significantly associated with higher risk of NEDA-3 loss (HR = 1.16, 95% CI 1.02–1.30, p = 0.025 for both). The effect of all other variables diminished after adjustment.

In PPMS patients, univariate Cox regression identified both baseline sNfL level (HR = 0.94, 95% CI 0.90–0.99, p = 0.01) and sNfL z-score > 2.0 (HR = 0.47, 95% CI 0.24–0.90, p = 0.026) as significant predictors of NEDA-3 status change. The MSRS analysis did not identify significant thresholds for either biomarker (κ-index = 64.11, p = 0.608; sNfL z-score = –1.41, p = 0.155), consistent with the weaker associations observed in Cox models. In the multivariable analysis, only elevated sNfL z-scores (> 2.0) remained independently associated with NEDA-3 maintenance during follow-up (HR = 0.47, 95% CI 0.24–0.90, p = 0.026).

In both subgroups, κ-index as a continuous variable was not a significant predictor of NEDA-3 loss (p = 0.137 in RRMS; p = 0.442 in PPMS). Conversely, in RRMS, a positive κ-index (> 6.4 or the data-driven cut-off 6.51) consistently emerged as an independent predictor of NEDA-3 loss. Although not statistically significant, exploratory Kaplan–Meier curves were generated for the κ-index dichotomized according to different thresholds for RRMS patients (6.4, 100, 106, and 130; Fig. 7).

In the prospectively enrolled population, correlations between baseline variables and NEDA+ loss after MS diagnosis could not be evaluated due to the limited number of events (3) given the short follow-up duration.

Table 4 - Univariate Cox regression for baseline data and risk of NEDA-3 loss

Univariate	**RRMS**		**PPMS**	
	HR (95% CI)	***p-value****	***HR (95% CI)***	***p-value****
Sex (M)	1.07 (0.87;1.31)	0.551	1.34 (0.76;2.36)	0.308
Age at onset	0.98 (0.97;0.99)	**0.002**	1.01 (0.98;1.04)	0.531
Type of clinical disease onset				
Optic neuritis (yes)	0.82 (0.66;1.02)	0.078	NA	NA
Supratentorial symptoms (yes)	1.07 (0.65;1.74)	0.794	0.8 (0.6;1.08)	0.145
Brainstem symptoms (yes)	1.07 (0.87;1.33)	0.517	1.39 (0.95;2.02)	0.089
Myelitis (yes)	1.11 (0.91;1.35)	0.323	1.25 (0.93;1.68)	0.145
No. of brain T2 lesions at baseline >=9	1.23 (1.0;1.5)	**0.049**	0.86 (0.57;1.28)	0.449
Presence of brain Gd-enhancing lesions	1.5 (1.2;1.8)	**<0.001**	1.01 (0.82;1.26)	0.898
No. of total T2 spinal cord lesions >=3	0.92 (0.73;1.16)	0.456	0.84 (0.56;1.27)	0.404
Presence of spinal Gd-enhancing lesions	0.95 (0.7;1.3)	0.750	0.75 (0.53;1.05)	0.097
EDSS at the time of LP	1.02 (0.91;1.14)	0.724	1.01 (0.86;1.19)	0.901
CSF white blood cell count	1.02 (1.0;1.04)	**0.032**	0.97 (0.93;1.01)	0.156
CSF proteins	1.0 (0.99;1.01)	0.935	1.0 (0.99;1.02)	0.536
Albumin quotient	1.0 (0.96;1.05)	0.921	1.06 (0.97;1.17)	0.213

IgG index	1.1 (0.88;1.35)	0.424	0.56 (0.31;1.04)	0.065
OCB (patterns 2 and 3)	1.3 (0.93;1.84)	0.123	NA	NA
κ-index	1.0 (1.0;1.0)	0.137	1 (1;1)	0.442
ref 6.4	1.5 (1.1;1.9)	**0.008**	0.935 (0.681;1.283)	0.675
ref 6.51 (MSRS RRMS)	1.5 (1.1;1.9)	**0.008**	-	-
ref 64.11 (MSRS PPMS)	-	-	0.94 (0.7;1.3)	0.675
ref 100	1.03 (0.8;1.3)	0.786	0.63 (0.38;1.03)	0.064
ref 106	1.1 (0.84;1.38)	0.585	0.63 (0.38;1.03)	0.064
ref 130	1.05 (0.8;1.4)	0.692	0.65 (0.37;1.08)	0.095
sNfL	1.0 (0.99;1.01)	0.308	0.94 (0.9;0.99)	**0.01**
sNfL z-score	1.1 (1.02;1.17)	**0.014**	0.83 (0.69;1)	0.05
ref 1.5	1.2 (0.96;1.4)	0.112	0.61 (0.35;1.05)	0.072
ref 1.98 (MSRS RRMS)	1.4 (1.13;1.75)	**0.002**	-	-
ref 2.0	1.4 (1.13;1.75)	**0.002**	0.23 (0.06;0.8)	**0.022**

Gd = gadolinium; EDSS = Expanded Disability Status Scale; LP = lumbar puncture; CSF = cerebrospinal fluid; OCB = oligoclonal bands; MSRS = Maximally Selected Rank Statistics; sNfL: serum neurofilament light chain.
** Statistically significant at a $p < 0.05$ level. Bold text indicates $p < 0.05$.*

Table 5 - Multivariate Cox regression for baseline data and risk of NEDA-3 loss in RRMS patients

Multivariate	*HR (95% CI)*	*p-value**
Age at onset	0.99 (0.99;1.00)	0.165
Optic neuritis at disease onset	0.88 (0.7;1.08)	0.227
No. of brain T2 lesions at baseline >=9	1.1 (0.90;1.31)	0.401
Presence of brain Gd-enhancing lesions	1.4 (1.13;1.64)	**0.001**
CSF white blood cell count	1.01 (0.99;1.02)	0.499
OCB (patterns 2 and 3)	1.16 (0.9;1.5)	0.272
κ-index	1.0 (1.0;1.0)	0.395
ref 6.4	1.2 (1.0;1.38)	**0.041**
ref 6.51 (MSRS)	1.2 (1.0;1.38)	**0.041**
sNfL z-score	1.01 (0.96;1.07)	0.674
ref 1.5	0.88 (0.74;1.03)	0.112
ref 1.98 (MSRS)	1.16 (1.02;1.3)	**0.023**
ref 2.0	1.16 (1.02;1.3)	**0.023**

Table 6 - Multivariate Cox regression for baseline data and risk of NEDA-3 loss in PPMS patients

Multivariate	*HR (95% CI)*	*p-value**
Type of clinical disease onset		
Supratentorial symptoms (yes)	0.76 (0.57;1.01)	0.060
Brainstem symptoms (yes)	1.36 (0.96;1.9)	0.083
Myelitis (yes)	1.3 (0.99;1.7)	0.060
Presence of spinal Gd-enhancing lesions	0.76 (0.54;1.06)	0.102
IgG index	0.76 (0.43;1.3)	0.322
sNfL	0.97 (0.94;1.0)	0.078
sNfL z-score	0.97 (0.86;1.1)	0.619
ref 1.5	0.89 (0.59;1.3)	0.529
ref 2.0	0.47 (0.24;0.9)	**0.026**
κ-index		
ref 106	0.86 (0.58;1.29)	0.473

Gd = gadolinium; EDSS = Expanded Disability Status Scale; LP = lumbar puncture; CSF = cerebrospinal fluid; OCB = oligoclonal bands; MSRS = Maximally Selected Rank Statistics; sNfL: serum neurofilament light chain.
** Statistically significant at a p < 0.05 level. Bold text indicates p < 0.0*

