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Diagnostic and prognostic biomarkers in Multiple Sclerosis: a multidimensional approach

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1. Introduction

Background on Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory and neurodegenerative disease of the Central Nervous System (CNS), which mainly affects young adults between the mid-twenties and late-thirties, with a male to female ratio close to 1:3 (1,2). Recent studies reported a worldwide prevalence of MS by 2.22 million, with a 10.4% increase in the age-standardized prevalence since 1990 (3). Prevalence positively correlates with latitude, being highest in North America (164.6 cases per 100,000 population), western Europe (127/100,000) and Australasia (91.1/100,000), while lowest in eastern sub-Saharan Africa (3.3/100,000), central sub-Saharan African (2.8/100,000) and Oceania (2/100,000) (3).

Despite the cause of MS is still unknown, several factors, including genetic and environmental ones, have been implicated in either triggering the disease or modulating its subsequent course, from the mother's pregnancy to adulthood (4). Despite not being a hereditary disease, a genetic influence on the susceptibility to develop MS exists and one out of eight patients has a family history of MS (5). Heterozygosis for HLA-DRB1*15 and other loci in linkage with this allele confer a three-fold increased risk of MS, while homozygosis a six-fold one. Moreover, more than 150 single nucleotide

polymorphisms (SNPs), variously correlating with the immune function, have been associated with an increased MS susceptibility.

According to several migration studies, the exposure to environmental factors during childhood and adolescence may play a critical role, since an increased risk has been observed in people who immigrate from low- to high-risk regions before adolescence, but not when migration occurs in adulthood (6). The most known factor associated with MS prevalence and incidence is latitude, which correlates with several environmental factors. Particularly, numerous epidemiological studies have suggested that sunshine and vitamin D insufficiency contributes to MS risk in temperate countries (7). Smoking is an established risk factor for the developing of MS, a higher disease severity and a greater risk of conversion in secondary progressive phenotype (SPMS), in a dose-dependent manner (8). Being MS an autoimmune disease, its primary trigger has been thoroughly investigated, especially among infectious agents. Particularly, a 32-fold increased risk of MS had been associated with the Epstein-Barr virus (EBV) infection in a cohort of more than 10 million young adults, while the risk was not increased after infection with other viruses, including the similarly transmitted cytomegalovirus (9).

The diagnosis of MS relies on the demonstration of the typical demyelinating lesions disseminated in space (DIS) and time (DIT),

supported by clinical findings alone or combined with paraclinical findings. The clinical presentation may be extremely different, depending on the localization of the eloquent lesions (Table 1).

Table 1. Presenting symptoms in Multiple Sclerosis patients**.** Adapted from *M. Olek, 2007*

Symptom	Males (%)	Females (%)	Total (%)
Sensory disturbance–limbs	25.1	33.2	30.7
Visual loss	15.1	16.3	15.9
Motor (subacute)	10.4	8.3	8.9
Diplopia	8.5	6.0	6.8
Gait disturbance	8.3	3.2	4.8
Motor (acute)	4.2	4.4	4.3
Balance problems	4.0	2.5	2.9
Sensory disturbance–face	2.5	2.9	2.8
Lhermitte's phenomenon	2.3	1.6	1.8
Vertigo	1.5	1.8	1.7
Bladder disturbance	1.1	0.9	1.0
Limb ataxia	1.3	0.9	1.0
Acute transverse myelopathy	0.6	0.8	0.7
Pain	0.8	0.3	0.5
Unclassified	2.5	2.6	2.5
Polysymptomatic onset	11.9	14.5	13.7

MS is typically suspected after a first episode of neurologic symptoms (clinically isolated syndrome, CIS), often optic neuritis, myelitis, brainstem or cerebellar syndrome, lasting at least 24 hours in absence of fever or infection (10). Symptoms of clinical relapses usually exhibit a subacute onset, worsen over days or weeks reaching a peak severity within 2–3 weeks, and usually recover spontaneously within 4 weeks. The

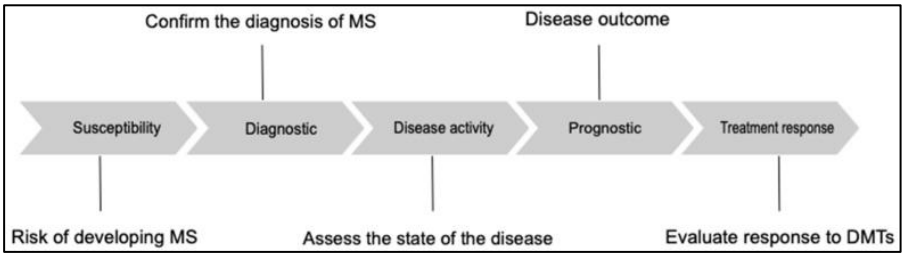
alternation of relapses and remissions, typical of relapsing-remitting MS (RRMS), characterizes the disease in about 85% of cases. According to the natural history of the disease, almost 60% of patients with RRMS exhibit a transition to SPMS within 20-25 years (11). A smaller number of patients, about 10%, exhibits a progressive disease course with deterioration of neurological functions from the beginning (primary progressive MS, PPMS). A distinct condition, named radiologically isolated syndrome (RIS), include those patients who show MRI findings that are strongly suggestive of MS lesions, with no neurological manifestations or other clear-cut explanation (12).

2. Biomarkers in Multiple Sclerosis

Biological markers, or biomarkers, are defined as objectively measurable characteristics reflecting underlying biological or pathological processes. They can be extremely different in nature, including molecular, histologic and radiologic ones, among others (13).

In the field of inflammatory diseases, biomarkers can provide useful information, defining the involved actors of the immune response and potential therapeutic targets, allowing to understand the aetiopathogenesis and to monitor disease-activity and treatment-response (14). Among neurodegenerative diseases, often lacking of effective therapeutic options, biomarkers can be helpful to make earlier diagnosis, to predict prognosis and to identify possible treatment options (15). Since both inflammation and neurodegeneration occur in MS from the very early phases of the disease, the use of biomarkers holds great potential for its diagnosis and management (16). The FDA-NIH Biomarker Working Group proposed a functional classification, which distinguishes among susceptibility, diagnostic, monitoring, prognostic, safety and treatment-response biomarkers (13,17,18) (Fig. 1).

Fig. 1. Types of biomarkers in multiple sclerosis. Adapted from *Nociti et al, 2022*



All categories of biomarkers have been and are still being studied in the field of MS.

Susceptibility biomarkers, which aim to detect individuals at risk of developing MS among healthy subjects, potentially include genetic investigation in first-degree relatives of MS patients (13).

Diagnostic biomarkers can be helpful to support the diagnosis of MS and to exclude differential diagnosis in the appropriate clinical and radiological context. They could also be able to detect patients with CIS and RIS and to identify different clinical phenotypes of MS (14). The biomarkers of intrathecal synthesis, including IgG Index, IgG OCB and κFLC index, are included in this functional group (19).

Monitoring, or disease-activity, biomarkers include most of the currently investigated biomarkers, as nitric oxide (NO) metabolites, osteopontin, C-X-C motif ligand 13 (CXCL13), matrix metalloproteinase-9 (MMP-9), myelin basic protein (MBP), neuronal cell adhesion molecule (N-

CAM), chitinase-3-like-1 (CHI3L1) and neurofilaments light chains (NfL) (19). The detection of high disease-activity in the early phases of MS is crucial to identify the proper therapeutic approach (20,21). Further, correlating with clinical and radiological activity, disease-activity biomarkers can provide indirect evidence of low therapeutic response in patients under disease-modifying treatment (DMT) (22).

Prognostic biomarkers in MS should predict either the risk of relapses or progression or both, and be able to identify transitional forms of RRMS, evolving to SPMS (20). However, the identification of a separate class for prognostic markers is controversial, since a prognostic impact has been attributed to other functional groups of biomarkers, as disease-activity ones (22). Actually, the term "prognostic" is often attributed to those molecules which are expression of axonal damage, astrocyte activation and remyelination, prevailing in the progressive phases of the disease (15). Nevertheless, IgM OCB, neurofilaments heavy chains (NfH) and NfL have been traditionally considered in this group.

Treatment monitoring biomarkers, including safety and pharmacodynamic/response biomarkers, can be useful to personalize treatment strategies and plan therapeutic switches (23). Among them, neutralizing antibodies against natalizumab or interferon-beta and

antibodies against the John Cunningham (JC) virus, are extensively employed in clinical practice (24–26).

Only a small fraction of the potential biomarkers investigated in MS has been validated, and an even lower number has been implemented in clinical practice so far (Table 2).

The clinical validity and usefulness of a biomarker is assessed through a multistage process, going from preclinical exploratory studies to retrospective and then prospective ones, to disease burden reduction studies (27). Further, the level of evidence for a given biomarker is related to the number of supporting studies exploring the independence of the results in independent cohorts and the number of patients included (28).

Only those biomarkers whose validity relies on strong level of evidence, whose detection is reproducible and cost-effective, and whose impact on the diagnostic-therapeutic workout is significant, are lastly implemented in clinical practice (14).

The cerebrospinal fluid (CSF), among body fluids, is the main and ideal source of potential biomarkers for MS, due to both anatomic and physiological reasons (15). Indeed, its composition reflects the impairment of brain metabolism, the breakdown of the blood-brain

barrier and many ongoing processes occurring in the CNS with consequent production of catabolites (29). Nevertheless, serum samples are more and more used and explored as a source of biomarkers (22), due to the invasiveness of CSF withdrawal through lumbar puncture, which is not suitable for repeated measurements over time.

Table 2. Clinically useful and validated CSF biomarker in MS. Adapted from *Toscano et al 2020*

	Status	Function	Evidence
IgG OCB	Clinically useful	Diagnostic	Nearly 86% specificity and more than 95% sensitivity for the diagnosis of MS[19]. Implemented in 2017 McDonald criteria as indicator of DIT[20]
		Prognostic for conversion	Associated with higher risk of conversion in MS when detected in CIS[28,29] and RIS[30-32]
IgG index	Clinically useful	Diagnostic	Positive values found in 70-80% of MS patients[18]. Useful as a complementary tool, without replacing CSF IgG OCB[41]
		Disease-activity	Associated with MRI activity[43]
		Prognostic for conversion	Associated with higher risk of conversion in MS when detected in CIS[43]
		Prognostic for progression	Associated with disability progression[44]
KFLC	Validated	Diagnostic	Useful for the diagnosis of MS[49,51,53,54,58]. Increased levels detected in MS patients with no IgG OCB[50,55,62]
		Prognostic for conversion	Associated with higher risk of conversion in MS when detected in CIS[43,60]
		Prognostic for progression	Associated with disability progression[60,64-66]
IgM OCB	Validated	Disease-activity	Associated with aggressive disease course[248,250]
		Prognostic for conversion	Lipid-specific IgM OCB are associated with higher risk of conversion in CIS patients[252,253]
		Prognostic for progression	Associated with disability progression and conversion to SPMS[247,248,256]
		Treatment-response	Lipid-specific IgM OCB predict a decreased response to IFN-β[256]
N-CAM	Validated	Diagnostic	Lower levels detected in MS patients and in PPMS compared with RRMS ones. Considered as an indicator of poor remyelination and repair[180,181]
		Disease-activity	Increased levels detected after relapses, especially under steroid treatment, and related to clinical remission[183]
CHI3L1	Validated	Diagnostic	Increased levels in MS and NMO patients[185,188,189]
		Prognostic for conversion	Associated with higher risk of conversion to MS in CIS patients[190,192]
		Disease-activity	Increased levels associated with higher clinical and MRI disease-activity[190,193]
		Treatment-response	Increased levels in non-responder patients under IFN-β treatment compared with responders[193]
CXCL13	Validated	Diagnostic	Higher levels in MS patients compared with controls, though low specificity[126-128]
		Prognostic for conversion	Associated with higher risk of conversion to MS in CIS patients[130]
		Disease-activity	Associated with clinical and radiological activity[126,127]. Decreased levels after steroid treatment[127]
		Treatment-response	Decreased levels after treatment with NTZ[127,132], RTX[129,131]

Table 2 (continued).

NFs	Validated	Prognostic for conversion	In RIS increased CSF NF-L are an independent risk factor for the conversion into CIS and MS, with greater values related to shorter times of conversion[32]. Associated with higher risk of conversion to MS in CIS patients[224,234]
		Disease-activity	Double NF-L levels in relapsing patients compared with remitting ones[228]. CSF NF-L levels correlate with NEDA-3, MRI activity and brain atrophy[31]. Serum NF-L in early phases contributed to predict the lesion load and brain volume loss over a period of 10 years[238]
		Prognostic for progression	High NF-L concentrations associated with progression in both clinically stable patients and relapsing ones[226,227]. In CIS patients with optic neuritis, CSF NF-L predicted long-term cognitive and physical disability over a follow-up period ranging between 9-19 years[235]. Higher NF-H levels in SPMS patients[224,225]
		Treatment-response	NF-L concentrations decreased after 12-24 months of immunosuppressive therapy in active progressive MS patients[239], after switching from first-line therapies to fingolimod[240] and after 12 months of NTZ[241,242]
MBP	Validated	Disease-activity	Higher values detected in active RRMS compared with stable patients and progressive MS. Increased levels in MS are temporally related to relapses and detectable up to 5-6 weeks after, with greater values in polysymptomatic and severe exacerbations[158,159,166-168]. Reduced levels after steroid treatment[168,169]
GFAP	Validated	Prognostic for progression	Elevated levels in MS compared with controls[265-267], with higher values in patients with EDSS greater than 6.5[266]. Associated with greater EDSS score, longer disease duration and progressive course[268]. Increased levels of GFAP in MS predictive for the disability achieved 8-10 years later[267]
		Disease-activity	Associated with MRI parameters as infratentorial chronic lesion load and the intensity of Gd+ in both CIS and RRMS patients[269]
MMP-9	Validated	Disease-activity	Elevated values during clinical relapses, related to a greater number of MRI Gd+ lesions[144]. Higher values in MS compared with controls and in RRMS compared with PPMS[148]
		Treatment-response	Decreased levels after treatment with IFN-β[152-154] and NTZ[155]
OPN	Validated	Diagnostic	Significantly greater levels in MS patients compared with controls[102,107,108,110]
		Disease-activity	In RRMS patients, higher levels detected in active disease compared with stable disease and during relapses compared with remission phases[100-103]
NO metabolites	Validated	Disease-activity	Increased levels in body fluids of MS patients, particularly RRMS compare with SPMS. Higher values detected during relapses[78,90]
MRZ reaction	Validated	Diagnostic	A humoral response against at least 2 of 3 viruses is detected in 78% of patients with MS with high specificity[73]
		Prognostic for conversion	Associated with higher risk of conversion in MS when detected in CIS[69,70]

MS: multiple sclerosis; CIS: clinically isolated syndrome; RIS: radiologically isolated syndrome; MRI: magnetic resonance imaging; OCB: oligoclonal bands; RRMS: relapsing-remitting multiple sclerosis; SPMS: secondary progressive multiple sclerosis; NMO: neuromyelitis optica; NEDA: no evidence of disease-activity; N-CAM: neuronal cell adhesion molecule; CHI3L1: chitinase-3-like-1; MBP: myelin basic protein; GFAP: glial fibrillary acidic protein; Gd+: gadolinium-enhancing; MMP-9: matrix metalloproteinase-9; CXCL13: C-X-C motif ligand 13; NFs: neurofilaments; NF-L: light chains of neurofilaments; NF-H: heavy chains of neurofilaments; OPN: osteopontin; NO: nitric oxid; MRZ: measles-rubella-varicella; NTZ: natalizumab; RTX: rituximab

2.1 Biomarkers of intrathecal synthesis and diagnosis of Multiple Sclerosis

The diagnosis of MS relies on clinical findings, eventually supported by paraclinical investigations, providing evidence of DIS and DIT (10). Two clinical attacks are sufficient to formulate a clinical diagnosis of RRMS, without needing additional data. In this case, at least one relapse has to be corroborated by findings on neurological examination, or provided by magnetic resonance imaging (MRI), consistent with demyelination in the area of CNS presumably implicated in the historical report of neurological symptoms (10).