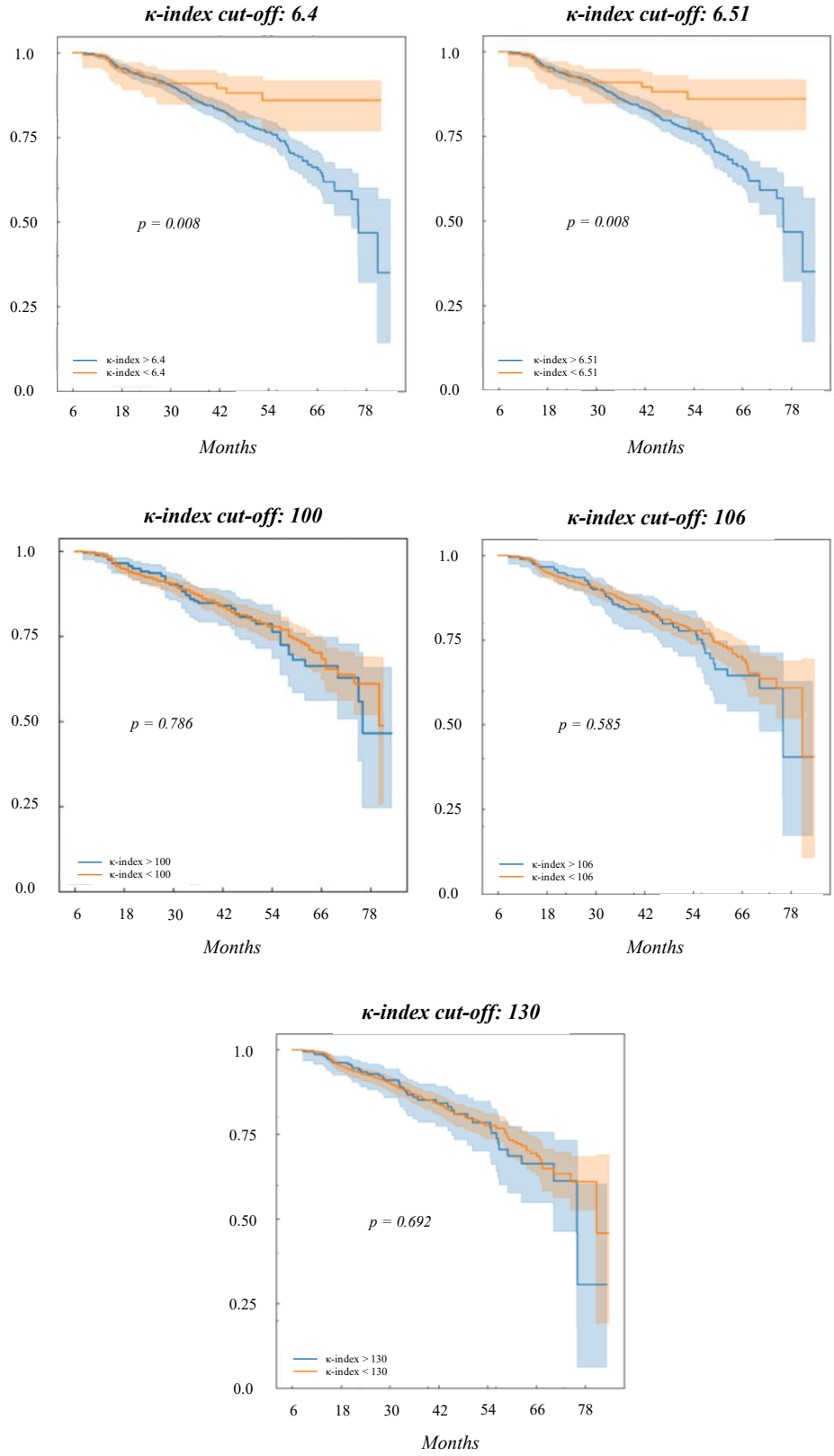

Fig. 7 - ***Kaplan–Meier curves showing the probability of NEDA-3 maintenance in RRMS patients according to different κ-index cut-offs (6.4, 6.51, 100, 106, and 130).*** *P-values are from log-rank tests comparing groups above and below each threshold. Shaded areas represent 95% confidence intervals.*

Exploratory survival analyses on NEDA-3 components and time to NEDA-3 loss in the RRMS subgroup

Given the higher number of RRMS patients and the heterogeneity of disease activity patterns, additional analyses were conducted within this subgroup to further explore baseline predictors of NEDA-3 loss and its individual components. Specifically, separate Cox regression models were performed to assess the risk of clinical relapses, MRI activity, and PIRA. Moreover, among patients who lost NEDA-3 during follow-up, baseline predictors of the time to NEDA-3 loss were investigated.

In the univariable Cox regression analysis (Table 7), several baseline variables were significantly associated with the individual components of NEDA-3, particularly with MRI activity. These included κ-index > 6.4 (HR = 1.58, 95% CI 1.18–2.10, p = 0.002) and sNfL z-score > 2.0 (HR = 1.26, 95% CI 1.01–1.60, p = 0.042). However, after adjustment for potential confounders, only the presence of gadolinium-enhancing brain lesions and albumin quotient remained independently associated with the risk of clinical relapses (HR = 2.10, 95% CI 1.20–3.60, p = 0.01, and HR = 1.20, 95% CI 1.10–1.30, p < 0.001, respectively). None of the baseline variables retained a significant association with MRI activity or PIRA in the multivariable models (Table 8).

Finally, in the subgroup of patients who experienced evidence of disease activity (EDA), a univariable analysis was performed to identify predictors of time to NEDA-3 loss (Table 9). Baseline gadolinium-enhancing brain lesions, a higher overall brain lesion load, particularly involving juxta-cortical areas, and elevated sNfL z-scores emerged as predictors of a shorter time to disease reactivation and consequently to NEDA-3 loss.

Table 7 - Univariate Cox regression for baseline data and risk of NEDA-3 components in RRMS patients

	Clinical relapse		MRI activity		PIRA	
Univariate	***HR (95% CI)***	***p-value****	***HR (95% CI)***	***p-value****	***HR (95% CI)***	***p-value****
Sex (M)	1.3 (0.73;2.3)	0.378	1.06 (0.86;1.3)	0.584	0.9 (0.77;1.06)	0.195
Age at onset	1.01 (0.98;1.04)	0.661	1.0 (0.98;1.0)	**0.03**	1.01 (1.0;1.02)	0.111
Type of clinical disease onset						
Optic neuritis (yes)	0.83 (0.41;1.69)	0.612	0.93 (0.7;1.2)	0.545	0.93 (0.78;1.1)	0.384
Supratentorial symptoms (yes)	1.5 (0.9;2.35)	0.113	1.3 (0.78;2.3)	0.295	1.05 (0.9;1.23)	0.604
Brainstem symptoms (yes)	1.3 (0.68;2.6)	0.402	0.96 (0.77;1.2)	0.695	1.06 (0.88;1.27)	0.563
Myelitis (yes)	0.87 (0.49;1.54)	0.622	1.1 (0.9;1.35)	0.368	0.99 (0.84;1.17)	0.915
No. of brain T2 lesions at baseline >=9	1.52 (0.86;2.7)	0.147	1.34 (1.1;1.64)	**0.005**	0.97 (0.82;1.13)	0.667
Presence of brain Gd-enhancing lesions	2.4 (1.36;4.1)	**0.002**	1.23 (1.1;1.6)	**0.014**	1.1 (0.94;1.3)	0.237
No. of total T2 spinal cord lesions >=3	1.1 (0.57;2.1)	0.767	0.78 (0.62;1.0)	**0.048**	1.02 (0.84;1.24)	0.863
Presence of spinal Gd-enhancing lesions	1.02 (0.62;1.7)	0.925	1.25 (0.94;1.7)	0.131	1.01 (0.78;1.3)	0.929
EDSS at the time of LP	1.01 (0.7;1.48)	0.97	0.92 (0.81;1.1)	0.227	1.02 (0.93;1.13)	0.641
CSF white blood cell count	1.02 (0.98;1.07)	0.326	1.03 (1.01;1.05)	**0.006**	0.99 (0.98;1.01)	0.238
CSF proteins	1.01 (1.0;1.03)	0.096	1.0 (0.99;1.01)	0.762	1 (0.9;1.01)	0.938
Albumin quotient	1.1 (1.01;1.16)	**0.017**	1.0 (0.96;1.04)	0.945	1.10 (0.98;1.05)	0.369
IgG index	1.6 (0.95;2.7)	0.077	0.92 (0.75;1.14)	0.468	0.91 (0.74;1.13)	0.407

OCB (pattern 2 and 3)	1.06 (0.53;2.1)	0.878	1.4 (0.85;2.4)	0.185	0.89 (0.66;1.2)	0.434
κ-index	1.0 (1.0;1.0)	0.462	1.0 (0.9;1.0)	0.532	1.0 (1.0;1.0)	0.173
ref 6.4	0.87 (0.5;1.54)	0.625	1.58 (1.18;2.1)	**0.002**	1.13 (0.9;1.4)	0.297
ref 100	1.02 (0.6;1.9)	0.958	1.07 (0.84;1.4)	0.588	0.84 (0.7;1.0)	0.053
ref 106	1.02 (0.6;1.9)	0.958	1.1 (0.82;1.36)	0.667	0.84 (0.7;1.0)	0.053
ref 130	1.01 (0.43;2.4)	0.977	1.0 (0.8;1.3)	0.993	0.82 (0.7;0.97)	**0.024**
sNfL	1.03 (1.0;1.06)	**0.023**	1.0 (1.0;1.01)	**0.015**	1.0 (1.0;1.0)	0.699
sNfL z-score	1.37 (1.14;1.66)	**0.001**	1.1 (0.97;1.15)	0.201	0.98 (0.92;1.03)	0.414
ref 1.5	1.99 (1.1;3.6)	**0.020**	1.16 (0.94;1.43)	0.171	1.03 (0.9;1.2)	0.761
ref 2.0	1.95 (1.1;3.5)	**0.022**	1.26 (1.01;1.6)	**0.042**	0.92 (0.8;1.08)	0.308

Gd = gadolinium; EDSS = Expanded Disability Status Scale; LP = lumbar puncture; CSF = cerebrospinal fluid; OCB = oligoclonal bands; sNfL: serum neurofilament light chain.
** Statistically significant at a $p < 0.05$ level. Bold text indicates $p < 0.05$.*

Table 8 - Multivariate Cox regression for baseline data and risk of NEDA-3 components in RRMS patients

Multivariate	Clinical relapses		MRI activity		PIRA	
	HR (95% CI)	*p-value**	*HR (95% CI)*	*p-value**	*HR (95% CI)*	*p-value**
Age at onset	-	-	1.0 (0.99;1.01)	0.6810	1.0 (0.99;1.0)	0.354
Type of clinical disease onset Supratentorial symptoms (yes)	1.4 (0.54;3.5)	0.517	-	-	-	-
Presence of brain Gd-enhancing lesions	2.1 (1.2;3.6)	**0.01**	1.07 (0.9;1.3)	0.436	-	-
Presence of spinal Gd-enhancing lesions	-	-	1.23 (0.99;1.5)	0.062	-	-
No. of brain T2 lesions at baseline >=9	0.62 (0.3;1.3)	0.210	1.04 (0.9;1.2)	0.643	-	-
No. of total T2 spinal cord lesions >=3	-	-	0.95 (0.8;1.13)	0.574	-	-
CSF white blood cell count	-	-	0.99 (0.98;1.01)	0.451	-	-
CSF proteins	0.99 (0.97;1.01)	0.252	-	-	-	-
Albumin quotient	1.2 (1.1;1.3)	**<0.001**	-	-	-	-
IgG index	1.56 (0.8;3.1)	0.186	-	-	-	-
κ-index						
ref 6.4	-	-	1.0 (0.8;1.25)	0.993	-	-
ref 100	-	-	-	-	1.12 (0.9;1.4)	0.284
ref 106	-	-	-	-	0.97 (0.85;1.1)	0.663
ref 130	-	-	-	-	0.8 (0.6;1.2)	0.292

sNfL	0.99 (0.96;1.03)	0.608	1.0 (1.0;1.0)	0.279	-	-
sNfL z-score	1.17 (0.9;1.5)	0.255	-	-	-	-
ref 1.5	1.17 (0.6;2.2)	0.614	-	-	-	-
ref 2.0	1.75 (0.9;3.4)	0.097	1.03 (0.93;1.14)	0.551	-	-