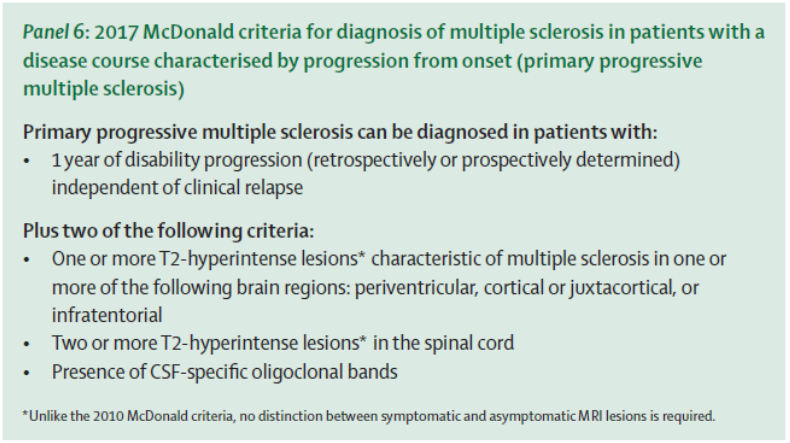
For patients who experienced a first clinical episode (CIS), radiological or laboratory data can be used to provide evidence for DIS and DIT, according to the latest revision of McDonald's criteria (30) (Fig. 2).

Fig. 2. The 2017 McDonald criteria for diagnosis of multiple sclerosis in patients with an attack at onset. Adapted from *Thompson et al 2018*

	Number of lesions with objective clinical evidence	Additional data needed for a diagnosis of multiple sclerosis
≥2 clinical attacks	≥2	None*
≥2 clinical attacks	1 (as well as clear-cut historical evidence of a previous attack involving a lesion in a distinct anatomical location†)	None*
≥2 clinical attacks	1	Dissemination in space demonstrated by an additional clinical attack implicating a different CNS site or by MRI‡
1 clinical attack	≥2	Dissemination in time demonstrated by an additional clinical attack or by MRI§ OR demonstration of CSF-specific oligoclonal bands¶
1 clinical attack	1	Dissemination in space demonstrated by an additional clinical attack implicating a different CNS site or by MRI‡ AND Dissemination in time demonstrated by an additional clinical attack or by MRI§ OR demonstration of CSF-specific oligoclonal bands¶

Differently, the diagnosis of PPMS requires at least a year-long history of neurological progressive deterioration, prospectively or retrospectively assessed, supported by radiological and laboratory data (30) (Fig. 3).

Fig.3. 2017 McDonald criteria for diagnosis of multiple sclerosis in patients with a disease course characterized by progression from onset (primary progressive multiple sclerosis). Adapted from *Thompson et al 2018*



Panel 6: 2017 McDonald criteria for diagnosis of multiple sclerosis in patients with a disease course characterised by progression from onset (primary progressive multiple sclerosis)

Primary progressive multiple sclerosis can be diagnosed in patients with:

- 1 year of disability progression (retrospectively or prospectively determined) independent of clinical relapse

Plus two of the following criteria:

- One or more T2-hyperintense lesions* characteristic of multiple sclerosis in one or more of the following brain regions: periventricular, cortical or juxtacortical, or infratentorial
- Two or more T2-hyperintense lesions* in the spinal cord
- Presence of CSF-specific oligoclonal bands

*Unlike the 2010 McDonald criteria, no distinction between symptomatic and asymptomatic MRI lesions is required.

The introduction of MRI in the 2001 McDonald's criteria, and in subsequent revisions, has strengthened the possibility of making an early diagnosis of MS, providing evidence of DIS and DIT in patients with CIS (31). The inflammatory lesions appear as focal hyperintensities in T2-weighted and FLAIR images, and are "enhanced" after gadolinium administration in post-contrast T1-weighted scans in the acute phase.

Some acute lesions may persist or become hypointense on T1-weighted scans (black holes) (32). Typical MS-lesions are round or ovoid in shape, at least 3 mm in their long axis and visible in at least two consecutive slices. Periventricular, infratentorial, juxtacortical/cortical and subcortical lesions in the brain and lesions in the spinal cord are usually detected in patients with MS and the presence of lesions in these areas contributes to DIS according to the current diagnostic criteria (30). Differently, lesions of the optic nerve, well observed in 2D STIR and post-contrast fat-suppressed T1-weighted coronal and axial MRI scans, are suggestive of MS but currently do not contribute to DIS (32).

Although being highly sensitive, MRI is not equally specific in detecting MS lesions, especially in older patients with vascular risk factors or patients with other comorbidities (32). The use of neurophysiological tests, including visual (VEPs), brainstem auditory (BAEPs), somatosensory (SSEPs) and motor (MEP) evoked potentials, despite not included in the diagnostic criteria, can be helpful in the diagnostic workout of MS, supporting the evaluation of DIS (33–35).

2.1.1 IgG Oligoclonal Bands

IgG OCB are identified when at least two bands of IgG are detected in CSF with no corresponding bands in the serum pattern, thus implying an intrathecal synthesis due to a clonally restricted B-cell activity (36). CSF IgG OCB are detected in up to 90% of patients with MS and in nearly 70% of patients with CIS (37). Among several techniques, isoelectric focusing followed by immunofixation in parallel of CSF and serum samples is mainly employed to their detection due to a high sensitivity (38,39).

CSF IgG OCB analysis is currently considered a more reliable test than any quantitative assessments of intrathecal synthesis, with values of sensitivity between

83-95% and specificity between 86-95% (39–42). Its validity relies on numerous confirmatory studies conducted on more than 200 patients, thus providing a strong level of evidence (28). However, CSF IgG OCB do not represent a pathognomonic finding of MS, since other inflammatory diseases with neurological involvement can be associated to their presence, as neuromyelitis optica, disseminated encephalomyelitis, HTLV-1 infection, AIDS-related encephalitis, systemic lupus erythematosus, brucellosis, sarcoidosis, sclerosing

subacute encephalomyelitis (36).

In 2017, the latest revised McDonald's criteria gave great significance to CSF IgG OCB as a substitute for DIT, increasing sensitivity in the diagnosis of RRMS in patients with a first clinical event (30,43). CSF IgG OCB were first introduced as a diagnostic biomarker in Poser criteria (1983), in order to formulate the diagnosis of "laboratory supported" definite/probable MS, as an alternative to a quantitative measurement of intrathecal synthesis (44). The 2001 McDonald's criteria (31) and the 2005 Polman's revision (45) considered CSF IgG OCB as a support to the demonstration of DIS, together with the presence of at least two MRI lesions consistent with MS. While not being included in 2010 revised criteria (10), CSF analysis with IgG OCB detection kept on being a common step of the diagnostic workout for MS in the following years, particularly due to the high specificity of this biomarker, which can be helpful to distinguish between MS and mimics at early stages (46). Additionally, CSF IgG OCB have continued to be considered as a diagnostic criterium for the diagnosis of PPMS (10,30,31,45).

Besides being the result of intrathecal inflammation, CSF IgG OCB seem to play a pathogenic role in MS, directly and indirectly perpetuating the inflammatory damage through the chronic stimulation of microglia via

immunoglobulin and immunocomplexes, even after the acute perivascular inflammation has stopped (47). In addition to its diagnostic value for RRMS and PPMS, the detection of CSF IgG OCB in patients with CIS increases the risk of conversion to clinically definite MS with a negative predictive value (NPV) of 88% (48). In a prospective study involving 572 patients with CIS, the presence of CSF IgG OCB almost doubled the risk of a second relapse, regardless of baseline MRI, without affecting disability outcomes during a follow-up of 50 months (49). Indeed, it does not seem that the presence of CSF IgG OCB is associated with more aggressive disease course or faster disease progression to SPMS (50,51). Although CSF IgG OCB also increase the risk to develop MS in patients with RIS (52–54), specific criteria have not been established in the 2017 latest revision (30).

2.1.2 IgG Index

IgG Index is calculated as the ratio between CSF/serum IgG and CSF/serum albumin quotient (QAlb) (55), representing a quantitative evaluation of intrathecal synthesis in MS. Values higher than 0.7, which is almost universally considered as the best cut-off value, are detected in 70-80% of patients with clinically definite MS and exhibit a positive predictive value (PPV) of 60% for the diagnosis of demyelinating CNS disease (38,56). In previous studies, increasing IgG index values exhibited an almost linear correlation with greater probabilities of MS diagnosis and a good correlation with the detection of IgG OCB (57).

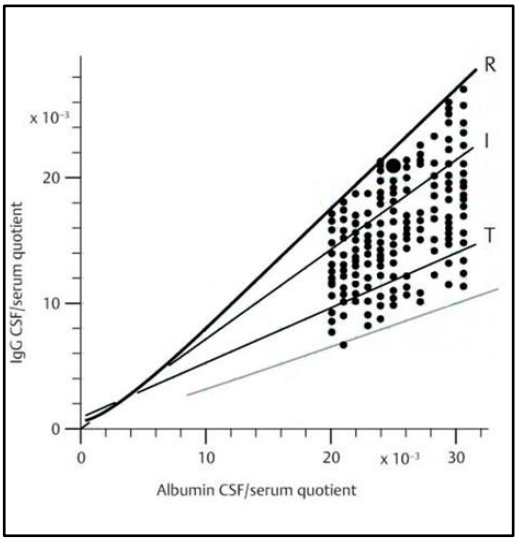
IgG Index is currently used in clinical practice as a complementary screening test for the diagnosis of MS, with the advantage of an easy and rapid detection, though it cannot replace CSF IgG OCB in terms of diagnostic accuracy (57,58). In this respect, despite previous diagnostic criteria considered IgG Index as an alternative method to IgG OCB analysis (31,44,45), the 2017 revision of McDonald criteria and two consensus statements established that IgG index and other quantitative assessments can be used only as complementary tests, being less sensitive than the qualitative detection of CSF IgG OCB (30,38,39).

Nephelometric immunoassays are usually employed to measure albumin

concentrations in CSF and serum, in order to calculate a ratio (QAlb) which reflects the integrity of the blood-CSF barrier (38,39). Similarly, a CSF/serum IgG quotient is usually calculated in order to reduce inter-individual variability in IgG concentrations (38). Several indexes have been computed applying different mathematical models, including Tourtellotte's, Reiber's, Link's and intrathecal IgG fraction (59,60) (Fig. 4). Despite IgG index is the most commonly used in clinical practice worldwide, other indexes employing hyperbolic mathematical functions, as Reiber's, are considered more accurate, resulting in a minor number of false positives (38,39,60).

In previous studies, IgG Index showed a sensitivity of 43% and a specificity of 64% in predicting the conversion of CIS to clinically definite MS, with PPV of 53% and NPV of 54% (61). In a retrospective study involving 149 patients with CIS and MS, IgG index strongly correlated with the detection of new cerebral lesions at MRI scans and proved to be an independent predictor of future MRI activity (62).

Fig. 4. Comparison of different discrimination lines (upper border of the reference range for blood-derived IgG in CSF) to detect intrathecal IgG Quotient diagrams. Adapted from *Reiber and Peter, 2001*.



R, Reiber's hyperbolic discrimination line. I, Link's IgG Index. T, Tourtellotte's IgG synthesis rate.

2.1.3 κ Free Light Chains Index

κ Free Light Chains (κFLC) are components of human Ig structure, which is a tetramer composed by an identical pair of FLC (kappa or lambda) and an identical pair of heavy chains (γ, δ, α, μ and ε) (63). Consequently, κFLC tend to accumulate in inflammatory disease of CNS, as a result of the intrathecal humoral activity of plasma cells, and increased concentration have been reported in the CSF of patients with MS (42,64,65).

The use of a ratio between CSF/serum κFLC and QAlb is currently considered the best method to represent the intrathecal synthesis of κFLC. As for IgG index, nephelometry, ELISA, or Western blot can be employed to measure κFLC concentrations in CSF and serum, assuring a rapid, easy and cost-effective analysis (65,66).

κFLC index potential as a diagnostic biomarker has been explored in previous studies, but there is no consensus on the threshold that should be adopted, with consequent difficulty in comparing results from different studies. Comprehensively, most of studies reported cut-off values ranging from 4.25 to 12.3 (42,67–72), reporting higher sensitivity but less specificity values for κFLC index compared with CSF IgG OCB for the diagnosis of MS. κFLC index may thus replace IgG index as a

first-line screening test, but there are still some concerns about the need to perform both κFLC and IgG OCB in patients with suspected MS or to use them sequentially (42,73). Since κFLC index exhibited not only good diagnostic accuracy, but also the advantage of being a quantitative measure, assessed with a rapid, operator-independent and cost-effective method, the implementation of this diagnostic biomarker in clinical practice could be of great work and should be closely considered.

Although κFLC proved to be highly accurate for the diagnosis of MS, its implementation is partially hampered by the inclusion of IgG OCB in the latest revision of McDonald's criteria, as a substitute for DIT (30). Nevertheless, maybe κFLC itself should be considered in the future revisions of diagnostic criteria, since it not only prognosticated the conversion to clinically definite MS within 2 years in CIS patients (61,74), but also exhibited higher sensitivity and only slight lower specificity than IgG OCB in predicting the occurrence of new T2 lesions and clinical relapses in CIS and RIS (75).

Article 1

A dynamic interpretation of κFLC index for the diagnosis of multiple sclerosis: a change of perspective

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Abstract

Background: Previous studies attempted to define the best threshold for κ free light chains (κFLC) index, confirming higher sensitivity (Se) but less specificity (Sp) compared with IgG oligoclonal bands (OCB) for the diagnosis of MS.

Objective: To evaluate the diagnostic accuracy of different κFLC index intervals in a miscellaneous cohort of neurological patients, proposing a procedural flowchart for MS diagnosis.

Methods: We analyzed data from 607 patients diagnosed with MS (179), CIS (116), other inflammatory (94) or non-inflammatory neurological diseases (218). Measures of diagnostic accuracy were reported for different potential thresholds of κFLC index, and for IgG OCB and IgG index. Binary logistic regression was used to calculate the odds of being diagnosed with MS based on each increase of κFLC index.

Results: CSF IgG OCB showed 72.2% Se (CI 95% 68.4–75.7) and 95.2% Sp (CI 95% 93.1–96.7) in discriminating between MS/CIS and controls,

with an AUC of 0.84 (CI 95% 0.80–0.87). The highest diagnostic accuracy was reported for κFLC index cut-off of 5.0 (Se = 85.4%, Sp = 90.4%, AUC = 0.88), while a threshold of 11.0 exhibited higher Sp (95.5%, 95% CI 93.1–97.1) than IgG OCB. AUCs for all thresholds between 4.25 and 6.6 were not significantly different from each other,
but were significantly higher than the AUC of IgG OCB ($p < 0.05$). The odds of being diagnosed with MS/CIS increased by 17.1% for each unit increase of κFLC index (OR = 1.17; 95% CI 1.12–1.23; $p < 0.001$).

Conclusion: κFLC index performed better than CSF IgG OCB in supporting the diagnosis of MS/CIS, with the advantage of being a cost-effective and quantitative analysis.

Keywords: Multiple sclerosis; Diagnosis; κ free light chain; Case-control study; Biomarkers; Oligoclonal bands

Introduction

CSF κ free light chains (κFLC) and the resulting κFLC index, calculated as the ratio between CSF/serum κFLC and albumin quotient, have been explored for years as an expression of the intrathecal humoral activity of plasma cells and a diagnostic biomarker for Multiple Sclerosis (MS) [1–3]. Several studies supported the high diagnostic accuracy of κFLC index, even when compared with CSF IgG Oligoclonal Bands (OCB), whose use in clinical practice as a diagnostic biomarker for MS relies on a strong level of evidence [4, 5]. Particularly, κFLC index has shown a higher sensitivity (Se) but a less specificity (Sp) compared with CSF IgG OCB in discriminating between MS and other neurological diseases [3, 6–11]. Noteworthy, κFLC index proved to be increased in up to 25% of MS patients with no evidence of CSF IgG OCB, who represent almost 5% of MS [7, 12, 13]. However, a recent meta-analysis highlighted no significant differences between these biomarkers in terms of diagnostic accuracy [14]. Different potential thresholds have been identified for κFLC index in literature, ranging from 4.25 [7] to 12.3 [6], representing a main limitation in comparing results from different studies. Since other inflammatory diseases of the central nervous system (CNS) can be characterized by a

certain amount of intrathecal synthesis [15], the choice of low cut-off values, though maximizing sensitivity, is not suitable to distinguish between MS and other mimics [16]. Moreover, the proposal to use two different κFLC index thresholds to distinguish MS from inflammatory or non-inflammatory diseases [3] is reasonable but difficult to implement in clinical practice, since CSF analysis is often required precisely to clarify the potential inflammatory nature of neurological symptoms.