Gd = gadolinium; EDSS = Expanded Disability Status Scale; LP = lumbar puncture; CSF = cerebrospinal fluid; OCB = oligoclonal bands; sNfL: serum neurofilament light chain.
** Statistically significant at a p < 0.05 level. Bold text indicates p < 0.05.*

Table 9 - Univariate correlations between time to EDA and baseline clinical, radiological and laboratory characteristics

Covariate	*Results*	*p-value**	*N*
Sex			
Male	10 [8;24]	0.82	69
Female	11 [8;24.25]		125
Age at MS onset	0.065	0.503	109
Type of clinical disease onset			
Optic neuritis	33 [26.2;42.1]	0.092	4
Supratentorial symptoms	18.2 [13.7;22.6]	0.503	2
Brainstem symptoms	25.2 [17:28.9]	0.499	14
Myelitis	16.9 [14.5;31.8]	0.347	21
No. of brain T2 lesions at baseline			
0	3 [2;4]	**0.008**	2
1-2	9 [6.5;25]		13
3-8	17 [9;35.5]		44
>=9	8.5 [7;13.75]		87
No. of periventricular T2 lesions at baseline			
0	8.0 [6.25;15]	0.479	7
1-2	16.0 [7.5;35]		23
>=3	10.0 [8;22]		75
No. of iuxta/cortical T2 lesions at baseline			
0	16.0 [8;29]	**0.001**	47
1	8.5 [8;9.25]		9
>1	9.0 [7;11]		53
No. of infratentorial T2 lesions at baseline			
0	14.0 [8;37]	0.114	39
1	10.0 [7;14]		38
>1	9.0 [7.5;20]		66
Brain Gd-enhancing lesions			
Yes	8.5 [7;10]	**<0.001**	66
No	17.5 [9;26.5]		112
No. of brain Gd-enhancing lesions	0.2	0.903	39
No. of total T2 spinal cord lesions			
0	12.0 [8.5;22]	0.895	31
1-2	10.0 [7;28]		71
>=3	15.0 [8;29]		48
No. of cervical T2 spinal cord lesions			
0	8.0 [3.5;13]	0.311	10
1-2	10.0 [7.5;29]		71
>=3	15.5 [8.5;28.75]		34

No. of dorsal T2 spinal cord lesions			
0	15.5 [9;19.75]	0.457	27
1-2	9.0 [5.75;19.5]		44
>=3	22.0 [8;34]		10
Spinal Gd-enhancing lesions			
Yes	10.0 [8;20.5]	0.409	32
No	11.0 [8;26]		111
No. of spinal Gd-enhancing lesions	0.034	0.907	14
EDSS at the time of LP	-0.051	0.598	109
Disease duration at the time of LP	0.117	0.226	109
CSF white blood cell count	-0.182	0.061	107
CSF proteins	-0.09	0.359	107
Albumin quotient	-0.138	0.153	109
IgG index	-0.046	0.635	109
Intrathecal OCB			
Pattern 1, 4 or 5	10.5 [8.75;20]	0.557	16
Pattern 2, 3	10.0 [8;24]		178
κ-index	0.002	0.983	107
sNfL	-0.159	0.111	102
sNfL z-score	-0.214	**0.031**	102

Results are shown as median [IQR] or Spearman rho depending on the type of variable. EDA: evidence of disease activity; MS: multiple sclerosis; LP: lumbar puncture; Gd: gadolinium; EDSS: expanded disability status scale; CSF: cerebrospinal fluid; OCB: oligoclonal bands; sNfL: serum neurofilament light chain.
** Statistically significant at a $p < 0.05$ level. Bold text indicates $p < 0.05$.*

Association between baseline κ-index and NEDA-3 outcomes across treatment timing and DMT classes

To investigate whether intrathecal B-cell activation may influence treatment response, additional analyses were performed within the RRMS subgroup to explore the association between baseline κ-index and NEDA-3 maintenance in relation to DMTs.

Before treatment initiation (Table 10), both the presence of more than three spinal cord lesions (HR = 0.40, 95% CI 0.23–0.90, p = 0.018) and κ-index > 6.4 (HR = 0.05, 95% CI 0.01–0.20, p < 0.001) were independently associated with a lower risk of early NEDA-3 loss. After DMT initiation, the presence of gadolinium-enhancing brain lesions and κ-index > 6.4 were independent predictors of a higher risk of disease activity, both in the entire RRMS cohort treated with DMT (HR = 1.30, 95% CI 1.07–1.60, p = 0.010, and HR = 1.30, 95% CI 1.10–1.60, p = 0.003, respectively) and in the subgroup receiving first line therapies (HR = 1.30, 95% CI 1.01–1.60, p = 0.04, and HR = 1.3, 95% CI 1.04–1.60, p = 0.023, respectively). In contrast, among patients treated with moderate-high efficacy DMTs, only brainstem symptoms at MS onset were independently and significantly associated with the risk of NEDA-3 loss (HR = 0.77, 95% CI 0.60–0.95, p = 0.017) (Tables 11–12).

To further assess whether κ-index was differentially associated with treatment response across specific DMT classes, point-biserial correlations between κ-index and NEDA-3 status were compared across RRMS patients treated with anti-CD20 therapies, other moderate-high efficacy DMTs and first line DMTs (Tab. 13). Fisher r-to-z tests did not detect significant differences in correlation strength between anti-CD20–treated patients and either comparator group (anti-CD20 vs high-efficacy: z = –0.709, p = 0.478; anti-CD20 vs first line: z = –0.607, p = 0.544), indicating that κ-index was not differentially associated with treatment response in patients receiving B-cell–depleting therapies.

Table 10 - Univariate and multivariate Cox regression for baseline data and risk of NEDA-3 loss <u>before</u> DMTs initiation in RRMS patients

Univariate	*HR (95% CI)*	*p-value**
Sex (M)	1.4 (0.43;4.6)	0.576
Age at onset	0.97 (0.9;1.05)	0.450
Type of clinical disease onset		
Optic neuritis (yes)	1.0 (0.3;3.5)	0.995
Supratentorial symptoms (yes)	1.3 (0.64;2.7)	0.462
Brainstem symptoms (yes)	3.2 (1.34;7.8)	**0.009**
Myelitis (yes)	0.49 (0.19;1.3)	0.145
No. of brain T2 lesions at baseline >=9	1.88 (0.64;5.5)	0.252
Presence of brain Gd-enhancing lesions	3.05 (1.2;7.6)	**0.017**
No. of total T2 spinal cord lesions >=3	0.3 (0.14;0.8)	**0.014**
Presence of spinal Gd-enhancing lesions	0.7 (0.4;1.3)	0.261
EDSS at the time of LP	2.1 (1.3;1.3.4)	**0.002**
CSF white blood cell count	1.09 (0.96;1.24)	0.201
CSF proteins	0.99 (0.94;1.03)	0.475
Albumin quotient	0.9 (0.67;1.2)	0.495
IgG index	1.07 (0.8;1.44)	0.661
OCB (pattern 2 and 3)	1.2 (0.9;1.7)	0.261
κ-index	1.0 (1.0;1.0)	0.751
ref 6.4	0.06 (0.02;0.2)	**<0.001**
ref 100	1.04 (0.5;2.3)	0.925
ref 106	1.04 (0.5;2.3)	0.925
ref 130	1.04 (0.5;2.3)	0.925
sNfL	1.06 (0.99;1.13)	0.087
sNfL z-score	1.18 (0.8;1.8)	0.441
ref 1.5	0.6 (0.23;1.3)	0.191
ref 2.0	5.3 (1.04;27.5)	**0.045**
Multivariate	*HR (95% CI)*	*p-value**
Type of clinical disease onset		
Brainstem symptoms (yes)	1.5 (0.42;5.1)	0.553
Myelitis (yes)	1.01 (0.41;2.5)	0.988

Presence of brain Gd-enhancing lesions	2.2 (0.9;5.1)	0.081
No. of total T2 spinal cord lesions >=3	0.4 (0.23;0.9)	**0.018**
EDSS at the time of LP	1.5 (0.96;2.4)	0.075
κ-index ref 6.4	0.05 (0.01;0.2)	**<0.001**
sNfL	0.97 (0.91;1.03)	0.263
sNfL z-score ref 2.0	0.97 (0.3;3.3)	0.741

Gd = gadolinium; EDSS = Expanded Disability Status Scale; LP = lumbar puncture; CSF = cerebrospinal fluid; OCB = oligoclonal bands; sNfL: serum neurofilament light chain.
** Statistically significant at a p < 0.05 level. Bold text indicates p < 0.05*

Table 11 - Univariate Cox regression for baseline data and risk of NEDA-3 loss <u>after</u> DMTs initiation in RRMS patients

Univariate	**All DMTs**		**First line DMTs**		**Moderate-high efficacy DMTs**	
	HR (95% CI)	***p-value****	***HR (95% CI)***	***p-value****	***HR (95% CI)***	***p-value****
Sex (M)	1.01 (0.82;1.25)	0.920	1.1 (0.87;1.4)	0.447	0.84 (0.65;1.1)	0.200
Age at onset	0.98 (0.98;1.00)	**0.009**	0.99 (0.98;1.0)	**0.006**	1.0 (0.98;1.01)	0.867
Type of clinical disease onset						
Optic neuritis (yes)	0.88 (0.7;1.1)	0.280	0.8 (0.62;1.03)	0.087	0.97 (0.74;1.27)	0.822
Supratentorial symptoms (yes)	1.0 (0.62;1.6)	0.992	1.05 (0.63;1.75)	0.847	1.03 (0.74;1.42)	0.871
Brainstem symptoms (yes)	0.95 (0.77;1.2)	0.665	1.07 (0.85;1.34)	0.562	0.75 (0.6;0.97)	**0.027**
Myelitis (yes)	1.14 (0.92;1.4)	0.225	1.1 (9;1.4)	0.376	1.26 (0.97;1.64)	0.090
No. of brain T2 lesions at baseline >=9	1.14 (0.92;1.42)	0.224	1.2 (0.94;1.5)	0.142	0.97 (0.75;1.27)	0.828
Presence of brain Gd-enhancing lesions	1.33 (1.08;1.65)	**0.009**	1.33 (1.05;1.7)	**0.018**	1.2 (0.9;1.5)	0.247
No. of total T2 spinal cord lesions >=3	0.9 (0.72;1.14)	0.410	0.96 (0.75;1.2)	**0.048**	0.83 (0.62;1.1)	0.203
Presence of spinal Gd-enhancing lesions	0.98 (0.72;1.3)	0.888	1.01 (0.7;1.5)	0.962	0.85 (0.6;1.2)	0.354
EDSS at the time of LP	1.01 (0.9;1.14)	0.839	1.13 (0.97;1.33)	0.110	0.94 (0.8;1.1)	0.371
CSF white blood cell count	1.01 (0.99;1.03)	0.274	1.02 (1.0;1.05)	**0.035**	1.0 (0.8;1.1)	0.606
CSF proteins	1.0 (0.99;1.01)	0.274	1.0 (0.99;1.01)	0.822	1.0 (0.99;1.01)	0.828
Albumin quotient	0.98 (0.94;1.03)	0.436	0.99 (0.95;1.03)	0.561	0.98 (0.93;1.04)	0.905

IgG index	1.07 (0.8;1.44)	0.661	1.07 (0.78;1.5)	0.688	0.96 (0.67;1.38)	0.808
OCB (pattern 2 and 3)	1.2 (0.9;1.7)	0.261	1.2 (0.86;1.7)	0.248	1.1 (0.72;1.7)	0.636
κ-index	1.0 (1.0;1.0)	0.135	1.0 (1.0;1.0)	0.364	1.0 (1.0;1.0)	0.388
ref 6.4	1.58 (1.2;1.2.1)	**0.001**	1.57 (1.18;2.1)	**0.002**	1.4 (0.9;2.1)	0.127
ref 100	1.12 (0.9;1.5)	0.404	1.13 (0.86;1.5)	0.387	1.1 (0.8;1.5)	0.656
ref 106	1.19 (0.91;1.5)	0.201	1.2 (0.9;1.56)	0.196	1.1 (0.8;1.6)	0.528
ref 130	1.14 (0.9;1.5)	0.355	1.04 (0.8;1.38)	0.769	1.1 (0.79;1.6)	0.560
sNfL	1.0 (1.0;1.01)	0.736	1.01 (1.0;1.02)	0.09	1.0 (0.99;1.0)	0.492
sNfL z-score	1.05 (0.98;1.13)	0.164	1.08 (1.0;1.16)	**0.041**	1.01 (0.9;1.1)	0.898
ref 1.5	1.1 (0.9;1.4)	0.367	1.16 (0.92;1.5)	0.212	0.97 (0.7;1.3)	0.805
ref 2.0	1.2 (0.95;1.5)	0.128	1.32 (1.03;1.7)	**0.030**	0.99 (0.7;1.3)	0.921

Gd = gadolinium; EDSS = Expanded Disability Status Scale; LP = lumbar puncture; CSF = cerebrospinal fluid; OCB = oligoclonal bands; sNfL: serum neurofilament light chain.
** Statistically significant at a $p < 0.05$ level. Bold text indicates $p < 0.05$.*

Table 12 - Multivariate Cox regression for baseline data and risk of NEDA-3 loss <u>after</u> DMTs initiation in RRMS patients

Multivariate	**All DMTs**		**First line DMTs**		**Moderate-high efficacy DMTs**	
	HR (95% CI)	***p-value****	***HR (95% CI)***	***p-value****	***HR (95% CI)***	***p-value****
Age at onset	1.0 (0.99;1.01)	0.584	0.99 (0.99;1.0)	0.180	-	-
Type of clinical disease onset						
Optic neuritis (yes)	-	-	0.9 (0.7;1.14)	0.397	-	-
Brainstem symptoms (yes)	-	-	-	-	0.77 (0.6;0.95)	**0.017**
Myelitis (yes)	-	-	-	-	1.11 (0.87;1.42)	0.385
No. of brain T2 lesions at baseline >=9	-	-	1.1 (0.9;1.34)	0.454	-	-
Presence of brain Gd-enhancing lesions	1.3 (1.07;1.6)	**0.010**	1.3 (1.01;1.6)	**0.040**	-	-
EDSS at the time of LP	-	-	1.1 (0.96;1.28)	0.160	-	-
CSF white blood cell count	-	-	1.0 (0.99;1.02)	0.722	-	-
κ-index	1.0 (1.0;1.0)	0.341	-	-	-	-
ref 6.4	1.3 (1.1;1.6)	**0.003**	1.3 (1.04;1.6)	**0.023**	1.3 (0.87;1.9)	0.207
sNfL	-	-	1.0 (0.99;1.01)	0.801	-	-
sNfL z-score	-	-	1.01 (0.96;1.07)	0.635	-	-
ref 2.0	1.05 (0.84;1.3)	0.677	1.04 (0.9;1.2)	0.598	-	-

Gd = gadolinium; EDSS = Expanded Disability Status Scale; LP = lumbar puncture; CSF = cerebrospinal fluid; OCB = oligoclonal bands; sNfL: serum neurofilament light chain. ** Statistically significant at a p < 0.05 level. Bold text indicates p < 0.05.*

Table 13 - Fisher r-to-z comparison of κ-index correlations with NEDA-3 loss across DMT subgroups

	N	*Point-biserial correlation*	*Z statistics*	*p-value*
Anti-CD20	19	-0.092	-	-
First line DMTs	131	0.096	-0.709	0.478
Other moderate-high efficacy DMTs	26	0.105	-0.607	0.544

Exploratory cut-off–based classification of κ-index and sNfL for NEDA-3 prediction

To ensure comparability with previous κ-index prognostic studies and to provide a reference point for subsequent modeling, an exploratory cut-off–based analysis was first performed in the RRMS subgroup. Optimal thresholds for κ-index and baseline sNfL z-scores were identified using the Youden Index derived from ROC analyses, and the resulting binary classifiers were combined according to both OR and AND logical rules to predict NEDA-3 loss after MS diagnosis.

Before DMT initiation, both biomarkers considered individually showed moderate discriminative performance (accuracy approximately 55–56%). The combined OR rule yielded a slightly improved balance between sensitivity and specificity (accuracy 61%, Youden Index 0.28), whereas the AND rule, although highly specific (93%), demonstrated poor sensitivity (28%) (Tab. 13). Similar findings were observed when the analysis was repeated with respect to the risk of NEDA-3 loss after DMT initiation, with overall accuracies ranging between 55% and 60% and again the OR rule performing best (Tab. 14).

To visualize how the combined κ-index and sNfL cut-offs stratified patients according to NEDA-3 loss, confusion matrices were generated for each classifier, illustrating the proportion of correctly and incorrectly classified cases (Fig. 8 and 10). In addition, the AND condition was graphically represented to display the proportion of patients losing NEDA-3 within each quadrant defined by κ-index and sNfL z-score thresholds (Fig. 9 and 11). Although less sensitive, this model offers a clearer visual interpretation of how concurrent abnormalities in both biomarkers identify a subgroup at particularly high risk of disease activity.