It could be argued that the interpretation of a κFLC index as a dichotomous variable, by choosing a rigid threshold, is likely to minimize the potentialities of this diagnostic biomarker, which has the inherent advantage to be a quantitative measure in contrast with the detection of CSF IgG OCB, which is based on a qualitative analysis. Possibly, a more dynamic interpretation of κFLC index, relying on a risk stratification or identification of different value ranges, can allow clinicians to restrict the use of CSF IgG OCB analysis to fewer cases, thus saving time, reducing costs and assuring an operator-independent evaluation.

For this purpose, we evaluated the diagnostic accuracy of CSF IgG OCB, IgG index and different cut-off values of κFLC index in a miscellaneous cohort of neurological patients, finally proposing a diagnostic procedural flowchart for the diagnosis of MS.

Patients and methods

Study population

We consecutively enrolled 615 patients admitted to the Neurology Clinic of the University Hospital "Policlinico G. Rodolico" of Catania, who underwent a diagnostic lumbar puncture (LP) in the period between 1st January 2017 and 7th February 2022. Patients were classified according to the diagnosis into four groups: MS, CIS, inflammatory neurological diseases other than CIS or MS (OIND), not inflammatory neurological diseases (NIND). MS and CIS were diagnosed according to the 2010 revision of McDonald's criteria(10). The study was approved by our local ethical committee. All patients signed a written informed consent before the execution of LP to authorize the procedure and to allow data collection and use for study purpose.

Cerebrospinal fluid and serum samples collection and analysis

All patients underwent LP and venipuncture as part of their diagnostic workup. LP were performed at the bedside, using 25 Gauge atraumatic needles whenever possible, or 22 Gauge needles otherwise. For each

patient, 2 ml of cerebrospinal fluid (CSF) divided into 0.5 ml aliquots and a serum 0.5 mL aliquot were collected in sterile polypropylene tubes and sent to the Central Laboratory of our University Hospital in order to be analysed. CSF and serum paired samples were analysed in order to determine κFLC index, IgG index and CSF IgG OCB.

κFLC index was determined by using an automated nephelometric immunoassay (Freelite LK016, The Binding Site Group Ltd). Monoclonal antibodies were used for the detection of FLC in serum and CSF. A 1:300 dilution was used for serum, while CSF is not diluted by default, but progressively increasing dilutions were used for progressively higher IgG concentrations (only for IgG > 5.0 mg/dL). κFLC index was calculated as the ratio between κFLC CSF/serum quotient (QKFLC) and albumin CSF/serum quotient (Qalb).

IgG index was calculated as the ratio between CSF/serum IgG corrected for Qalb, determined by nephelometry. We considered a threshold of 0.7, which is the most often used cut-off in clinical practice(76,77).

CSF IgG OCB were detected by agarose gel IEF followed by immunoblotting (Helena Biosciences SAS IgG IEF kit), considering the presence of patterns 2 (≥ 2 IgG OCB bands in CSF) or 3 (IgG OCB bands

in CSF and serum with at least 2 additional bands in CSF) as positive results(39).

Statistical analysis

Data were analysed with SPSS© (IBM Corp. IBM SPSS Statistics for Windows, Version 26.0). After assessed for normality with the Kolmogorov-Smirnov test, median and interquartile range (IQR) were provided for not normally distributed continuous variables. The Mann-Whitney U Test (U) was used to compare medians between groups. Categorical variables were reported as frequencies and percentages. Chi-square test (χ^2) and Cramer's phi (φ) coefficient were used to compare categorical variables distributions among groups. Se, Sp, positive predictive value (PPV) and negative predictive value (NPV) were calculated for each biomarker. The Area Under the Curve (AUC) of the Receiver Operator Characteristic (ROC) curve was calculated to assess the diagnostic accuracy of the biomarkers. A z-test was used to compare AUCs of different κFLC index values and IgG OCB in a paired design(78).

Youden's index was calculated for the chosen cut-off values for each biomarker and for other cut-off values tested in other studies, using the

formula J=Se + Sp -1. The point-biserial correlation coefficient (r_{pb}) was used to measure the association between continuous and dichotomous variables. Binary logistic regression was used to analyze the relationship between κFLC index and the probability of being diagnosed with MS/CIS, with IgG Index and IgG OCB as covariates. A p value of <0.05 was considered significant for all tests, which were 2-sided.

Results

Patients' characteristics

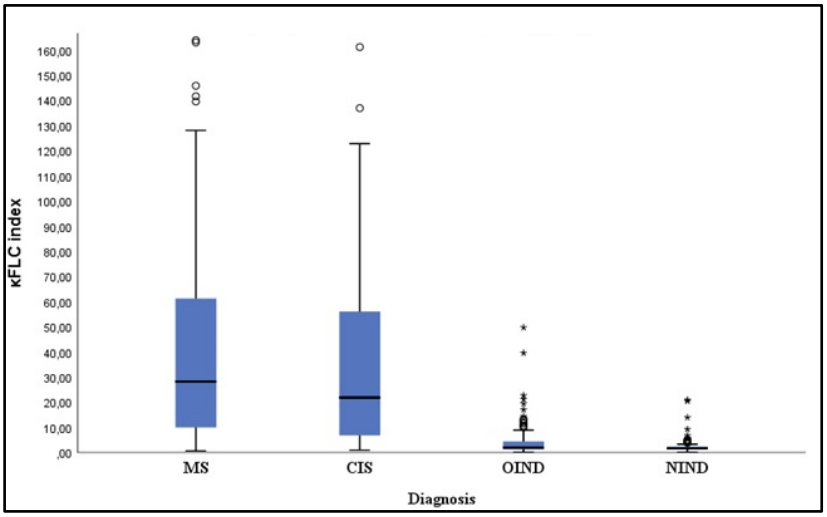
We analysed paired CSF and serum samples of 607 patients (Table 1, Fig.1). Among them, 179 patients were diagnosed with MS and 116 with CIS, while 94 and 218 patients were respectively affected by OIND and NIND. Patients with MS and CIS were considered together as cases, while those diagnosed with OIND and NIND were comprehensively considered as controls (Table 2).

Table 1. Characteristics of the study population and results from cerebrospinal fluid analysis (607 patients). MS: multiple sclerosis; CIS: clinically isolated syndrome; OIND: other inflammatory neurological diseases; NIND: not inflammatory neurological diseases; OCB: oligoclonal bands; IQR: interquartile range; κFLC: kappa free light chains.

	MS	CIS	OIND	NIND
N	179	116	94	218
Female *N* (%)	107 (59.8)	85 (72.4)	52 (55.3)	121 (55.5)
Age at diagnosis mean ± SD	40.0 ± 13.1	38.9 ± 14.3	43.1 ± 11.2	61.3 ± 9.8
IgG OCB *N* (%)	141 (78.8)	72 (62.1)	12 (12.8)	3 (1.4)
IgG index (median, IQR)	0.68 (0.56–0.88)	0.59 (0.51–0.84)	0.52 (0.47–0.60)	0.48 (0.44–0.51)
κFLC index (median, IQR)	28.19 (9.81–61.52)	21.84 (6.52–56.19)	1.94 (1.37–4.63)	1.67 (1.36–2.28)

MS multiple sclerosis, *CIS* clinically isolated syndrome, *OIND* other inflammatory neurological diseases, *NIND* not inflammatory neurological diseases, *OCB* oligoclonal bands, *IQR* interquartile range, *κFLC* kappa free light chains

Figure 1. Box plots of κFLC index values according to diagnosis.



κFLC: kappa free light chains; MS: multiple sclerosis; CIS: clinically isolated syndrome; OIND: other inflammatory neurological diseases; NIND: non-inflammatory neurological diseases.

Table 2 Diagnosis of patients with NIND and OIND

Diagnosis	*N*
NIND	**218**
CVD	69
Headache	20
Compressive myelopathy	22
Epilepsy	6
Neurodegenerative	44
Noninflammatory neuropathies	28
Psychogenic	14
Aspecific sensory symptoms	15
Diagnosis	*N*
OIND	**94**
NMOSD	14
Inflammatory neuropathies	36
Autoimmune encephalitis	3
Infectious encephalitis	6
Infectious myelopathies	13
Other inflammatory diseases	22

Bold values indicate the total number of patients for NIND (this group amounts to 218 patients and include the underlying categories in the table: CVD, Headache etc) and OIND (this group amounts to 94 patients and include the categories below: NMOSD, Inflammatory neuropathies, etc.)

NIND not inflammatory neurological diseases, *CVD* cerebrovascular diseases, *OIND* other inflammatory neurological diseases, *NMOSD* neuromyelitis optica spectrum disorder

Diagnostic accuracy of CSF IgG OCB and IgG Index for the diagnosis of MS/CIS

Among a population of 607 patients (295 MS/CIS, 312 controls), 228 (37.6%) exhibited the presence of CSF IgG OCB. IgG OCB were positive in 213 MS/CIS patients (72.2%) and only in 15 controls (4.8%) (χ^2=293.7, p<0.001). Notably, 82 out of 295 MS/CIS patients (27.8%) were OCB-negative.

CSF IgG OCB showed 72.2% Se (CI 95% 68.4-75.7) and 95.2% Sp (CI 95% 93.1-96.7) in discriminating between MS/CIS and controls, with PPV

of 93.4% (CI 95% 91.1-95.2) and NPV of 78.4% (CI 95% 74.8-81.5) (Table 3). The diagnostic accuracy of CSF IgG OCB was defined by an AUC of 0.84 (CI 95% 0.80-0.87) and by J=0.67.

IgG Index values in MS/CIS patients (median=0.65, IQR=0.53-0.87) were significantly higher than in controls (median=0.49, IQR=0.45-0.54) ($p<0.001$).

IgG Index exhibited 44.4% Se (CI 95% 38.5-50.4) and 95.2% Sp (CI 95% 93.1-96.7), with PPV of 89.7% (CI 95% 87.0-92.0) and NPV of 64.4% (CI 95% 60.5-68.2) for the diagnosis of MS/CIS. The AUC was equal to 0.70 (CI 95% 0.66-0.74) and J=0.39. There was a moderate positive correlation between IgG Index and IgG OCB ($r_{pb}=0.53$, n=607, $p<0.001$). The odds of being diagnosed with MS/CIS was 5-fold increased (OR=5.04; 95% CI 2.41-10.56; $p<0.001$) when IgG OCB were detected, while IgG Index was not a significant risk predictor for the same outcome.

Table 3 Diagnostic performance of different thresholds of κFLC index and IgG OCB for the diagnosis of MS/CIS in our study population (607 patients)

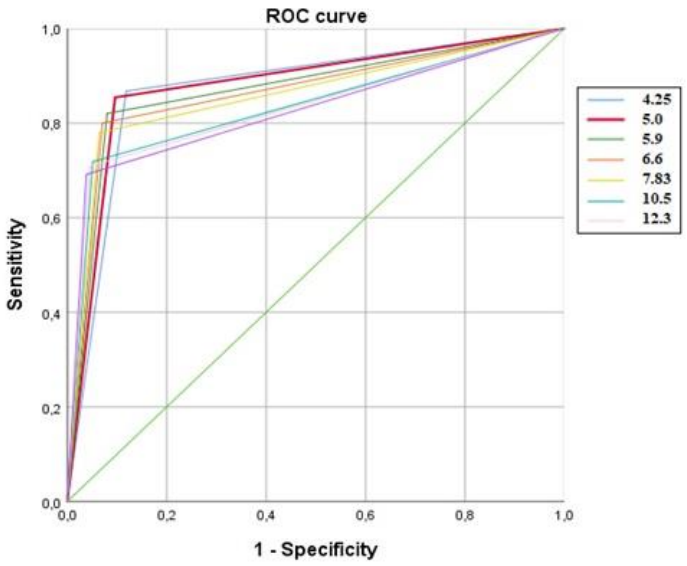
	Se, % (95% CI)	Sp, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)	*J*	AUC (95% CI)
4.25	86.8 (83.8–89.3)	88.1 (85.2–90.6)	87.4 (84.4–89.9)	87.6 (84.6–90.0)	0.75	0.875 (0.844–0.905)
5.00	85.4 (82.3–88.1)	90.4 (87.7–92.6)	89.4 (86.6–91.6)	86.8 (83.8–89.3)	0.76	0.879 (0.849–0.909)
5.90	82.0 (78.7–85.0)	92.0 (89.5–94.0)	90.6 (88.0–92.8)	84.4 (81.2–87.2)	0.74	0.870 (0.839–0.901)
6.60	80.0 (76.5–83.1)	92.9 (90.5–94.8)	91.5 (88.9–93.5)	83.1 (79.8–85.9)	0.73	0.865 (0.833–0.869)
7.83	78.0 (74.4–81.2)	93.6 (91.3–95.3)	92.0 (89.5–94.0)	81.8 (78.4–84.7)	0.72	0.858 (0.825–0.890)
10.5	71.9 (67.4–76.0)	94.9 (92.7–96.5)	93.0 (90.6–94.8)	78.1 (74.6–81.3)	0.67	0.834 (0.799–0.868)
11.0	73.2 (68.3–77.7)	95.5 (93.1–97.1)	90.3 (87.3–92.7)	86.1 (82.7–89.0)	0.69	0.832 (0.797–0.866)
12.3	69.2 (65.3–72.8)	96.2 (94.2–97.5)	94.4 (92.2–96.1)	76.7 (73.1–80.0)	0.65	0.827 (0.791–0.862)
IgG OCB	72.2 (68.4–75.7)	95.2 (93.1–96.7)	93.4 (91.1–95.2)	78.4 (74.8–81.5)	0.67	0.84 (0.80–0.87)

κFLC kappa free light chains, *OCB* oligoclonal bands, *MS* multiple sclerosis, *CIS* clinically isolated syndrome, *Se* sensitivity, *Sp* specificity, *VPP* positive predictive value, *NPV* negative predictive value, *J* Youden's index, *AUC* area under the curve, *CI* confidence interval

Diagnostic accuracy of κFLC index for the diagnosis of MS/CIS

κFLC index in MS/CIS patients (median=26.3, IQR=9.1-59.5) was significantly higher than in controls (median=1.7, IQR=1.4-2.5) ($p<0.001$). Measures of diagnostic accuracy for different κFLC index thresholds proposed in literature and ROC curves are reported in Table 3 and Fig. 2.

Figure 2. ROC curves for different potential thresholds of κFLC index.



Among different thresholds proposed in literature, the cut-off value of 5.0 emerged as the one which maximized the AUC (0.879, CI 95% 0.849-

0.909) and the J (0.75) in our study population (Table 3). Se and Sp were respectively 85.4% (CI 95% 82.3-88.1) and 90.4% (CI 95% 87.7-92.6), with PPV of 89.4% (CI 95% 86.6-91.6) and NPV of 86.8% (CI 95% 83.8-89.3). κFLC index >5.0 was detected in 43 out of 82 (52.4%) OCB-negative and in 209 out of 213 (98.1%) OCB-positive patients with MS/CIS. Among all proposed thresholds, κFLC index specificity exceeded that of other diagnostic biomarkers for a cut-off of 11.0 (Sp=95.5%, CI 95% 93.1-97.1), and PPV peaked to 90.3% (CI 95% 87.3-92.7), though reducing Se (73.2%, CI 95% 68.3-77.7) and NPV (86.1%, CI 95% 82.7-89.0).

AUCs for all thresholds between 4.25 and 6.6 were higher than the AUCs of cut-off ≥10.5, while they were not significantly different from each other (Table 4). The interval of κFLC index values between 4.25 and 6.6 was characterized by Se values between 80.0-86.8% and Sp between 88.1-92.9% (Table 3). AUCs for thresholds between 4.25 and 6.6 were significantly higher than the AUC of IgG OCB (Table 5). Positive κFLC index values, according to the chosen threshold between 4.25-6.6, were detected in 37.8-54.9% of OCB-negative patients with MS/CIS.