	κ-index cut-off	*sNfL z-score cut-off*	*Accuracy*	*Sensitivity*	*Specificity*	*Youden's index*
κ-index based	51.680	-	0.559	0.500	0.643	0.211
sNfL z-score based	-	1.980	0.559	0.340	0.871	0.211
κ-index OR sNfL z-score based	151.948	1.994	0.612	0.490	0.786	0.276
κ-index AND sNfL z-score based	6.079	2.195	0.547	0.280	0.929	0.209

Table 13 - Cut-off–based performance metrics for κ-index and sNfL z-score in predicting NEDA-3 loss from the time of lumbar puncture. *The table reports optimal thresholds, accuracy, sensitivity, specificity, and Youden's index for individual and combined classifiers using OR and AND rules.*

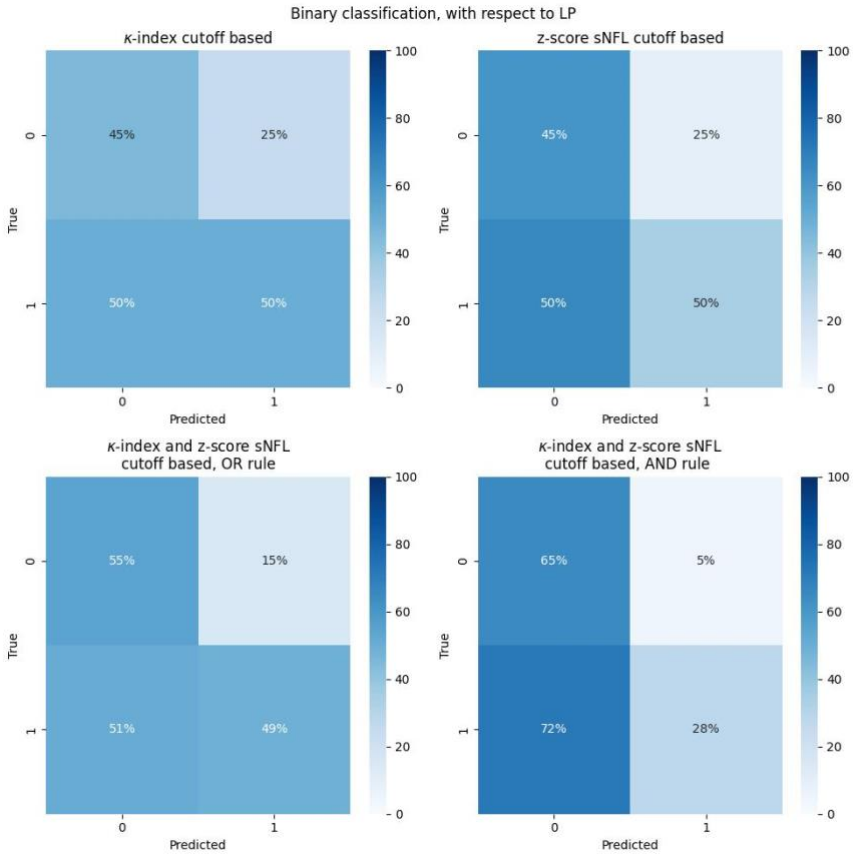

Fig. 8 ***- Confusion matrices showing the classification performance of κ-index–based, sNfL z-score–based and combined (OR and AND) binary models in predicting NEDA-3 loss from MS diagnosis.*** *The proportion of true and false classifications is expressed as a percentage of the total sample.*

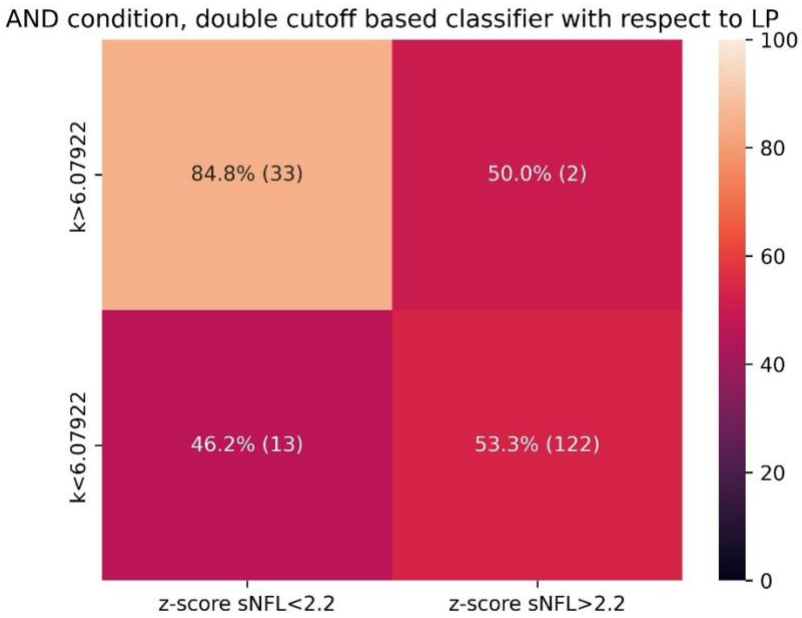

Fig. 9 ***- Proportion of patients losing NEDA-3 according to the double cut-off classifier (AND condition) with respect to the time of MS diagnosis.*** *Each quadrant represents the combination of κ-index and sNfL z-score thresholds; numbers in parentheses indicate the number of patients per subgroup.*

	κ-index cut-off	***sNfL z-score cut-off***	***Accuracy***	***Sensitivity***	***Specificity***	***Youden's index***
κ-index based	53.410	-	0.547	0.483	0.617	0.134
sNfL z-score based	-	2.260	0.553	0.270	0.864	0.134
κ-index OR sNfL z-score based	79.014	2.397	0.588	0.562	0.617	0.179
κ-index AND sNfL z-score based	6.079	2.195	0.553	0.258	0.877	0.135

Tab. 14 - ***Cut-off–based performance metrics for κ-index and sNfL z-score in predicting NEDA-3 loss after DMT initiation.*** *Optimal thresholds, accuracy, sensitivity, specificity, and Youden's index are reported for individual and combined classifiers.*

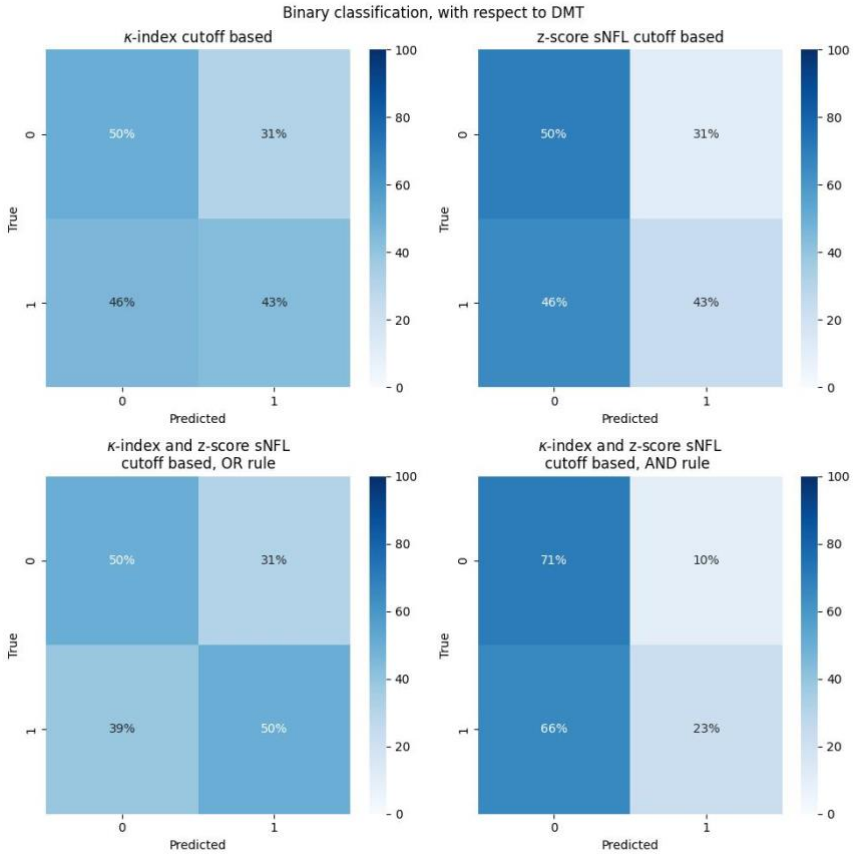

Fig. 10 ***- Confusion matrices showing the classification performance of κ-index–based, sNfL z-score–base and combined (OR and AND) binary models in predicting NEDA-3 loss after DMT initiation.***

Fig. 11 - ***Proportion of patients losing NEDA-3 according to the double cut-off classifier (AND condition) with respect to DMT initiation.*** *Percentages within each quadrant represent the proportion of patients losing NEDA-3; numbers in parentheses correspond to the size of each subgroup.*

These results, while modest in predictive accuracy, served as a useful descriptive benchmark and highlighted the limitations of dichotomous threshold-based models. This motivated the subsequent use of continuous-variable Cox models with spline functions to more accurately characterize the potentially non-linear relationship between κ-index, sNfL, and the risk of NEDA-3 loss.

Combined effect of baseline κ-index and sNFL z-score on the risk of NEDA-3 loss

Given the modest discriminative performance of the cut-off–based classifiers (accuracy 55–60%), κ-index and sNfL were subsequently analyzed as continuous predictors in flexible Cox models with spline functions, to better characterize their joint and potentially non-linear association with NEDA-3 loss.

The relationship between baseline κ-index and the risk of NEDA-3 loss during follow-up was first explored through univariate Cox regression models using B-splines. Several models with different degrees of freedom were tested, and the 5-degree-of-freedom (5-df) model was selected as the most appropriate according to concordance index (C-index), log-likelihood ratio test and AIC comparisons (Tab. 15). For this model, Schoenfeld residuals confirmed that the estimated relationship between κ-index and NEDA-3 loss remained stable throughout the follow-up period, satisfying the proportional hazards assumption.

No. of degree-of-freedom	***Concordance***	***Log-likelihood ratio test***	***AIC***
3	0.526	4.663	2637.970
4	0.522	8.466	2636.167
5	0.540	15.272	2631.361
6	0.538	15.147	2622.486

Table 15 - ***Performance metrics of B-spline Cox models with 3–6 degrees of freedom for κ-index as a continuous predictor of NEDA-3 loss****. Model performance was evaluated using concordance index (C-index), log-likelihood ratio test, and Akaike Information Criterion (AIC).*

Importantly, the 5-df spline model revealed a statistically significant non-linear association between κ-index and the risk of NEDA-3 loss, outperforming the corresponding linear Cox model (likelihood ratio test $p = 0.02$). This finding indicates that the relationship between κ-index and disease activity is not adequately described by a simple linear trend.