Table 4 Paired comparison between AUCs of different κFLC index thresholds for the diagnosis of MS/CIS in our study population (607 patients)

κFLC index	*z*	*p*	Delta AUC	95% CI	
				Lower limit	Upper limit
4.25–5.0	− 0.824	0.410	− 0.004	− 0.015	0.006
4.25–5.90	0.545	0.586	0.004	− 0.012	0.021
4.25–6.60	1.036	0.300	0.010	− 0.009	0.029
4.25–7.83	1.606	0.108	0.017	− 0.004	0.037
4.25–10.5	3.252	**0.001**	0.041	0.016	0.066
4.25–11.0	3.295	**0.001**	0.043	0.017	0.068
4.25–12.3	3.556	**0.000**	0.048	0.022	0.075
5.0–5.90	1.404	0.160	0.009	− 0.004	0.021
5.0–6.60	1.791	0.073	0.014	− 0.001	0.030
5.0–7.83	2.325	**0.020**	0.021	0.003	0.039
5.0–10.5	3.917	**0.000**	0.045	0.023	0.068
5.0–11.0	3.923	**0.000**	0.047	0.024	0.071
5.0–12.3	4.157	**0.000**	0.053	0.028	0.077
5.90–6.60	1.081	0.280	0.005	− 0.004	0.015
5.90–7.83	1.820	0.069	0.012	− 0.001	0.026
5.90–10.5	3.639	**0.000**	0.036	0.017	0.056
5.90–11.0	3.622	**0.000**	0.038	0.018	0.059
5.90–12.3	3.859	**0.000**	0.044	0.021	0.066
6.60–7.83	1.483	0.138	0.007	− 0.002	0.016
6.60–10.5	3.501	**0.000**	0.031	0.014	0.048
6.60–11.0	3.457	**0.001**	0.033	0.014	0.052
6.60–12.3	3.691	**0.000**	0.038	0.018	0.059
7.83–10.5	3.140	**0.002**	0.024	0.009	0.039
7.83–11.0	3.075	**0.002**	0.026	0.009	0.043
7.83–12.3	3.323	**0.001**	0.031	0.013	0.050
10.5–11.0	0.508	0.611	0.002	− 0.005	0.009
10.5–12.3	1.252	0.211	0.007	− 0.004	0.018
11.0–12.3	1.200	0.230	0.005	− 0.003	0.014

Bold values indicate the statistically significant *p* values

κFLC kappa free light chains, *AUC* area under the curve, *CI* confidence interval

Table 5 Paired comparison between AUCs of CSF IgG OCB and different κFLC index thresholds for the diagnosis of MS/CIS in our study population (607 patients)

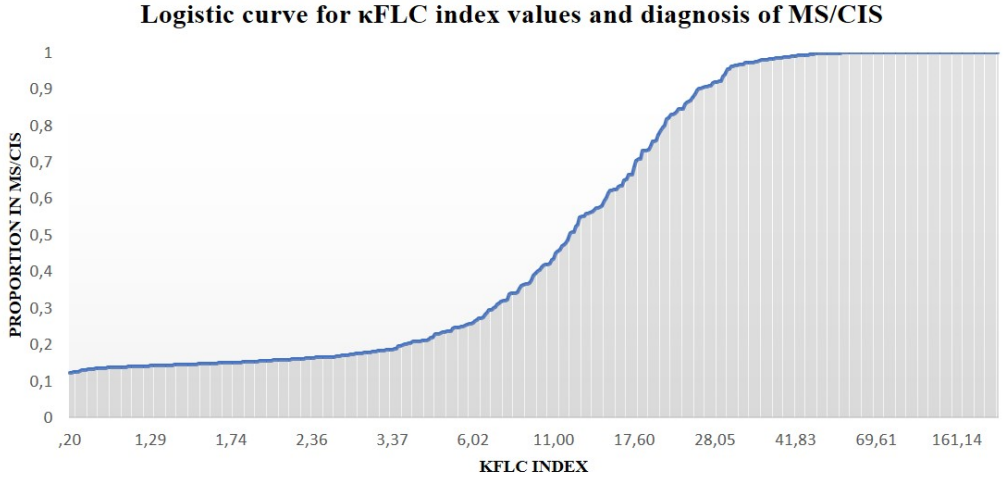
IgG OCB-κFLC	*z*	*p*	Delta AUC	95% CI	
				Lower limit	Upper limit
4.25	− 2.725	**0.006**	− 0.038	− 0.065	− 0.011
5.0	− 3.153	**0.002**	− 0.042	− 0.068	− 0.016
5.90	− 2.656	**0.008**	− 0.033	− 0.058	− 0.009
6.60	− 2.262	**0.024**	− 0.028	− 0.052	− 0.004
7.83	− 1.744	0.081	− 0.021	− 0.044	0.003
10.5	0.263	0.792	0.003	− 0.021	0.028
11.0	0.410	0.682	0.005	− 0.020	0.030
12.3	0.851	0.395	0.010	− 0.014	0.035

Bold values indicate the statistically significant *p* values

κFLC kappa free light chains, *AUC* area under the curve, *CI* confidence interval

The binary logistic regression analysis, even when IgG index and IgG OCB were used as covariates, confirmed that the odds of being diagnosed with MS/CIS increased by 17.1% for each unit increase of κFLC index (OR=1.17; 95% CI 1.12-1.23; p<0.001) (Fig. 3). For each increase of 5 units in κFLC index values, OR is expected to increase by 2.2 times [OR = $(1.17)^5$].

Fig. 3. Probability of diagnosis of MS/CIS based on the values of the independent variable KFLC index.



	B	S.E.	Sign.	Exp(B)	95% C.I. Exp(B)
KFLC index	**0.16**	0.03	0.000	**1.17**	**1.115 – 1.231**
IgG index	0.13	0.85	0.875	1.14	0.215 – 6.087
IgG OCB	1.62	0.38	0.000	5.04	2.409 – 10.556
Constant	-2.00	0.45	0.000	0.14	

Discussion

CSF IgG OCB detection has generally been considered the gold standard to assess intrathecal synthesis in patients with MS and its introduction in the latest revision of McDonald's criteria as a substitute for dissemination in time (DIT) has further enhanced its diagnostic role [4]. This recent acquisition highlighted even more the importance of performing CSF collection and analysis, already implemented in clinical practice, in patients suspected with MS. In our analysis, CSF IgG OCB showed Se of 72.2% and Sp of 95.2% in distinguishing between patients diagnosed with MS/CIS and patients with other neurological diseases, regardless of their inflammatory or not inflammatory nature. This is in agreement with a high number of results from previous studies, which reported for IgG OCB sensitivity values ranging from 83% and 95% [3, 20, 22–24] and Sp ranging from 86% to 95% [3, 20, 25] for the diagnosis of MS. Further, we detected CSF IgG OCB in 78.8% of MS patients and 62.1% of CIS, values similar to those found by Dobson and co-workers in a large meta-analysis of 71 articles, involving more than 12,000 patients with MS (87.7% of MS, 68.6% of CIS) [22].

An IgG index higher than 0.7 was detected in 49.2% of our MS subgroup and in 37.1% of CIS, roughly in line with previous literature data reporting values between 50% and 75% [19, 26]. Furthermore, it showed good Sp in our analysis (95.2%) when comparing MS/CIS with other neurological diseases, but very low Se (44.4%). Other studies reported good Sp for IgG index, together with a good concordance with the detection of CSF IgG OCB [18, 19]. Differently, the correlation between IgG Index and IgG OCB was only moderate in our analysis.

κFLC index showed a higher sensitivity than CSF IgG OCB in all comparisons. When distinguishing patients with MS/CIS from controls, by choosing a threshold of 5.0, κFLC index showed a sensitivity of 85.4% (vs 72.2% of CSF IgG OCB), NPV of 86.8% (vs 78.4% of CSF IgG OCB) and good specificity and PPV, despite lower than values reported for CSF IgG OCB (90.4% vs 95.2% and 89.4% vs 93.4%, respectively). Other studies reported a higher sensitivity of κFLC index compared with CSF IgG OCB, but a lower specificity, as in our analysis [3, 11]. However, this result is not univocal and the lack of an established cut-off may limit the comparison among literature data [27, 28] (Table 6).

Table 6. Sensitivity and specificity values for different thresholds of κFLC index reported in previous studies and characteristics of the study cohorts.

Cut-Off	Sensitivity	Specificity	Patients	Cases	McDonald's criteria	
≥4.25	94%	100%	137	MS (70)	2017	**Puthenparampil et al, 2018**
≥5	96%	78%	385	MS (127)	2017	**Crespi et al, 2019**
≥5.9	96%	86%	438	CIS/MS (70)	2010	**Presslauer et al, 2016**
≥6.6	93%	83%	745	CIS, MS (526)	2010	**Leurs et al, 2019**
≥7.83	89%	81%	170	RIS, CIS, MS (64)	2010	**Gaetani et al, 2020**
≥10.5	87%	76%	320	RIS, CIS, MS (67)	2010	**Gurtner et al, 2018**
≥12.3	93%	100%	176	MS (71)	2010	**Pieri et al, 2017**

Compared with a cut-off value of 5.0, which maximized the AUC (0.879, CI 95% 0.849-0.909) and J index (0.75), thresholds higher than 5 (5.9 [10], 6.6 [11], 7.83 [3], 10.5 [9], 12.3 [6]) showed higher specificity but lower sensitivity in our study cohort, with generally lower AUC and J. Of note, as shown in Table 3, different κFLC index potential cut-off values explored in our analysis exhibited AUCs higher than the one of OCB (0.84), but all have lower values for Sp, as found in other studies [14].

The threshold of 4.23 suggested by Puthenparampil and co-workers [7] showed slightly increased sensitivity and decreased specificity in our sample, with lower J index and similar AUC. Moreover, Crespi and co-workers [29] identified the same threshold of 5.0 chosen in our study, though finding different sensitivity and specificity values (96% vs our 85.4% and 78% vs our 90.4%, respectively).

Comparisons among different studies are certainly limited by several factors. First, different revisions of McDonald's criteria were used by different authors and patients with CIS have not always been considered together with MS as "cases" (Table 6). Second, the use of different commercial assays to detect κFLC in CSF and serum in different laboratories can hamper the repeatability of results. This could be also due

to the different protein sources adopted by different commercial suppliers and therefore also by different laboratories. In order to partially overcome these limitations, we tested and applied all the thresholds proposed in literature in our study population, recruited according to the latest revision of McDonald's the same criteria and tested with a unique technical procedure, including the use of the same monoclonal antibodies and dilutions of test samples. However, other potential sources of error include the underestimation or overestimation of FLC concentrations due to antigen excess and polymerisation effects [30]. On the one hand, this could be a further stimulus to overcome the concept of choosing a unique threshold and consider a more "dynamic" interpretation of κFLC index. On the other hand, since extensive data have been provided so far from several studies on quite similar cut-off values for κFLC index without conclusive results, multicenter studies using different platforms and assays should be performed to definitively confirm these thresholds, and certified reference materials should be developed. As expected, patients with MS/CIS exhibited significantly higher κFLC index values than controls. Se values between 80.0-86.8% and Sp between 88.1-92.9% were reported for κFLC index interval 4.25-6.6, with no significant differences in the AUCs of the explored thresholds 4.25, 5.0, 5.9, 6.6. Based on our results,

this prevents in fact to assert that one cut-off value is superior to another for values between 4.25 and 6.6, suggesting that the lack of a univocal cut-off, which is currently the main limitation for the use of κFLC index in clinical practice, is not an insurmountable problem. Further, κFLC index AUC was higher than IgG OCB AUC when considering thresholds between 4.25-6.6, while no differences emerged for values ≥7.83. Therefore, we should take in account that IgG OCB exhibit a lower or at least equal diagnostic accuracy compared with κFLC index.

Several previous studies reported a higher Se of κFLC index compared with CSF IgG OCB, but a lower Sp [3, 11]. To overcome this issue, Gaetani and colleagues suggested the choice of a higher κFLC index cut-off when discriminating between MS/CIS and OIND, in order to increase Sp [3]. However, these results are not univocal and the lack of an established cut-off has partially limited the comparison among literature data [27, 28] (Table 6). Finally, a recent metanalysis, including results from 32 studies, identified a value of 6.1 as the better discriminatory cut-off, but found no significant differences between κFLC index and IgG OCB in terms of diagnostic accuracy [14].

Evidently, being a quantitative continuous variable, κFLC index exhibits

an intrinsic advantage compared with the analysis of IgG OCB, since values are much more informative about the risk of being diagnosed with MS/CIS. As a consequence, the use of IEF could be restricted only to cases actually characterized by elements of uncertainty, including atypical MRI lesions, non-specific symptoms or κFLC index values close to the lower limit of the interval (i.e., values between 4.25-6.6).

It is known that IgG OCB are currently the gold standard as a biomarker of intrathecal synthesis in MS and that their detection can substitute for DIT according to the 2017 revision of McDonald's criteria [4], actually limiting the use of other diagnostic biomarkers for MS. Further, this limitation also relies on the fact that quantitative determinations (e.g., IgG index, κFLC index) are less reliable than qualitative ones, since they depend on the specificity of the antiserum used and are more subject to variability of results among laboratories [31].

However, κFLC index reflects the intrathecal synthesis of CSF κFLC, which are produced in excess during the synthesis of Ig, consequently sharing the same physiopathological substrate with OCB. If technical limitations were exceeded, κFLC index could then represent a valuable instrument to substitute for DIT, or to support the diagnosis of MS in OCB-

negative patients or when DIS and DIT are already satisfied by clinical and radiological criteria. It might be interesting to evaluate OCB-negative CIS patients and high κFLC index values over time, in order to assess whether they might benefit of an earlier diagnosis of MS, with consequent therapeutic implications, assuming κFLC index as a substitute for DIT.

If the identification of a threshold is important to exclude the diagnostic suspicion in controls, the increase in the risk of being diagnosed with MS/CIS along with the increase of κFLC index values is even more crucial. Indeed, evidence from clinical practice confirm that lots of patients diagnosed with MS exhibit very high κFLC index values, much higher than the possible cut-off explored, and that they are more likely to be diagnosed with MS/CIS. However, this observation would have no specific meaning when a dichotomous interpretation of κFLC index is used.

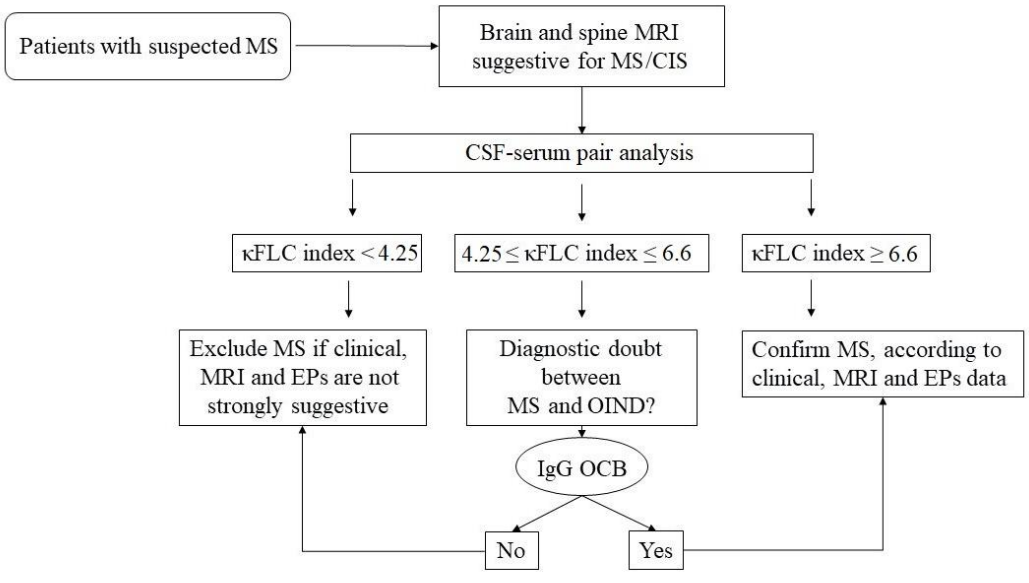
In our population, each increase of 5 units in κFLC index value corresponded to a 2.2-fold higher risk of being diagnosed with MS/CIS. In other words, for progressively increasing κFLC index values, the probability of being diagnosed with MS/CIS can be represented by an exponential curve (Fig. 3).

Based on our findings, κFLC index is not only highly sensitive in excluding a diagnosis of MS and precursory conditions during the diagnostic workout, but also exhibits the irreplaceable advantage of being a quantitative variable, which lends itself to a flexible interpretation. Additionally, it is notably less time-consuming and less expensive than OCB analysis. It has been estimated that the cost of isoelectric focusing immunoassay (IEF) for the detection of IgG OCB amounts to 23.5 euros/patient (including materials, controls, antisera), which adds to personnel cost (about 15 euros/hour), for a total of approximately 46 euros/patient. Further, three working hours are required to evaluate IgG OCB in CSF of two patients [32]. Differently, about 16 euros/patient for material costs are required for the analysis of κFLC index and only 10 minutes are needed for evaluating two patients, thus significantly reducing personnel cost as well (for a total of about 17.25 euros/patient). Consequently, the exclusive use of κFLC index for diagnostic purpose would have saved about 62.5% of costs and have taken about 18 times less than the analysis of IgG OCB for the entire study population, in line with data reported by Crespi and colleagues [32]. Indeed, the analysis of CSF IgG OCB implies a costly multistep method requiring paired CSF and serum specimens to be run in parallel, with a subjective visual

interpretation, and an average time for the analytical processing of over 3 hours. Moreover, IEF is a qualitative assessment and there is no standard definition of the IgG OCB amounts required for a clinically positive result (anything from 1 to 4 unique CSF bands). In this regard, package inserts suggest establishing an individual laboratory reference interval within its own population, despite the FDA approval of IEF testing [9].

Comprehensively, we propose to use κFLC index as a preliminary test, which can be useful not only to exclude the diagnosis of MS/CIS in the appropriate clinical context when values below the considered range are detected, but also to predict the probability of MS/CIS diagnosis with greater confidence the higher κFLC index values. The use of IgG OCB, which currently remains the gold standard for the diagnosis of MS, could be restricted to patients with κFLC index values between 4.25-6.6 or according to clinical judgement, to provide further confirmation in doubtful cases (Fig. 4). Additionally, the analysis of CSF IgG OCB should be performed when DIT cannot be provided otherwise, according to the latest revision of McDonald's criteria.

Fig. 4. Procedural algorithm for the diagnosis of Multiple Sclerosis.



.

It should also be noted that κFLC index can correctly identify OCB-negative MS and CIS patients, who amounted to 21.2% and 37.9%, respectively, in our sample. Particularly, 37.8-54.9% of OCB-negative MS/CIS patients exhibited positive κFLC index values in our study, according to the chosen thresholds between 4.25-6.6. This was quite in line with data reported by Ferraro and coworkers in a recent study, showing that a κFLC index ≥5.8 was detected in 25% of OCB-negative MS patients and in 98% of OCB-positive ones [33].

Based on our results, the use of κFLC index in clinical practice could be highly beneficial, providing an easily and quickly achieved, cost-effective and helpful support for the diagnosis of MS, leading itself to a flexible interpretation in the appropriate clinical context.

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2.2 Prognostic biomarkers in Multiple Sclerosis

For many years, MS has been considered a biphasic-course disease, dominated by inflammation and demyelination and clinically characterized by a relapsing-remitting course in its early phases, and by axonal loss and neurodegeneration in the subsequent progressive phase.

Actually, several studies demonstrated that the axonal damage, consequent or concurrent to demyelination, occurs since the early phases of the disease and accumulates over time leading to sustained disability (79). Additionally, it seems that inflammation and neurodegeneration prevail differently among clinical phenotypes (79). In the last few years, the concept of "progression independent from relapse activity" (PIRA) has further changed what was known on this topic (80), confirming that the accrual of disability can occur at any stage and in all phenotypes, independently from the accumulation of relapse-associated worsening (RAW) (81), which predominates in the early phases in RRMS and pediatric MS (80,82).

Early diagnosis and effective treatment seem to be crucial to impact the long-term prognosis of patients with MS, especially since the currently available disease-modifying drugs (DMDs) have been produced and

approved to be effective on the inflammatory-related outcomes, including relapse rate and RAW. In this view, the availability of prognostic biomarkers, able to predict the short- and long-term prognosis of MS disease course, and to provide a risk stratification of patients, could be extremely valuable (28).

2.2.1 Demographic and clinical factors

Several studies explored the relation between demographic characteristics, including sex, age at symptom onset, ethnicity, and MS pathogenesis and subsequent evolution (83). A lot of studies identified the male sex as a negative prognostic factor in patients with RRMS, predicting an earlier onset of transition to SPMS and a shorter time to reach disability milestones. Further, while the female sex exhibits a higher prevalence of RRMS, the sex ratio balances out in PPMS (84). An older age at onset has been associated with faster disability progression in RRMS. Indeed, despite patients who were younger at the time of first relapse tend to reach disability milestones at a younger absolute age, those with later onset exhibit shorter intervals between MS onset and disability outcomes (85,86).

Finally, higher rates of disability accumulation have been observed among African-American, Hispanic-American and North-African people (83).

Clinical factors have been widely investigated, particularly with the purpose of identifying those patients who should be treated with high-efficacy DMDs from the onset, due to the presence of unfavorable prognostic factors. A higher relapse rate and shorter intervals between

relapses, often with subsequent incomplete recovery, have been identified as additional risk factors for the identification of an "aggressive" phenotype (87,88). Further, multifocal relapses or those affecting motor, cerebellar, cognitive or sphincter functions, and severe relapses resulting in ≥1-point increase in EDSS or ≥2-points in any functional system, should be carefully considered and guide the therapeutic choice towards highly-effective DMDs since the early phases of the disease. Still, the presence of pyramidal signs, or the achievement of EDSS ≥3.0 within the first year of disease evolution, can be considered as negative prognostic factor for an earlier transition to SPMS (87,89). Although 3.4-14.0% of the MS population could present the characteristics of an "aggressive" or "malignant" phenotype, the identification of this condition is not simple and is often defined in retrospect, since there is no consensus on its exact definition. Additionally, we still do not have sensitive tools in recognizing the transition phase from RRMS to SPMS (90). Despite MS is still an incurable and chronic disease, the therapeutic scenario has expanded greatly in the latest years, considerably improving the quality of life and physical conditions of patients. Together with the increase of available treatment options, the treatment goal in MS has evolved as well, from reducing the relapse rate to achieving the No Evidence of Disease Activity

(NEDA), which consists of clinical and radiological remission together with the absence of disability progression (91), and is expected to change further with the introduction of the concept of PIRA. Treatment in MS patients has to be started as soon as possible, since it is now well known that impacting on the inflammatory processes in the early phases of the disease can lead to a minor accrual of axonal loss and neurodegeneration, and thus of sustained disability (92). Two different treatment strategies exist, including the "escalation" and the "induction". The "escalation" approach relies on the use of first-line DMDs as starting treatment, planning therapeutic switches to increasingly effective second-line or third-line DMDs in case of treatment failure (20). The "induction" strategy should strictly include only those treatments able to induce a long-term remission after a short and intermittent time of administration, which consequent profound change and reset in the immune system, followed by gradual lymphocyte repopulation through a modified pathway (93). However, the term is more extensively used to indicate an initial therapeutic approach with the use of high-efficacy DMDs since the very early phases of the disease. Accumulating evidence supports the use of high-efficacy DMDs from the time of MS diagnosis, especially in patients with negative prognostic factors, despite this therapeutic approach is

mainly limited by safety concerns (26). However, some questions remain open: what is the right time to identify a suboptimal response to the chosen treatment and how can this impact on the long-term prognosis?

Article 2

First-year treatment response predicts the following 5-year disease course in patients with Relapsing-Remitting Multiple Sclerosis

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Abstract

Background: Predicting long-term prognosis and choosing the appropriate therapeutic approach in patients with Multiple Sclerosis (MS) at the time of diagnosis is crucial in view of a personalized medicine. We investigated the impact of early therapeutic response on the 5-year prognosis of patients with relapsing-remitting MS (RRMS).

Methods: We recruited patients from MSBase Registry covering the period between 1996-2022. All patients were diagnosed with RRMS and actively followed-up for at least 5 years to explore the following outcomes: clinical relapses, confirmed disability worsening (CDW) and improvement (CDI), EDSS 3.0, EDSS 6.0, conversion to secondary progressive MS (SPMS), new MRI lesions, Progression Independent of Relapse Activity (PIRA). Predictors included demographic, clinical and radiological data, and sub-optimal response (SR) within the first year of treatment.

Results: Female sex (HR 1.27; 95% CI 1.16-1.40) and EDSS at baseline (HR 1.19; 95% CI 1.15-1.23) were independent risk factors for the occurrence of relapses during the first 5 years after diagnosis, while HET (HR 0.79; 95% CI 0.67-0.92) and age at diagnosis (HR 0.83; 95% CI 0.79-0.86) significantly reduced the risk. SR predicted clinical relapses

(HR=3.77; 95% CI 3.46-4.11), CDW (HR=2.56; 95% CI 2.16-3.02), conversion to SPMS (HR=1.85; 95% CI 1.17-2.92), EDSS 3.0 (HR=2.99; 95% CI 2.58-3.47), EDSS 6.0 (HR=1.76; 95% CI 1.43-2.17) and new brain (HR=2.37; 95% CI 2.09-2.69) and spinal (HR 1.85; 95% CI 1.17-2.92) MRI lesions.

Conclusions: This study highlights the importance of selecting the appropriate DMT for each patient soon after MS diagnosis, also providing clinicians with a practical tool able to calculate personalized risk estimates for different outcomes.

Keywords: multiple sclerosis, disease-modifying treatment, prognosis, nomogram, high-efficacy drugs

INTRODUCTION

Multiple Sclerosis (MS) is a chronic immune-mediated disease of the central nervous system characterized by high complexity and extreme heterogeneity in terms of clinical presentation and course. The relapsing-remitting phenotype (RRMS), that accounts for 80-85% of cases, is associated with both demyelination and neurodegeneration since its early phases (1) and the accumulation of disability may occur at any stage of the disease, associated with the occurrence of relapses (relapse-associated worsening, RAW) or in the absence of relapses (progression independent of relapse activity, PIRA) (2). While RAW predominates in the early phases of the disease and mostly in RRMS and pediatric MS, PIRA seems to affect disability worsening in all phenotypes of MS and can start at different points during the disease course, even precociously (3,4). Nevertheless, RRMS course can be extremely variable and profoundly affected by the introduction of highly effective disease-modifying treatments (HET). In this context, a prognostic stratification since disease onset is not simple and a lot is yet to be understood about the long-term disease course and the timing of transition into a secondary

progressive phenotype (SPMS).

A minority of patients, ranging between 3.4-14.0% of the whole MS population, exhibit a “malignant” or “aggressive” disease course and several attempts have been made to reach their early identification. This condition is often recognized in retrospect in patients who achieve a score of 6.0 at the Expanded Disability Status Scale (EDSS) within 5 years from the onset (5–7) or by the age of 40 years (8), or in those who turned to SPMS phenotype within 3 years from the onset (8). Alternatively, an aggressive course has also been defined as the occurrence within the first year after onset of at least two gadolinium-enhancing lesions at brain MRI together with at least two clinical relapses, or even one relapse if resulting in sustained EDSS score of 3.0 (9).

A worse prognosis has been attributed to some demographic features, including male sex, older age at symptom onset, Afro-Americans and Hispanic ethnicity (10). A higher relapse rate and shorter intervals between relapses, often with subsequent incomplete recovery have been identified as additional risk factors. Further, PPMS phenotype and the presence of spinal cord and brainstem lesions at MRI at clinical presentation are often predictors of poor clinical outcome (5). In a two-

stage model for disability progression in MS (11), gender, age at onset, the occurrence of relapses during the first 2 years after onset and an incomplete recovery after relapses were found to be predictive factors only for the achievement of EDSS 3.0. According to the model, the subsequent phase and reaching an EDSS 6.0 were independent in terms of duration (median 6-9 years) from the time needed to reach an EDSS 3.0.

In this context, we collected clinical and radiological data of a large population of patients with RRMS, actively followed-up at different MS Centers, in order to investigate the impact of both the first disease-modifying treatment (DMT) choice and the treatment response in the first year after diagnosis on the 5-years prognosis. As a secondary aim, prognostic nomograms were built to predict the disease course at 5 years based on early clinical markers.

METHODS

Study Population

In this multicenter retrospective study, we collected demographic and

clinical data of patients with RRMS covering the period 1996-2022 from MSBase, a large international Registry recording routine clinical data inserted in iMed© from MS Centers in over 30 countries worldwide. Inclusion criteria were: a diagnosis of RRMS based on the existing McDonald's criteria according to epoch and country, a diagnostic delay ≤ 12 months, start of DMT within 12 months from diagnosis, availability of demographic, clinical and radiological data within 12 months from diagnosis and for at least 5 years after diagnosis (Table 1).

Table 1. Inclusion criteria of the study population

	N.
All patients in MSBase from recruiting centers	83978
RRMS	68470
Time between onset and diagnosis ≤12 months	30943
First DMT started within 12-months of diagnosis	15145
Minimum 5 years post diagnosis registry follow-up	7955
Baseline clinical and MRI data recorded within 12 months from diagnosis	3797

MS: Multiple Sclerosis; RRMS: Relapsing-remitting MS; DMT: disease-modifying treatment; MRI: magnetic resonance imaging

Age at onset and sex were considered as demographics. Clinical

variables included EDSS and pyramidal Functional System (FS) scores, number of relapses. Radiological data included the number of lesions counted in T2-weighted and T1-weighted post-gadolinium (Gd^{+}) scans in brain and spinal MRI, performed by patients as for clinical routine. Treatment with DMT was reported for all patients. Particularly, interferon, glatiramer acetate, dimethyl fumarate, teriflunomide were considered as mild-to-moderate-efficacy DMT (MET), while cladribine, natalizumab, ocrelizumab, alemtuzumab, fingolimod and mitoxantrone were considered as HET. Data were extracted from a computerized database, iMed© (Merck Serono SA; Geneva), which contains clinical information inserted in real-time during outpatient visits.

Outcomes and definitions

Primary outcomes were defined over a period of 5 years from the time of diagnosis (Table S1). Time to first relapse, confirmed worsening, conversion to SPMS and PIRA were analysed as the primary study endpoints. Time to disability improvement, milestone EDSS, and new lesions on brain MRI were analysed as exploratory outcomes only.

Baseline was defined as the date of MS diagnosis. Diagnosis year was

split into epochs as follows: pre-2000, 2000-2004, 2005-2009, 2010-2014 and 2015 onwards.

Predictor variables included demographic (age at diagnosis, sex), clinical (disease duration from onset, EDSS and pyramidal FS at baseline) and radiological data (number of T2 brain lesions, ≥ 1 spinal lesion, ≥ 1 gadolinium-enhancing brain lesions). EDSS at baseline was considered as the EDSS score recorded within 1 to 3 months from the last relapse occurred. Additionally, we considered as a predictor the suboptimal response after 1-year treatment with a DMT (SR), defined by the contextual occurrence of ≥1 gadolinium-enhanced lesions at brain or spine Magnetic Resonance Imaging (MRI) scans, or ≥ 1 relapse.