Specifically, when considering the simultaneous confidence bands, significantly higher hazards were observed both at lower κ-index values (0–194) and at higher

values (565–600), whereas intermediate levels were associated with a comparatively lower and more stable risk (Tab. 16, Fig. 12). Nevertheless, the estimated spline curve for κ-index remained consistently above the null line (log(HR) = 0), indicating an overall trend toward increased risk of NEDA-3 loss.

Despite the relatively modest overall performance of the Cox regression model, the inclusion of the spline function for κ-index significantly improved model fit compared with the linear specification. This result indicates that κ-index is globally associated with the risk of NEDA-3 loss and supports the use of non-linear modeling approaches to more accurately describe this relationship.

Covariate	***log(HR)***	***HR***	***SE (log(HR))***	***logHR, 95%CI lower***	***logHR, 95%CI upper***	***HR lower 95%***	***HR upper 95%***	***p-value****
bs(κ-index, df=5)[1]	1.49	4.437	0.509	0.493	2.487	1.637	12.028	**0.003**
bs(κ-index, df=5)[2]	0.611	1.842	0.395	-0.164	1.385	0.849	3.996	0.122
bs(κ-index, df=5)[3]	1.414	4.112	0.946	-0.440	3.268	0.644	26.259	0.135
bs(κ-index, df=5)[4]	0.056	1.058	1.768	-3.409	3.522	0.033	33.842	0.975
bs(κ-index, df=5)[5]	2.301	9.981	0.720	0.890	3.711	2.435	40.910	**0.001**

Table 16 - ***Coefficients and related statistics of the Cox regression model with B-spline transformation (df = 5) for κ-index.*** *The table reports the estimated log-hazard ratios (log(HR)), corresponding hazard ratios (HR), standard errors (SE), 95% confidence intervals, and p-values for each spline basis function.*
**Statistically significant at a $p < 0.05$ level. Bold text indicates $p < 0.05$.*

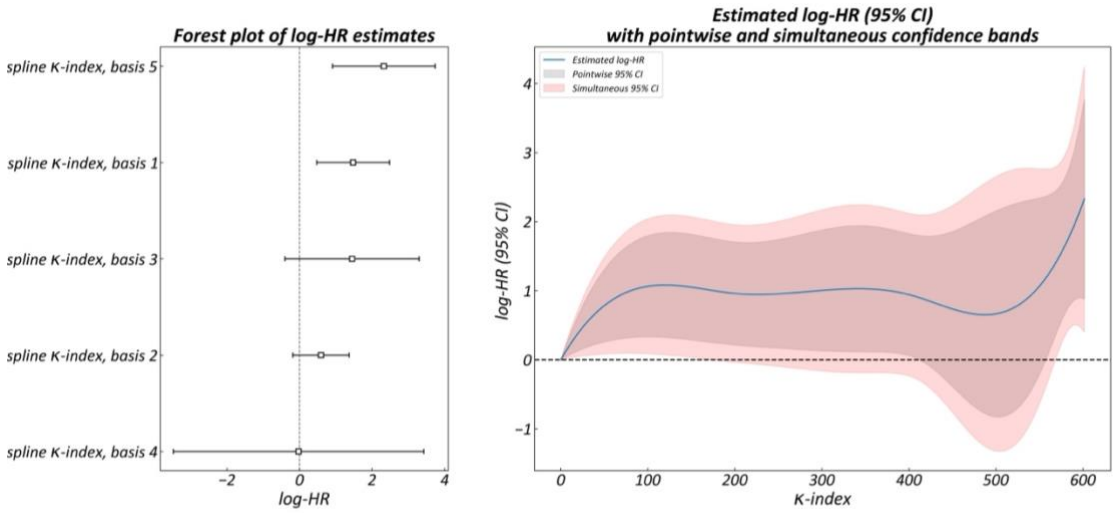

Fig. 12 - ***B-spline Cox model with 5 degrees of freedom.*** *Left panel: 95% confidence intervals of the estimated log-hazard ratio. Right panel: Estimated spline function showing the relationship between κ-index and the log-hazard of NEDA-3 loss, displayed with both simultaneous and pointwise confidence bands.*

To further refine prognostic modeling and account for potential joint effects, a bivariate Cox regression model was developed including κ-index, modeled flexibly with B-splines, and baseline sNfL z-score, entered as a linear covariate based on its previously demonstrated significance in linear Cox models (see Section "Correlation between baseline data and NEDA status during follow up"). The 5-df model again showed the best performance and no violation of the proportional hazards assumption, indicating that the inclusion of the linear sNfL z-score improved overall model fit (Table 17).

No. of degree-of-freedom	***Concordance***	***Log-likelihood ratio test***	***AIC***
3	0.579	11.310	2633.323
4	0.581	15.523	2631.110
5	0.587	23.689	2624.944
6	0.587	22.928	2627.705

Table 17 - ***Performance metrics of bivariate B-spline Cox models with 3–6 degrees of freedom for κ-index (modeled as a spline) and baseline sNfL z-score (modeled linearly) as predictors of NEDA-3 loss.*** *Model performance was evaluated using concordance index (C-index), log-likelihood ratio test, and Akaike Information Criterion (AIC).*

In the bivariate model including both κ-index and baseline sNfL z-score (Table 18, Fig. 13), two spline components of κ-index (bs[κ-index, df=5][1] and bs[κ-index, df=5][5]) showed statistically significant associations with the risk of NEDA-3 loss (p = 0.002 for both), confirming a non-linear pattern consistent with the univariate analysis. In addition, baseline sNfL z-score was independently and linearly associated with an increased hazard of NEDA-3 loss (HR = 1.16, 95% CI 1.04–1.29, p = 0.008).

Covariate	***log(HR)***	***HR***	***SE (log(HR))***	***logHR, 95%CI lower***	***logHR, 95%CI upper***	***HR lower 95%***	***HR upper 95%***	***p-value****
bs(κ-index, df=5)[1]	1.632	5.117	0.528	0.598	2.666	1.819	14.389	**0.002**
bs(κ-index, df=5)[2]	0.614	1.849	0.401	-0.171	1.4	0.843	4.056	0.125
bs(κ-index, df=5)[3]	1.534	4.638	0.946	-0.319	3.388	0.727	29.602	0.105
bs(κ-index, df=5)[4]	0.294	1.342	1.621	-2.882	3.471	0.056	32.162	0.856
bs(κ-index, df=5)[5]	2.194	8.972	0.718	0.787	3.601	2.197	36.632	**0.002**
sNfL z-score	0.148	1.159	0.055	0.039	0.256	1.04	1.292	**0.008**

Table 18 - ***Coefficients and related statistics of the bivariate Cox regression model including κ-index (B-spline transformation, df = 5) and baseline sNfL z-score (linear term).*** *The table reports the estimated log-hazard ratios (log(HR)), corresponding hazard ratios (HR), standard errors (SE), 95% confidence intervals, and p-values for each spline basis function and for the sNfL z-score covariate.*
** Statistically significant at a p < 0.05 level. Bold text indicates p < 0.05.*

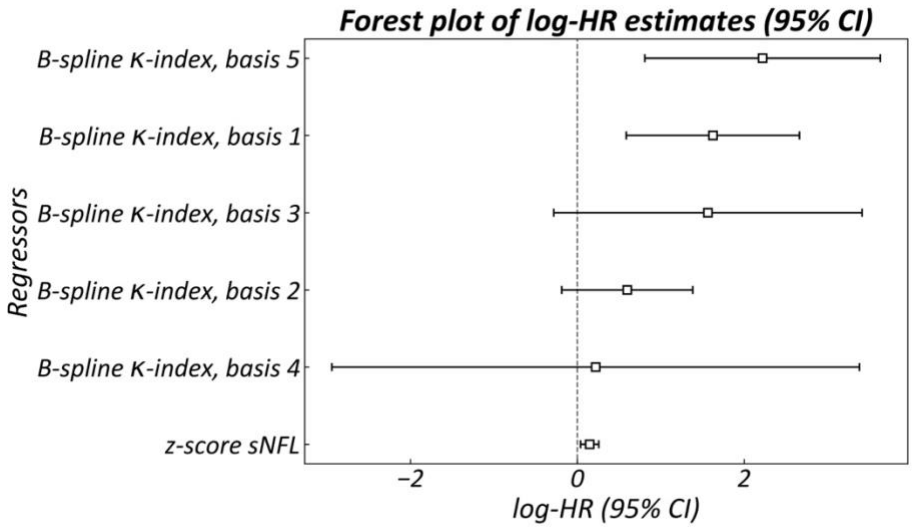

Fig. 13 - ***Forest plot from the bivariate Cox model illustrating log-hazard ratios (95% CI) for κ-index (modeled with 5-degree B-splines) and sNfL z-score (linear term) in predicting NEDA-3 loss.***

The graphical representation of the combined model is reported in Fig. 14 and 15, providing a visual summary of the estimated effects. In the bivariate representation, the estimated spline function for κ-index appears noticeably flatter compared with the univariate model, with wide 95% confidence bands largely overlapping the null line (log(HR) = 0). This pattern indicates reduced precision in the estimation of κ-index–related effects after adjustment for baseline sNfL z-score.

To better visualize the estimated non-linear component of κ-index within the bivariate model, an additional plot with only point-wise 95% confidence intervals was generated (Fig. 15). Compared with the representation including simultaneous confidence bands, this figure provides a clearer depiction of the underlying shape of the estimated effect, as the wider simultaneous intervals tend to flatten the curve when global uncertainty is high. The point-wise plot confirms a non-linear trend broadly comparable to that observed in the univariate model, with modest variations in the estimated log-hazard across the κ-index range.