Statistical analysis

Categorical variables were summarized using frequency and percentage. Continuous variables were summarized using mean and standard deviation (SD) or median and interquartile range (IQR) as appropriate. The identification of demographic, clinical and investigational correlates of five-year clinical outcomes were undertaken using a multilevel mixed effects parametric survival model presuming an underlying Weibull

distribution. Age, sex, EDSS, time since onset, MRI lesions and SR were defined as fixed effects, whilst country and diagnosis epoch were included in the model as random effects. Hazard Ratio (HR) and 95% confidence interval (CI) were provided for all variables explored and for each outcome. Independent prognostic correlates of five-year outcome identified in the multivariable parametric survival modelling were then used to derive the prognostic nomograms using the method described by Kattan et al (12, 13), using the nomogram function of the RMS package in R (14). Candidate multivariable models were assessed for collinearity and potential interactions between concurrent nomogram predictors. Quadratic transformations were incorporated into the models to test for the linearity of association between candidate explanatory variables and the clinical endpoints. The Akaike and Bayesian Information criteria were used to assess relative goodness of fit between multiple, competing multivariable model solutions prior to the selection of the final model for the development of the final prognostic nomogram. Internal validation of each nomogram was conducted via derivation of concordance indices and evaluation of nomogram calibration. Calibration was conducted by taking 500 bootstrapped resamples. Clinical outcome probability (as

predicted by the nomogram) and the mean scores of these probability groups were then compared to the empirically observed non-response estimates on a calibration curve. All analyses were conducted in R version 4.0.5 (R Foundation for Statistical Computing) and Stata version 16.1 (StataCorp, College Station, Texas).

RESULTS

Study population

From a total of 83978 patients recorded in the Registry from participating centers, 3797 subjects met the inclusion criteria and were enrolled in the study. Of those, 2682 (70.9%) were female, and the mean age at onset was 32.15±9.79 years. The characteristics of the study population are reported in Table 2.

Risk of clinical relapses

Results from the multivariate analysis confirmed SR [HR 3.77 (95% CI 3.46-4.11), p<0.001], female sex [HR 1.27 (95% CI 1.16-1.40), p<0.001] and baseline EDSS [HR 1.19 (95% CI 1.15-1.23), p<0.001] as

independent risk factors for the occurrence of at least one clinical relapse within 5 years after the diagnosis of MS. HET as the first therapeutic choice [HR 0.79 (95% CI 0.67-0.92), p=0.002] and an older age at baseline [HR 0.83 (95% CI 0.79-0.86), p<0.001] were protective factors towards the explored outcome (Table 3; Table S2; Fig. 1).

Table 2. Baseline characteristics of the study population

			Cohort with a legitimate baseline EDSS and MRI[a]
Factor	**Category**	**n=7955**	**n=3797**
Age at baseline (years) - mean (SD)		31.43 (9.79)	32.15 (9.79)
Sex - n (%)	Female	5652 (71.1)	2682 (29.3)
	Male	2302 (28.9)	1114 (29.3)
	Not recorded	1 (0.0)	1 (0.0)
Months since first symptoms - mean (SD)		4.20 (3.55)	4.27 (3.35)
Diagnosis year - n (%)	Pre-2000	501 (6.3)	91 (2.4)
	2000-2004	1440 (18.1)	444 (11.7)
	2005-2009	2426 (30.5)	1086 (28.6)
	2010-2014	2671 (33.6)	1503 (39.6)
	2015 onwards	917 (11.5)	673 (17.7)
Country - n (%)	Australia	1632 (20.5)	694 (18.3)
	Turkey	1439 (18.1)	636 (16.8)
	Italy	867 (10.9)	612 (16.1)
	Canada	676 (8.5)	479 (12.6)
	Spain	666 (8.4)	454 (12.0)
	Kuwait	453 (5.7)	182 (4.8)
	Belgium	285 (3.6)	130 (3.4)
	Iran	266 (3.3)	34 (0.9)
	Netherlands	257 (3.2)	137 (3.6)
	Portugal	157 (2.0)	107 (2.8)
	Lebanon	156 (2.0)	72 (1.9)
	United States	153 (1.9)	35 (0.9)
	Switzerland	141 (1.8)	14 (0.4)
	Egypt	92 (1.2)	1 (0.0)
	Argentina	86 (1.1)	35 (0.9)
	United Kingdom	73 (0.9)	18 (0.5)
	Tunisia	67 (0.8)	19 (0.5)
	Ireland	61 (0.8)	1 (0.0)

	Croatia	54 (0.7)	0 (0.0)
	Brazil	51 (0.6)	40 (1.1)
	UAE	47 (0.6)	0 (0.0)
	Oman	45 (0.6)	18 (0.5)
	Czechia	36 (0.5)	21 (0.6)
	Denmark	32 (0.4)	9 (0.2)
	Hungary	32 (0.4)	13 (0.3)
	Other	131 (1.7)	36 (0.9)
Baseline EDSS - median (IQR)*		2 (1, 2.5)	N/A
Baseline EDSS - median (IQR)**		2 (1, 2.5)	2 (1, 2.5)
Baseline*** MRI - T1 Gd+ lesions - n (%)	0	1263 (15.9)	971 (25.6)
	1+	825 (10.4)	658 (17.3)
	MRI performed, lesions not recorded	3117 (39.2)	2168 (57.1)
	No baseline MRI	2750 (34.6)	N/A
Baseline*** MRI - T2 lesions - n (%)	0	22 (0.3)	14 (0.4)
	1-2	95 (1.2)	79 (2.1)
	3-8	884 (11.1)	649 (17.1)
	9+	1571 (19.8)	1220 (32.1)
	MRI performed, lesions not recorded	2633 (33.1)	1835 (48.3)
	No baseline MRI	2750 (34.6)	N/A
First DMT - n (%)	Rebif	2148 (27.0)	1046 (27.8)
	Betaferon	1754 (22.1)	690 (18.2)
	Avonex	1732 (21.8)	640 (16.9)
	Glatiramer acetate	1145 (14.4)	652 (17.2)
	Natalizumab	375 (4.7)	253 (6.7)
	Fingolimod	340 (4.3)	216 (5.7)
	DMF	186 (2.3)	127 (3.3)
	Teriflunomide	112 (1.4)	74 (2.0)
	Mitoxantrone	59 (0.7)	29 (0.8)
	Alemtuzumab	34 (0.4)	20 (0.5)
	Rituximab	22 (0.3)	12 (0.3)
	Cladribine	16 (0.2)	12 (0.3)
	Plegridy	15 (0.2)	12 (0.3)
	Daclizumab	12 (0.2)	10 (0.3)
	Ocrelizumab	5 (0.1)	4 (0.1)

Table 2 (continued). SD: standard deviation; IQR: interquartile range; EDSS: Expanded Disability Status Scale; DMT: disease-modifying treatment; MRI: magnetic resonance imaging.

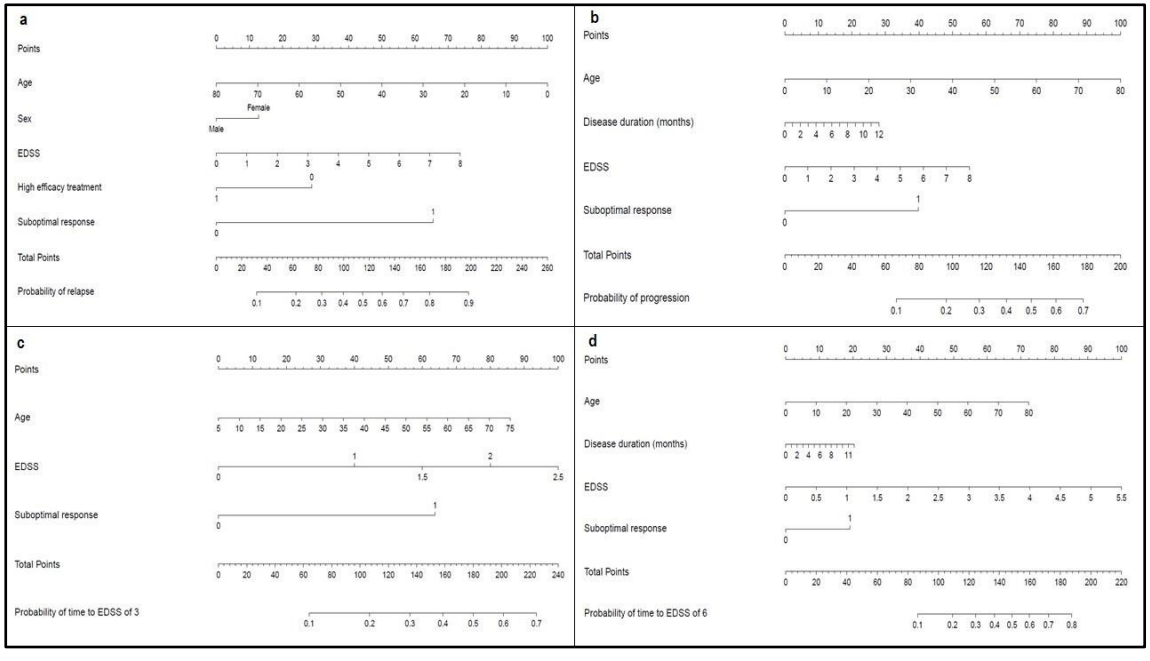
Table 3. Multivariate survival model for all outcomes.

		First relapse	Disability progression	EDSS 3.0	EDSS 6.0	Conversion to SPMS	New brain MRI lesions	New spine MRI lesions	PIRA
Explanatory variable	Category								
Age at baseline (units=10 years)		0.83 (0.79, 0.86) <0.001	1.19 (1.10, 1.29) <0.001	1.21 (1.12, 1.31) <0.001	1.30 (1.17, 1.44) <0.001	2.05 (1.64, 2.55) <0.001	0.79 (0.73, 0.84) <0.001	0.89 (0.78, 1.00) 0.051	1.84 (1.56, 2.17) <0.001
Sex	Female	1.27 (1.16, 1.40) <0.001	1.06 (0.88, 1.26) 0.559	1.03 (0.88, 1.22) 0.681	0.95 (0.76, 1.19) 0.641	0.53 (0.34, 0.84) 0.007	1.02 (0.89, 1.18) 0.733	1.00 (0.78, 1.29) 0.983	0.80 (0.56, 1.16) 0.242
	Male	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Months since first symptoms		0.99 (0.97, 1.00) 0.045	1.03 (1.01, 1.06) 0.012	1.02 (1.00, 1.04) 0.063	1.03 (1.00, 1.07) 0.029	1.04 (0.97, 1.11) 0.278	1.00 (0.98, 1.02) 0.821	0.99 (0.96, 1.03) 0.642	1.07 (1.02, 1.12) 0.007
First DMT - high efficacy	Yes	0.79 (0.67, 0.92) 0.002	1.12 (0.89, 1.42) 0.336	1.01 (0.78, 1.31) 0.959	1.12 (0.80, 1.57) 0.520	0.56 (0.26, 1.20) 0.138	0.78 (0.63, 0.96) 0.021	0.69 (0.47, 1.03) 0.072	1.25 (0.78, 2.00) 0.361
	No	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Baseline EDSS		1.19 (1.15, 1.23) <0.001	1.25 (1.16, 1.34) <0.001	1.93 (1.72, 2.16) <0.001	1.60 (1.45, 1.76) <0.001	1.59 (1.35, 1.86) <0.001	1.02 (0.96, 1.08) 0.526	1.00 (0.90, 1.12) 0.936	1.12 (1.01, 1.27)

									0.039
Pyramidal FS ≥ 2 - n (%)	<2	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
	≥2	1.02 (0.90, 1.15) 0.811	1.23 (0.98, 1.56) 0.078	1.10 (0.88, 1.38) 0.392	1.37 (1.04, 1.81) 0.025	1.36 (0.75, 2.47) 0.306	1.04 (0.86, 1.26) 0.711	1.25 (0.90, 1.74) 0.191	-
	No baseline pyramidal KFS	1.01 (0.89, 1.14) 0.886	1.08 (0.86, 1.36) 0.492	0.96 (0.78, 1.19) 0.734	1.28 (0.94, 1.74) 0.117	1.71 (0.88, 3.33) 0.116	0.65 (0.52, 0.81) <0.001	0.49 (0.29, 0.84) 0.010	-
Baseline Brain MRI - T1 Gd+ lesions	0	Reference	Reference	Reference	Reference	Reference	Reference	Reference	-
	1+	1.04 (0.92, 1.19) 0.512	0.95 (0.74, 1.23) 0.710	0.97 (0.77, 1.22) 0.804	1.11 (0.80, 1.55) 0.526	0.99 (0.51, 1.92) 0.979	1.00 (0.82, 1.21) 0.961	0.86 (0.61, 1.21) 0.380	-
	MRI performed, lesions not recorded	0.96 (0.86, 1.08) 0.495	0.83 (0.68, 1.03) 0.085	1.04 (0.85, 1.26) 0.703	0.97 (0.73, 1.28) 0.819	0.55 (0.32, 0.97) 0.038	0.93 (0.78, 1.10) 0.389	0.72 (0.52, 0.99) 0.044	-
Baseline Brain MRI - T2 lesions	0	Reference	Reference	Reference	Reference	Reference	Reference	Reference	-
	1-2	1.58 (0.77, 3.24) 0.215	1.81 (0.53, 6.23) 0.347	0.80 (0.23, 2.80) 0.731	1.15 (0.28, 4.67) 0.849	0.32 (0.02, 5.26) 0.425	2.53 (0.59, 10.87) 0.211	2.25 (0.28, 17.99) 0.444	-
	3-8	1.32 (0.68, 2.59) 0.412	1.37 (0.43, 4.32) 0.596	0.92 (0.29, 2.93) 0.884	1.03 (0.32, 3.35) 0.957	0.49(0.06, 3.84) 0.493	2.82 (0.69, 11.59) 0.150	1.10 (0.15, 0.25) 0.926	-
	9+	1.30 (0.67, 2.54) 0.440	1.20 (0.38, 3.79) 0.750	0.77 (0.24, 2.46) 0.662	0.86 (0.27, 2.77) 0.798	0.59 (0.08, 4.51) 0.608	3.16 (0.77, 12.93) 0.109	1.34 (0.18, 9.97) 0.774	-
	MRI performed, lesions not recorded	1.45 (0.75, 2.83) 0.273	1.38 (0.44, 4.32) 0.579	0.81 (0.26, 2.56) 0.718	1.06 (0.34, 3.38) 0.916	0.60 (0.08, 4.53) 0.619	1.74 (0.43, 7.14) 0.440	0.72 (0.10, 5.42) 0.753	-
Sub-optimal response in first year of treatment	Yes	3.77 (3.46, 4.11) <0.001	2.56 (2.16, 3.02) <0.001	2.99 (2.58, 3.47) <0.001	1.76 (1.43, 2.17) <0.001	1.85 (1.17, 2.92) 0.008	2.37 (2.09, 2.69) <0.001	1.63 (1.30, 2.06) <0.001	-
	No	Reference	Reference	Reference	Reference	Reference	Reference	Reference	-

Table 3 (continued). EDSS: Expanded Disability Status Scale; SPMS: secondary progressive multiple sclerosis; PIRA: progression independent from relapse activity; DMT: disease-modifying treatment; MRI: magnetic resonance imaging

Fig. 1 Nomograms used to determine the risk of relapses, disability progression and achievement of EDSS milestones



Each predictor has to be matched with the corresponding number of points on the top "Points" scale (vertical lines). **a)** Nomogram used to determine the risk of relapses within 5 years. **b)** Nomogram used to determine the risk of confirmed disability progression within 5 years. **c)** Nomogram used to determine the risk of reaching EDSS 3.0 within 5 years. **d)** Nomogram used to determine the risk of reaching EDSS 6.0 within 5 years.

Confirmed disability worsening and improvement

In the overall study population, a higher baseline EDSS [HR 1.25 (95% CI 1.16-1.34), p<0.001], an older age at baseline [HR 1.19 (95% CI 1.10-1.29), p<0.001] and a longer disease duration [HR 1.03 (95% CI 1.01-1.06), p=0.012] were associated with an increased risk of 6-month confirmed disability worsening. Patients with SR exhibited a significantly higher risk of disability worsening [HR 2.56 (95% CI 2.16-3.02), p<0.001] (Table 3; Table S3; Fig. 1). Accordingly, the aforementioned variables, except for EDSS at baseline, were also associated with a lower probability of disability improvement in the subgroup of patients with a baseline EDSS ≥2.0 (Table 3; Table S4; Fig. 2).

Reaching EDSS 3.0 and 6.0

SR [HR 2.62 (95% CI 2.26-3.04), p <0.001], a higher EDSS at baseline [HR 1.86 (95% CI 1.68-2.06), p <0.001] and an older age at diagnosis [HR 1.25 (95% CI 1.16-1.35), p <0.001] were independent risk factors for the achievement of EDSS 3.0 within 5 years in patients who exhibited EDSS <3.0 at baseline (Table 3; Table S5; Fig. 1). The abovementioned variables were also significantly associated with the achievement of EDSS 6.0

(Table 3; Table S6; Fig. 1). The Pyramidal FS score ≥2 was a significant risk factor for EDSS milestone 6.0 [HR 1.37 (95% CI 1.04-1.81), p =0.025], but not for EDSS milestone 3.0. The number of T2 and Gd+ lesions at brain and spinal MRI did not predict the achievement of EDSS 3.0 or 6.0.

Conversion to SPMS

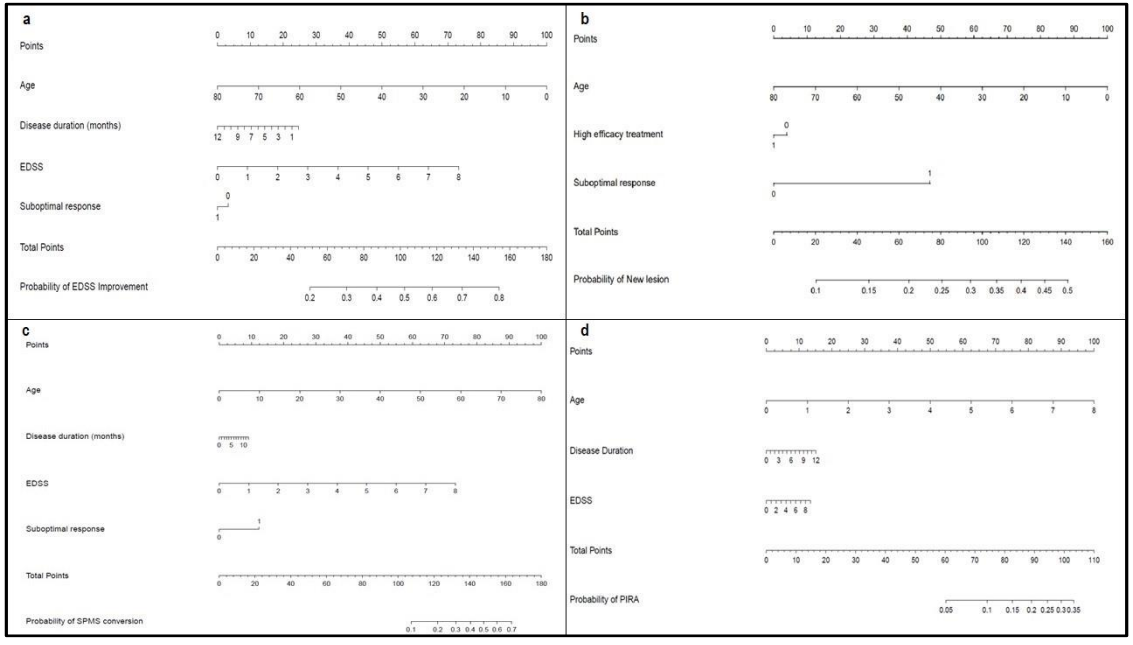
The main predictors for the risk of conversion into SPMS were age at baseline [HR 2.05 (95% CI 1.64-2.55), p<0.001], SR [HR 1.85 (95% CI 1.17-2.92), p=0.008] and EDSS [HR 1.59 (95% CI 1.35-1.86), p <0.001] at baseline. Conversely, the female sex was a protective factor for the explored outcome [HR 0.53 (95% CI 0.34-0.84), p=0.007] (Table 3; Table S7; Fig. 2).

Development of new brain or spinal lesions at MRI scans

The risk of detecting new lesions at brain MRI scans was lower in patients treated with HET as first therapeutic option [HR 0.78 (95% CI 0.63-0.96), p=0.021] and in those who were older at the time of diagnosis [HR 0.79 (95% CI 0.73-0.84), p<0.001]. Conversely, the probability of developing

new lesions at brain MRI scans was higher in patients exhibiting SR [HR=2.37 (95% CI 2.09-2.69), p<0.001] (Table 3; Table S8). None of the variables explored predicted the occurrence of new lesions at spinal MRI, except for SR [HR 1.85 (95% CI 1.17-2.92), p=0.008] (Table 3; Table S9; Fig. 2).

Fig. 2 Nomograms used to determine the risk of EDSS improvement, development of new brain lesions, conversion to SPMS and PIRA



Each predictor has to be matched with the corresponding number of points on the top "Points" scale (vertical lines). **a)** Nomogram used to predict the risk of EDSS improvement within 5 years. **b)** Nomogram used to determine the risk of developing new brain lesions at MRI within 5 years. **c)** Nomogram used to determine the risk of conversion to SPMS within 5 years. **d)** Nomogram used to determine the risk of PIRA within 5 years.

PIRA

Among all variables explored, age [HR 1.84 (95% CI 1.56-2.17), p<0.001], SR [HR 1.12 (95% CI 1.01-1.27), p=0.039] and EDSS at baseline [HR 1.07 (95% CI 1.02-1. 21), p=0.007] were independent risk factors for the development of PIRA (Table 3; Table S10; Fig. 2). The number of T2 and Gd^+ lesions at brain and spinal MRI were not predictive for the explored outcome.

DISCUSSION

Our study confirms the crucial role of the first therapeutic choice and early treatment response on the 5-year prognosis of patients with MS.

Particularly, the choice of HET as the first DMT started at the time of diagnosis is associated with a 20% decrease in the risk of relapses and new brain MRI lesions within 5 years, regardless of other risk factors. It is known that the immediate initiation of HET is superior to treatment escalation strategy in reducing the rate of relapses and disability progression (15). Further, the timing for the introduction of HET seems to be equally important. Data from the MSBase registry and Swedish MS

registry confirmed that HET started within 2 years from disease onset is protective towards the development of disability within 6-10 years (16). Additionally, an Italian MS Registry study assessed the effects of early and late start of HET in patients with RRMS, reporting significantly higher mean annual delta-EDSS values in the escalation group compared with the early intensive treatment group at all timepoints and more markedly in the long-term, up to 10 years (17).

Our results confirmed that early treatment response to the first therapeutic choice is a predictor for all outcomes explored. In this regard, a sub-optimal response within the first year of treatment was associated with an increased risk more than 3-fold for relapses and 2-fold for developing new brain lesions at MRI scans. Additionally, an incomplete response to the first DMT not only predicted clinical and radiological signs of disease activity, but was also associated with a higher risk of disease progression (HR=2.56), conversion into SPMS (HR=1.85), and achievement of EDSS 3.0 (HR=2.99) and 6.0 (HR=1.76). This is particularly relevant, considering the two-stage model for disability progression proposed by Leray and colleagues (11). In this view, demographic and clinical factors can only affect the time needed to reach EDSS 3.0, while the disability

progression from this milestone to EDSS 6.0 lasted from 6 to 9 years irrespective of the previous phase duration. As a consequence, efforts should be concentrated in delaying the achievement of EDSS 3.0. In our study, a sub-optimal treatment response in the first year after treatment start was the most relevant independent predictor for reaching EDSS 3.0, being associated with a nearly 3-fold higher risk to achieve the outcome within 5 years from the time of diagnosis.

An older age at the time of diagnosis and a higher EDSS at baseline were also predictive for 6-month confirmed disability progression, EDSS milestones 3.0 and 6.0, in line with results from previous studies (18). On the other hand, an older age at baseline was a protective factor towards clinical and radiological activity, reducing by 20% the risk of relapses and detection of new brain MRI lesions within 5 years. Recent data reported a decrease in clinical and subclinical disease activity, as shown in our study, together with a lower efficacy of DMT and poor post-relapse recovery with aging, most likely due to immune-senescence (19,20). An older age at baseline was associated with a doubling of risk of converting to SPMS within 5 years in our study, confirming evidence of common onset of the progressive phase in MS in the fifth decade (19). Our results confirmed the

role of sex in affecting disease activity and progression (21). Indeed, female sex was a risk factor for the occurrence of relapses within the first 5 years from diagnosis, confirming the higher frequency of autoimmune responses in women. However, female sex was a protective factor towards the transition into SPMS. Previous studies reported shorter times to achieve given disability levels and to convert into SPMS from MS onset in men compared with women (22–25).

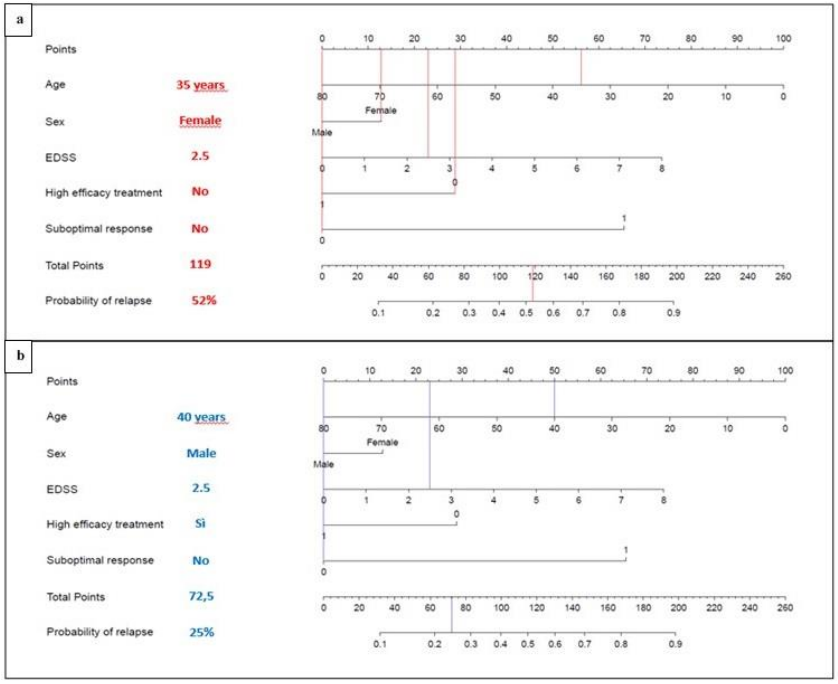
In our study, the risk of conversion to SPMS within 5 years was also predicted by an older age and a higher EDSS at baseline, as well as by male sex and a sub-optimal response in the first year of treatment. Despite several studies exploring predictive factors of conversion to SPMS have not been conclusive as yet, most reported results similar to ours (26–29). Particularly, older age seems to increase the risk of progression to SPMS regardless of disease duration (27). It should be noted that universally accepted criteria for SPMS diagnosis do not yet exist and that, in our study, different criteria were probably used by MS Centers to establish the timing of SPMS diagnosis. Indeed, the difficulty in identifying the moment of transition from RRMS to SPMS remains a major challenge and can cause a diagnostic delay of up to 3 years, due to our inadequate measuring tools

(e.g., EDSS) (27). Indeed, the traditional biphasic view of MS, as mainly characterized by inflammation before and neurodegeneration later, is being questioned by new evidence and modern imaging techniques. Imaging markers of chronic inflammation, as slowly expanding lesions, paramagnetic rim sign, and microglial activation, are already present in the relapsing-remitting phase (30–32), as well as the histopathological evidence of axonal damage (33), documenting a "silent progression" occurring in patients who meet the criteria for RRMS (34). PIRA seems to be the main driver of disease worsening in all phenotypes of MS, and can start even after the first demyelinating event (2,4). Thus, it seems that MS progresses as a continuum from relapsing to progressive disease and progression, even if difficult to identify, is present from the very early phases of the disease. We investigated the impact of the variables explored on PIRA. Age and EDSS at baseline, and disease duration from symptoms onset were the only predictors in our model, with a greater impact exerted by age [HR=1.84 (95% CI 1.56-2.17), $p<0.001$). This highlights the need to better understand the underlying mechanisms and to develop tools and new biomarkers which can be sensitive in detecting insidious disease progression. Of note, HET did not reduce the risk of PIRA, suggesting that

a change in the therapeutic approach could be needed, including the potential use of combination therapies.

For each outcome, nomograms were built including the significant predictors among all variables explored (Fig. 1, 2). Nomograms can provide a useful support in the decision-making process of MS management, allowing clinicians to obtain rapid and personalized risk estimates and thus facilitating patient therapeutic counselling. The risk estimates can be easily obtained by drawing vertical lines from each predictor upwards to the point axis, adding up the partial scores and drawing a vertical line from the total point axis downwards to the outcome probability axis. We hypothesized two different clinical scenarios to better explain the use of nomograms. For example, a hypothetic 35-year-old female patient, with baseline EDSS score of 2.5 and early optimal response to MET, would exhibit a 52% risk of relapses during the first 5 years, as illustrated in Fig. 3. Differently, a supposed 40-year- old male subject, with comparable EDSS and optimal response to HET, would experience a risk of 25% of clinical relapses within 5 years.

Fig. 3 Worked example of how to use nomograms to predict the risk of relapses during the first 5 years from diagnosis.



Each predictor has to be matched with the corresponding number of points on the top "Points" scale (vertical lines). **a)** The age of 35 years matches to 56 points, the female sex to 12.5 points, a baseline EDSS score of 2.5 matches to 22.5 points, the choice of DMT others than HET corresponds to 28 points and the absence of suboptimal response to 0 points. This sums to a cumulative total of 119 points. Drawing a line down from the "Total Points" scale to the corresponding "Probability of relapse" scale reveals that 119 total points corresponds to a probability of relapses of 52% for this hypothetic patient.

b) The age of 40 years (50 points), the male sex (0 points), a baseline EDSS score of 2.5 (22.5 points), the use of HET (0 points) and the absence of suboptimal response (0 points) sums to a cumulative score of 72.5 points, corresponding to a 25% probability of relapses for this hypothetic patient.

Even if the number of predictors included is limited, this can represent an advantage in terms of facilitated use in clinical practice, since all the variables considered are easily accessible during a routine neurological visit. Even if few previous studies reported nomograms as a valid tool to predict the risk of specific outcomes (35,36), and others proposed models to early predict conversion to SPMS (37,38), to our knowledge this is the first one which combines multiple prognostic factors, particularly focusing on the impact of the first therapeutic choice and of the very early treatment response on a mid-long-term prognosis for patients with MS.

This study exhibits some limitations. First, the incomplete reporting of data in iMed©, particularly MRI data and FS scores, almost certainly affected the results of the analysis. Indeed, the number of T2 and Gd^+ brain and spine lesions often were not recorded and were not retrievable to be used in the analysis. Additionally, lesion volumes and measures of brain atrophy were not available. This could explain why MRI data and the Pyramidal FS score, which have been reported as relevant prognostic factors in previous studies (28,39), did not reach statistical significance in our model and were not included in nomograms. Second, despite data are inserted real-time in iMed©, the study is observational and extends over a

long period of time, when diagnostic criteria have been revised more than once, progressively increasing in sensitivity.

Our study underscores and provides further evidence about the crucial role played by the initial treatment response to the first therapeutic approach. In addition, it confirms the relevance of demographic and clinical factors on the mid-term prognosis of patients with MS. A highly effective approach since the time of diagnosis is warranted, especially in patients with adverse prognostic factors, and risk stratification of patients with MS in every day practice may be guided by simple prognostic tools, as nomograms, procedural flowcharts and risk tables.

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2.2.2 Imaging biomarkers

Magnetic Resonance Imaging

The use of MRI has deeply modified the diagnostic workout and the management of MS, allowing to make earlier diagnosis in patients with CIS, monitoring disease-activity during treatment with DMDs, and providing prognostic information (94). In this respect, the use of conventional MRI can provide information about the lesion load in T2 scans, the presence of gadolinium-enhanced lesions and black holes in T1 scans, the location of lesions in the spinal cord or in infratentorial regions, the detection of cortical atrophy, this last requiring the use of post-processing techniques (95). However, conventional MRI is unable to detect the burden of cortical and juxtacortical lesions, which can be best detected with the use of double inversion recovery (DIR) sequences (96). Moreover, it is not able to recognize the damage occurring in the normal-appearing white matter (NAWM) (94). For this purpose, magnetization transfer (MT), diffusion tensor (DT), proton spectroscopy and functional MRI (fMRI) have been added to MRI protocols for study purpose, despite

not being implemented in clinical practice so far (97–100). These techniques have been able to detect the presence of both focal and diffuse damage in the white and cortical grey matter (96). Further, the use of TSPO-PET has identified the activation of microglia in the NAWM, which seems to be a good marker for the detection of the so called "smouldering" MS (101). Indeed, the activation of microglia has been detected not only in acute lesions, but also in chronic active ones, defined as slowly evolving lesions (SELs), characterized by focal smouldering inflammation with ongoing demyelination and chronic axonal loss (102). These lesions exhibit a paramagnetic iron rim, which can be observed with phase-contrast and susceptibility-weighted MRI (103). The combined use of these advanced MRI technique could provide crucial information on the pathogenesis and evolution of MS, better clarifying mechanisms which lead to the accrual of irreversible disability over time.