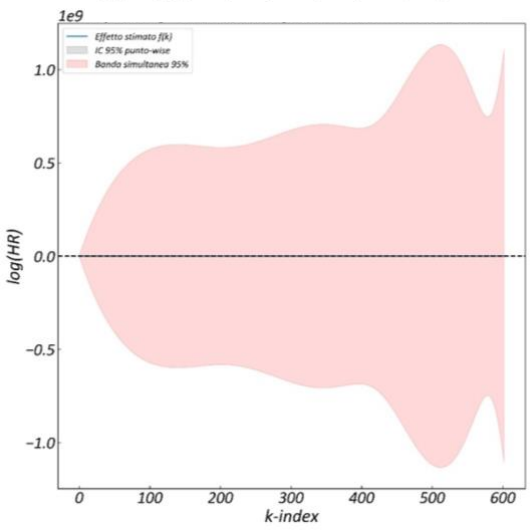

Fig. 14 - ***Estimated log-hazard ratio function for κ-index in the bivariate Cox regression model (B-spline transformation, df = 5) adjusted for median baseline sNfL z-score.*** *The plot shows the estimated effect (blue line) with 95% point-wise (dark pink) and simultaneous (light pink) confidence bands.*

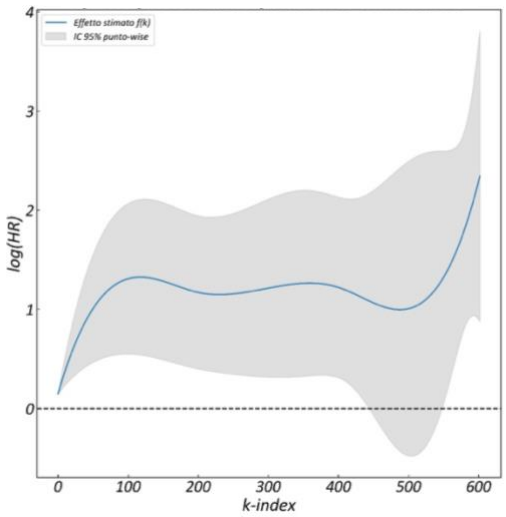

Fig. 15 - ***Estimated log-hazard ratio function for κ-index in the bivariate Cox regression model (B-spline transformation, df = 5) adjusted for median baseline sNfL z-score.*** *The plot displays the estimated effect (blue line) with 95% point-wise confidence intervals (grey area).*

The three-dimensional surface plot provides a complementary view of the joint effect of the two biomarkers (Fig. 16). Along the sNfL axis, the estimated log-hazard increases gradually and linearly, whereas the κ-index dimension retains a non-linear profile similar to that observed in the univariate model, with slightly higher estimated risks at both lower and higher κ-index values. Overall, the bivariate surface indicates that baseline sNfL z-score exerts a consistent linear effect on the risk of NEDA-3 loss, while κ-index contributes a less stable, non-monotonic modulation of the hazard function, characterized by wider uncertainty and a comparatively smaller overall impact within the combined model.

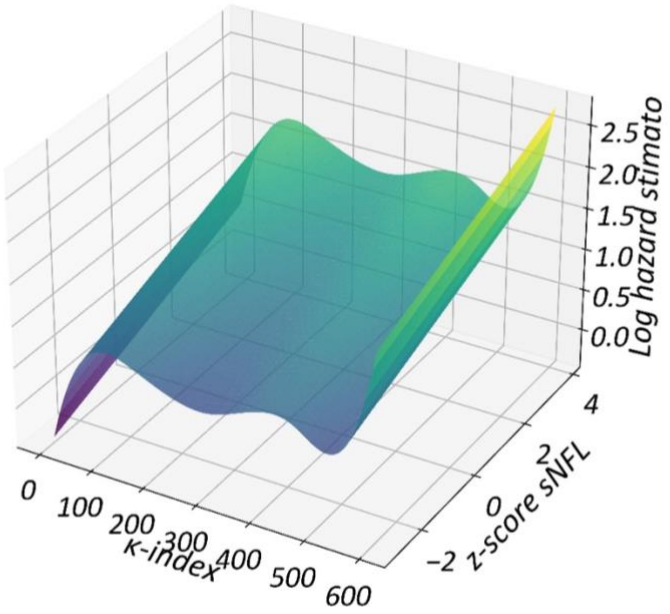

Fig. 16 - ***Three-dimensional surface plot of the estimated log-hazard in the bivariate Cox regression model including κ-index (B-spline transformation, df = 5) and baseline sNfL z-score (linear term)***.

DISCUSSION

This study investigated the prognostic value of the κ-index obtained at diagnostic lumbar puncture (LP) in patients with multiple sclerosis (MS), by assessing its relationship with disease activity and clinical-radiological outcomes during follow-up. The κ-index, reflecting intrathecal B-cell activation and immunoglobulin synthesis, was specifically evaluated in this study as a potential biomarker of long-term disease dynamics and risk of NEDA-3 loss over time. The study included a relatively large real-world cohort of MS patients with up to five years of follow-up (median 28.0 months), combining standardized laboratory data with detailed longitudinal clinical and MRI assessments. In addition to its primary aim, an exploratory analysis evaluated the integration of the κ-index with serum neurofilament light chain (sNfL) levels, to explore whether combining immunological and neuroaxonal markers could enhance prognostic stratification in MS.

The κ-index represents a quantitative measure of intrathecal free light chain κ production and a sensitive marker of sustained humoral immune activation within the CNS. Early work by Cepok et al.[78] demonstrated that short-lived plasmablasts constitute the main B-cell effector population in the CSF of patients with MS, correlating with intrathecal IgG synthesis and MRI inflammatory activity. More recently, Castillo-Villalba et al.[79] confirmed that high κ-index values reflect an expanded and persistent intrathecal B-cell compartment, strongly associated with intrathecal Ig synthesis and leptomeningeal contrast enhancement, features consistent with chronic, compartmentalized B-cell–driven inflammation. Altogether, these findings provide a biological framework supporting the interpretation of elevated κ-index values as a proxy for sustained intrathecal B-cell activation and, ultimately, for disease severity and progression risk in MS.

Within this context, the associations observed in our cohort between higher κ-index values and greater total T2-weighted lesion count, in line with previous reports by Berek et al. (2021)[67] and Cutellé et al.[63], and particularly with periventricular, infratentorial, and spinal lesion burden, suggest that enhanced intrathecal B-cell activation parallels a more extensive demyelinating load in regions particularly susceptible to inflammatory infiltration. In addition, the κ-index was associated with

the number of gadolinium-enhancing spinal lesions. Moreover, consistent with Duell et al.[62], the κ-index correlated in our cohort with other CSF markers of intrathecal inflammation, such as CSF white blood cell count, IgG index, and OCB positivity. Taken together, these findings support the view that the κ-index aligns with known markers of poor prognosis[2] and may reflect a convergent pathogenic pathway linking chronic B-cell activity to unfavorable disease evolution. Notably, in the smaller PPMS subgroup, significant correlations were limited to an inverse relationship with age and direct associations with CSF white blood cell count and IgG index, while no significant relationships were observed with MRI parameters. This pattern likely reflects the limited sample size and the lower degree of inflammatory activity typical of progressive forms, rather than the absence of an underlying link between intrathecal B-cell activation and tissue damage.

When assessing the risk of NEDA-3 loss, RRMS and PPMS were analyzed separately, given their distinct disease trajectories and pathophysiological profiles. Univariate Cox proportional hazards models were used to test multiple κ-index thresholds, including those proposed in the literature (100, 106, and 130), the diagnostic cut-off routinely adopted in our laboratory (6.4) and additional cohort-specific thresholds derived through maximally selected rank statistics. These values were originally derived from studies addressing single outcomes such as MRI activity[64], PIRA[65], or DMT failure[66], whereas our endpoint integrated multiple domains of disease activity.

Among patients with RRMS, baseline gadolinium-enhancing brain lesions, a κ-index exceeding either the diagnostic threshold of 6.4 or the data-driven threshold of 6.51, and an sNfL z-score > 2.0 emerged as independent risk factors for NEDA-3 loss. However, when analyzed as continuous variables, neither κ-index nor sNfL showed significant associations, nor did other κ-index thresholds (100, 106, or 130) derived from recent literature. In the PPMS subgroup, no significant associations were detected between κ-index and the risk of NEDA-3 loss, and neither total sNfL levels nor continuous z-scores showed statistically significant effects in multivariable models. However, when dichotomized at a z-score > 2.0, baseline sNfL displayed an inverse association with NEDA-3 loss, a finding that may reflect transient elevations unrelated to progressive neurodegeneration, possibly linked to residual inflammatory activity. Given the small sample size and limited number of events, this observation should be

interpreted with caution. These results are consistent with the distinct immunopathological substrates of RRMS and PPMS, the latter being characterized by low-grade, compartmentalized inflammation and neurodegeneration largely independent of acute immune activation.

Within the RRMS subgroup, baseline predictors of individual NEDA-3 components were further analyzed. Although a κ-index > 6.4 was significantly associated with the risk of MRI activity during follow-up in univariate analyses, it did not retain independent significance in the multivariate model. In contrast, the presence of gadolinium-enhancing brain lesions and increased albumin quotient were independently associated with a higher risk of clinical relapses. In patients who experienced disease reactivation during follow-up, only an sNfL z-score > 2.0 was significantly associated with a shorter time to event, suggesting that elevated neurofilament levels may identify patients with more active axonal damage and a faster transition to disease reactivation.

To investigate whether the prognostic role of κ-index may vary according to treatment timing and DMT class, additional analyses were performed in RRMS patients. Before treatment initiation, both the presence of >3 spinal lesions and κ-index > 6.4 were independently associated with a lower risk of early NEDA-3 loss, suggesting that high intrathecal immune activation at disease onset may reflect an inflammatory phase that is particularly responsive to early therapeutic intervention. After DMT initiation, κ-index > 6.4 and the presence of gadolinium-enhancing lesions were associated with a higher risk of disease activity, but this effect was driven exclusively by patients receiving first line DMTs, whereas no such association was observed among those treated with moderate-high efficacy therapies. This pattern raises the hypothesis that patients with positive κ-index may derive particular benefit from more potent immunomodulation.

To further investigate whether intrathecal B-cell activation was more strongly associated with treatment response in specific DMT groups, the association between κ-index and NEDA-3 outcomes was compared across RRMS patients treated with anti-CD20 therapies, other moderate-high efficacy DMTs and first line agents. Fisher r-to-z tests did not detect significant differences in correlation strength between anti-

CD20–treated patients and either comparator group, indicating that κ-index was not differentially associated with treatment response in patients receiving B-cell–depleting therapies. Given the relatively small number of anti-CD20–treated patients and the limited follow-up period, these results should be interpreted with caution. Although exploratory and limited by sample size, these findings suggest that κ-index reflects a general marker of inflammatory disease activity rather than a predictor of preferential response to specific DMT classes.

Given the heterogeneous performance of individual κ-index thresholds and the limited explanatory power of univariate models, an exploratory analysis was conducted to determine whether integrating κ-index with sNfL z-score could enhance prognostic discrimination. This approach, consistent with recent suggestions by Freedman et al.[35] advocating combined biomarker strategies in MS, also enabled direct methodological comparison with previous κ-index prognostic studies. Although the predictive performance of these models was modest, with accuracy around 55–60%, the combination of both biomarkers slightly improved discrimination, suggesting partially independent and complementary contributions. This finding may reflect the partially complementary biological meaning of the two biomarkers, with the κ-index capturing upstream intrathecal B-cell activation and sNfL reflecting downstream neuroaxonal damage. Nevertheless, the limited accuracy of threshold-based classifiers highlights a key limitation of this approach. Dichotomizing continuous biological variables can obscure non-linear or threshold-independent effects, leading to a loss of prognostic information and interpretative depth.

To further explore these relationships, κ-index and sNfL z-scores were analyzed as continuous predictors in Cox models. In RRMS, sNfL z-scores showed a significant linear association with the risk of NEDA-3 loss, whereas κ-index did not when constrained to a linear effect. This pattern suggests that the prognostic contribution of κ-index may not follow a constant, monotonic trend across its range and could instead display non-linear behavior.

On this basis, κ-index was modeled using flexible B-splines within a Cox proportional hazards framework, allowing for potential non-linear associations with time-to-event outcomes[76]. The adoption of this flexible approach was further supported by recent

methodological evidence[77] indicating that survival models imposing unnecessary linearity constraints may lose predictive accuracy and obscure true biological relationships.

The spline-based model revealed a non-linear risk profile, with an initial significant increase in hazard followed by a broad intermediate range where the risk curve plateaued, and a sharper upturn at the highest κ-index values. This pattern suggests that κ-index exerts a measurable impact on disease reactivation already within the lower-to-intermediate range, with additional risk amplification at very high levels possibly reflecting intensified intrathecal immune activation and a greater propensity for NEDA-3 loss. However, given the widening confidence bands at the distribution extremes, these rises should be interpreted with caution, as they may partly reflect limited data density and potential overfitting at the tails. Therefore, while the non-linear fit improved descriptive accuracy compared with dichotomized analyses, these results should be interpreted cautiously, acknowledging the risk of overfitting and limited generalizability. Although no universal threshold emerged, the κ-index consistently showed a trend toward increased risk of NEDA-3 loss at higher values, supporting its prognostic potential as a continuous biomarker rather than a dichotomous one.

Given the linear association observed for sNfL z-scores and the non-linear pattern estimated for κ-index, a bivariate Cox model was subsequently evaluated specifying κ-index as a spline term and sNfL z-score as a linear covariate, to assess their complementary contributions to NEDA-3 loss prediction. The obtained overall model retained a good global fit, but the shape of the κ-index function became flatter and less precisely estimated. This apparent "attenuation" of the κ-index effect should not be interpreted as a loss of biological significance, but rather as a statistical consequence of the increased model complexity and partial interdependence between the two biomarkers. Several factors may contribute to this phenomenon. First, the sample size available for the bivariate spline model may have limited its statistical power, leading to wide confidence intervals and local instability of the curve. Second, the inclusion of a highly flexible spline term with five degrees of freedom increases the risk of overfitting, particularly when the number of events is modest. In practice, the model may fit the training data very closely, but the estimated shape of the curve becomes

less generalizable and biologically interpretable. The widening of the simultaneous confidence bands, and the visual flattening of the estimated log-hazard function, represent typical signatures of this phenomenon.

Altogether, these results support a model in which κ-index and sNfL capture distinct but interconnected dimensions of MS pathophysiology, immune activation within the CNS and its downstream neuroaxonal consequences. This conceptual integration, in line with the framework proposed by Freedman et al., underscores the potential of combined biomarker strategies for improving individualized prognostic assessment in MS, while also highlighting the need for validation in larger, prospectively designed cohorts.

Our study presented several limitations. Despite being, to our knowledge, the largest prognostic study of κ-index conducted to date, the relatively small number of patients with complete longitudinal data may have contributed to wide confidence intervals in some models, particularly in spline-based analyses, where the number of events per degree of freedom was limited. Additional limitations include the partially retrospective design, the median follow-up of 28 months, and the heterogeneity in the timing between disease onset and diagnostic LP. The latter may have influenced biomarker levels, as κ-index values can vary with disease phase and recent inflammatory activity. Finally, the absence of an independent validation cohort limits the generalizability of the findings, which should be regarded as hypothesis-generating rather than definitive. Restricting future analyses to patients with recent disease onset (e.g., within six months of diagnosis) and prospectively harmonizing CSF and serum collection protocols could help delineate the early prognostic role of intrathecal B-cell activity more precisely.

Strengths of this study include the relatively large sample size for this research area and the rigorous, stepwise analytical strategy. Starting from previously validated linear and dichotomous models, we extended the investigation to flexible non-linear survival modeling, allowing a more realistic representation of biological variability. This methodological evolution represents one of the most innovative aspects of the work, offering a proof of concept for the use of spline-based survival models in biomarker prognostication in MS. By coupling this flexible approach with the integration of sNfL as a complementary neuroaxonal marker, the study provides a conceptual and

statistical framework that could be expanded in larger prospective cohorts to construct individualized risk prediction tools.

Future research should aim to validate these findings in multicenter, prospectively designed studies with harmonized methodologies, extended observation periods, and external replication of model performance. The absence of universally accepted κ-index thresholds, partly due to assay variability, methodological heterogeneity, and differences in population characteristics, underscores the need for standardization before clinical implementation for prognostic purposes. Moreover, preliminary data linking κ-index to genetic susceptibility markers such as HLA-DRB1*15:01[62] highlight promising directions for exploring the interplay between immunogenetic background, B-cell activation and disease trajectory.

In summary, our results support the κ-index as a biologically grounded and clinically relevant marker of intrathecal immune activation, associated with known predictors of poor prognosis and subsequent loss of disease control. The integration of κ-index with sNfL, though exploratory, demonstrates the potential of combining immune and neuroaxonal biomarkers to refine disease monitoring and risk stratification in multiple sclerosis. Beyond its biological insights, the present study also establishes a methodological foundation for future predictive modeling efforts aimed at translating complex biomarker data into individualized prognostic algorithms.

CONCLUSIONS

This work investigated the prognostic significance of the κ-index as a biomarker of intrathecal immune activation in multiple sclerosis (MS). Integrating clinical, radiological, and biochemical data we explored its association with disease activity and long-term NEDA-3 loss. The κ-index, which reflects κ free light chain synthesis within the CNS, was demonstrated as a quantitative correlate of chronic B-cell activation, linking the immunological compartmentalization typical of MS to the downstream risk of demyelination and neuroaxonal injury.

In relapsing forms, κ-index values above the laboratory-specific diagnostic threshold (6.4) were associated with greater lesion burden and other CSF indicators of intrathecal inflammation, as well as with an increased probability of NEDA-3 loss over time. When analyzed as a continuous variable, however, κ-index did not show a significant linear association with disease activity, suggesting a potentially non-linear relationship better captured by flexible modeling. These associations reinforce the biological interpretation of the κ-index as a surrogate of sustained humoral activity rather than a transient marker of acute inflammation. The complementary evaluation of serum neurofilament light chain (sNfL) further underscored the potential of combining immune and neuroaxonal biomarkers to capture different yet interconnected dimensions of disease pathophysiology.

From a methodological standpoint, this study advances previous work by adopting a flexible spline-based Cox modeling strategy to explore non-linear associations between κ-index and time-to-event outcomes, revealing a non-linear risk profile that supports its role as a continuous rather than threshold-defined prognostic variable. Together with the integration of sNfL as a linear covariate, this analysis establishes a methodological and conceptual framework for future predictive models grounded on biologically informed, data-driven structures.

Although exploratory and limited by sample size, these results delineate a coherent path for subsequent studies aimed at validating κ-index–based prognostic models in larger, harmonized cohorts. Standardization of analytical procedures and prospective evaluation across disease stages will be essential to define clinically actionable

reference values and to integrate κ-index assessment into individualized treatment algorithms.

In conclusion, this study supports the κ-index as a biologically grounded, clinically relevant marker of intrathecal immune activity and proposes a refined analytical paradigm for its prognostic evaluation. By bridging immunological activation and neuroaxonal injury through combined biomarker modeling, it provides both biological and methodological advances toward the development of precision prognostic frameworks in multiple sclerosis.

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