Optical Coherence Tomography

Optical Coherence Tomography (OCT) is a non-invasive, rapid and reproducible technique, investigating retinal structures and the first unmyelinated part of the optic nerve through high-resolution tomographic

sections (104,105). It can provide information about the morphology, reflectivity, thickness and volume of retinal layers, whose changes have been frequently detected in patients with MS (106). Particularly, the thickness of the peripapillary retinal nerve fiber layer (RNFL), which is the innermost retinal layer including the unmyelinated axons of ganglion cell neurons, can be measured on a cross-sectional retinal image, sampled along a 3.4 mm-diameter circle centered on the optic nerve head (107,108). The thickness of the macular ganglion cell layer (GCL), which contains the bodies of the retinal ganglion cells, can also be assessed with OCT, providing an additional measure of neuronal degeneration (109).

Due to the frequent involvement of the visual pathway and to the lack of myelin, RNFL has been investigated as a promising imaging biomarker of axonal loss and neurodegeneration, correlating with brain atrophy, independently from the occurrence of previous optic neuritis (110–112). Heterogeneous results emerged from studies exploring the prognostic value of OCT measurements. Particularly, several studies reported correlations between RNFL thickness and EDSS score and disability worsening (105,106,113–115), while others did not (116–118). Evidence of associations between RNFL thickness and MRI outcomes, including

white matter and grey matter volume, was also reported, more in PPMS than RRMS patients (112,113). Similarly, not univocal results were reported for macular volume, which was also found to be lower in patients with progressive phenotype than in RRMS (109,114,119,120).

Article 3

Early reduction of retinal thickness predicts physical and cognitive disability in newly diagnosed multiple sclerosis patients: results from a cross-sectional study

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Abstract

Introduction: Retinal nerve fiber layer (RNFL) thickness is a promising biomarker of axonal loss and a potential outcome predictor in Multiple Sclerosis (MS). Cognitive impairment (CoI) exhibits a high prevalence in patients with MS (pwMS), even in the early phases of the disease. Our aim was to explore the role of RNFL thickness as a predictor of physical and cognitive disability in pwMS.

Methods: All newly diagnosed pwMS referred to the MS centre of the University-Hospital "Policlinico-San Marco" between 2015-2019 were evaluated at baseline and at 3 years. RNFL and ganglionar cell layer (GCL) thickness for right (r.e.) and left eyes (l.e.) were measured with Optical Coherence Tomography (OCT). Disability level and cognitive profile were assessed, using the Expanded Disability status scale (EDSS) and the Brief International Cognitive Assessment for Multiple Sclerosis (BICAMS) battery, respectively.

Results: We consecutively enrolled 487 pwMS, including 68 (14.0%) with

primary progressive MS (PPMS). At baseline, RNFL and GCL were bilaterally thinner in PPMS (r.e. 90.4±12.7; l.e. 90.2±13.5, and r.e. 80.1±11.2; l.e. 80.3±12.6, respectively) compared to relapsing-remitting MS (RRMS) (r.e. 94.6±13.1; l.e. 94.3±14.8, and r.e. 85.1±9.5; l.e. 84.9±9.3, respectively) ($p<0.01$). Both groups exhibited reduced RNFL ang GCL thickness, worse cognitive performance and higher EDSS scores at 3-years follow-up compared with baseline. A RNFL thickness ≤88.0 μm was an independent predictor of CoI (OR=5.32; 95% CI=1.84-9.12; p=0.02) and disability worsening (OR=3.18; 95% CI=1.21-10.33; p=0.05).

Discussion: RNFL thickness, as a biomarker of neurodegeneration, could be considered a predictive biomarker of cognitive degeneration and physical disability in MS.

Keywords: multiple sclerosis, cognitive impairment, optical coherence tomography, retinal thickness, physical disability

Introduction

Multiple Sclerosis (MS) is a chronic immune-mediated inflammatory disease of the central nervous system (CNS), characterized by both inflammation and neurodegeneration since its early phases. Over time, the accrual of axonal damage, consequent to demyelination, leads to the accumulation of physical disability and cognitive deterioration (1). The assessment of the peripapillary retinal nerve fiber layer (RNFL) thickness with optical coherence tomography (OCT), a non-invasive instrument providing high-resolution tomographic sections of the retina, can be considered a reliable marker of axonal loss (2). RNFL is the innermost retinal layer, which is measured on a cross-sectional retinal image sampled along a 3.4-mm diameter circle centered on the optic nerve head (3).

Previous studies investigated the association between reduced RNFL and higher scores at the Expanded Disability Status Scale (EDSS), the most widely used clinical instrument to monitor disease severity and progression (4). Whilst significant associations were found by some authors (5–7), other studies failed in demonstrating a correlation between RNFL and physical disability regardless of a previous history of optic

neuritis (ON) or in detecting differences between relapsing and progressive MS phenotypes (8–12). Beyond RNFL, which includes the unmyelinated axons of ganglion cell neurons, the measurement of the macular ganglion cell layer (GCL), alone or combined with the inner plexiform layers (GCIPL), is an even more accurate marker of neurodegeneration and correlates with brain atrophy (13–15).

Both RNFL and GCL thickness were investigated as indirect markers of CoI (6,16). It is well known that CoI exhibits a prevalence between 45-70% in patients with MS, higher in older and more severely disabled patients and in progressive phenotypes (17). Further, CoI is associated with cortical atrophy at Magnetic Resonance Imaging (MRI) scans, which is itself a marker of neurodegeneration (18,19). Both RNFL thickness and GCL were found to be reduced in cognitively impaired patients and some studies found them to be reliable markers in predicting future CoI (6,16,20,21).

In this perspective, we aimed to assess the role of RNFL and GCL in predicting CoI and physical disability over a 3-year follow-up in a population of patients newly diagnosed with MS.

Methods

Study population

We recruited all patients admitted to the MS Centre of Neurology Clinic at the University Hospital "Policlinico G. Rodolico" of Catania in the period between January 2015 and December 2019. All patients received a diagnosis of MS according to 2010 McDonald's criteria (22) within 5 years from disease onset and were followed-up annually for at least 3 years. All patients gave written consent to allow data collection and use for study purpose. We collected data about demographics, MS onset and course, EDSS, MRI and disease-modifying treatment (DMT) from a computerized database, iMed© (Merck Serono SA; Geneva), including real-time inserted data. Neurological examination and the attribution of EDSS scores were performed by experienced and certified neurologists for all patients according to clinical practice at baseline and then annually up to 3 years.

Neuropsychological assessments

All enrolled subjects underwent a neuropsychological evaluation with the

use of the Italian version of the Brief International Cognitive Assessment (BICAMS) (25,26), a validated neuropsychological battery including the following tests:

1. Symbol Digit Modalities Test (SDMT), to provide a measure of speed in information processing. The total number of correct answers provided in 90 seconds is recorded and a threshold of 34.2 is commonly considered to pass the test (27);

2. California Verbal Learning Test-II (CVLT-II), which measures episodic verbal learning and memory by assessing encoding, recall and recognition in a single modality of item presentation (auditory-verbal). The test includes a 16-item word list, each belonging to one of four semantic categories, which is read aloud five times in the same order to the patient, asked to recall as many items as possible in any order (28);

3. Brief Visuospatial Memory Test Revised (BVMT-R), to evaluate visual learning. It consists of three learning trials for the patient, who is asked to reproduce on a sheet of paper six geometric figures previously shown for 10 seconds. Each drawing is evaluated according to accuracy and location and scored with 0-2 points, for a total score ranging from 0 to 12 for each trial. Delayed free recall of the same geometric figures is tested

after 25 minutes (BVMT-R Delayed Recall) (29).

The presence of depressive symtoms was detected by administering the Beck Depression Inventory (BDI), a self-administered 21-items clinical interview investigating on the psychological and somatic symptoms of depression (30).

CoI was confirmed when failure of at least one neuropsychological test was recorded, identified as a score lower than 2 SD from the normative values.

Optical Coherence Tomography

OCT was performed at baseline and after 3 years (T2) with Stratus OCT (model Cyrrus 5000, Carl Zeiss Meditec, Dublin, CA). If ON occurred, the examination was conducted at least two months after symptoms onset or at least one month after steroid administration. RNFL was acquired with the Optic Disc Cube 200×200 protocol that images the optic disc in a 6 mm×6 mm region. The mean RNFL and values referred to individual quadrants were calculated. The annualized RNFL loss was considered as the difference between 3-year follow-up and baseline values calculated in the whole observation period divided by the number of years of

observation.

Macular GCL was obtained using the Macular Cube 512×128 protocol that images a 6 mm×6 mm area centered at the fovea. The GCL was calculated automatically over an elliptical annulus (2 mm×2.4 mm radius), excluding the central foveal region (0.5 mm×0.6 mm radius). The inter-eye differences in OCT measures (non-affected eyes minus affected eyes) were evaluated. Only well-focused and centred scans with a signal strength of at least 7 were included. Quality control and APOSTEL recommendations according to published criteria will be followed (31,32).

Statistical Analysis

Data were analyzed with STATA© (StataCorp, College Station, TX, United States, version 16.1). After assessing quantitative variables for normality with the Kolmogorov-Smirnov test, continuous variables were reported as mean and standard deviation (SD), or median and interval, as appropriate. Student's t test and Mann-Whitney U Test (U) were used to compare continuous variables between groups. Categorical variables were expressed in frequencies and percentages, and compared between groups

with Chi-square test (χ^2).

A cut-off value of 88 μm was chosen as threshold for RNFL thickness, representing the lowest tertile of data distribution in our sample and a potential promising threshold according to a previous multicentre cohort study(121) (33). Cox proportional hazard models correcting for age, previous history of optic neuritis, disease duration and EDSS at baseline were used to test RNFL thickness ≤88 μm as a predictor of EDSS progression.

We tested all variables for collinearity by variance inflation factor (VIF) and excluded all variables from the regression analysis if VIF >2.0 corresponding to an R^2 of 0.60.

Linear regression analyses were used to test associations between the RNFL and cognitive performance, both at baseline (number of tests failed) and on follow-up testing (number of tests with a worse result at follow-up).

Logistic regression analyses were used to determine odds ratios for cognitive deficits and EDSS worsening at 3-year follow-up, including RNFL value as independent variable and correcting for potential confounders. We considered EDSS worsening as a change in EDSS by 1-

point from a baseline score up to 5.5 or a 0.5-point increase from a baseline higher than 5.5 (34). The same statistical analysis was also used to determine odds for RNFL and GCL thickness, dichotomized according to median values, including cognitive performance and EDSS as independent variables.

A p value of <0.05 was considered significant for all tests, which were 2-sided.

Results

Patients' characteristics

We enrolled 487 patients diagnosed with MS, according to 2010 McDonald criteria (122), during the period between January 2015-December 2019 (Table 1). Of them, 419 (86.0%) exhibited a relapsing-remitting phenotype (RRMS) and 68 (14.0%) a primary progressive one (PPMS). Patients with PPMS (pwPPMS) were older than those with RRMS (pwRRMS) at onset (46.7±11.7 vs 34.2±11.7 years, $p<0.05$) and at the time of diagnosis (52.7±10.8 vs 37.0±12.2 years, $p<0.001$), and exhibited longer disease duration (107.4±81.9 vs 66.3±57.8; $p<0.05$).

Median EDSS at the time of diagnosis was significantly different between pwRRMS (2.0; 0.0-8.0) and pwPPMS (5.5; 1.5-8.0) (p<0.001).

Table 1. Demographic and clinical characteristics of the study population (487 patients).

	RRMS	**PPMS**	**p**
N (%)	419 (86.0)	68 (14.0)	
Age at onset, y mean±SD	34.2 ± 11.7	46.7 ± 11.7	<0.05
Age at diagnosis, y mean±SD	37 ± 12.2	52.7 ± 10.8	<0.001
Female N (%)	279 (66.6)	29 (42.6)	<0.001
Disease duration, m mean±SD	66.3 ± 57.8	107.4 ± 81.9	<0.05
EDSS at onset median (range)	2 (0.0-8.0)	5.5 (1.5-8.0)	<0.001

N: number; y: years; m: months; SD: standard deviation; EDSS: Expanded Disability Status Scale; n.s.: not significant.

Clinical presentation at disease onset was different between groups, with pyramidal symptoms reported by 82.3% of PPMS and 59.4% of RRMS

patients (p<0.001), and visual symptoms described by 10.3% of PPMS and 24.1% of RRMS (p<0.05). MRI characteristics of the study population are shown in Table 2. Among patients, 371 (88.5%) RRMS and 51 (75.0%) PPMS received DMT (Table 3).

Table 2. MRI characteristics of the study population (487 patients) at baseline and at 3-year follow-up.

	RRMS	**PPMS**	**p**
Baseline MRI mean ± SD			
Brain			
T1	5.5 ± 7.7	9.5 ± 10	**<0.01**
T2	22.3 ± 21.1	31.5 ± 29.2	**<0.05**
Gd+	1 ± 2.6	0.3 ± 1.6	**<0.01**
Spinal cord			
T1	0.03 ± 0.2	5.7 ± 14.1	**<0.001**
T2	2.4 ± 2.1	6 ± 16.1	**<0.05**
Gd+	0.6 ± 0.9	0.3 ± 0.4	**<0.05**
3-year follow-up MRI mean ± SD			
Brain			
T1	7.8 ± 8.6	13.7 ± 13.7	**<0.05**
T2	25.1 ± 23.3	36.9 ± 33.9	**<0.05**
Gd+	0.2 ± 1.7	0.1 ± 0.3	**<0.05**
Spinal cord			
T1	0.02 ± 0.1	0	n.a.
T2	2.4 ± 2.5	3.9 ± 2.3	**<0.05**
Gd+	0.07 ± 0.3	0.1 ± 0.2	**<0.01**

MRI: Magnetic Resonance Imaging; Gd+: gadolinium-enhanced; SD: standard deviation; n.a.: not applicable.

Table 3. Disease-modifying treatment of the study population (422 patients).

	RRMS (371)		**PPMS** (51)	
DMT	**N** (%)	**Treatment duration, m** mean ± SD	**N (%)**	**Treatment duration, m** mean ± SD
IFN + GA	147 (39.6)	25.9 ± 24.9	12 (23.5)	27.7 ± 20.2
FTY	1 (0.3)	53	3 (5.9)	24.3 ± 7.1
NTZ	63 (17)	27.9 ± 15.4	5 (9.8)	36.6 ±15.3
DMF	64 (17.2)	26.9 ± 17	9 (17.6)	25.3 ± 17.2
TFN	55 (14.8)	23.4 ± 15.5	8 (15.7)	32.4 ± 12.6
IS	8 (2.2)	7.6 ± 6	2 (3.9)	44.5 ± 17.7
RTX	13 (3.5)	11.1 ± 8	3 (5.9)	8 ± 4.4
ALM	2 (0.5)	28.5 ± 13.4	0	0
OCRE	13 (3.5)	21.5 ± 5.3	9 (17.6)	13 ± 7.2
CDA	4 (1.1)	10 ± 5.6	0	0
DCZ	1 (0.3)	3*	0	0

DMT: disease-modifying treatment; RRMS: relapsing-remitting multiple sclerosis; PPMS: primary progressive multiple sclerosis; N: number; m: months; IFN: interferon beta; GA: glatiramer acetate; FTY: fingolimod; NTZ: natalizumab; DMF: dimethyl fumarate; TFN: teriflunomide; IS: immunosuppressant; RTX: rituximab; ALM: alemtuzumab; OCRE: ocrelizumab; CDA: cladribine; DCZ: daclizumab.

Baseline and follow-up OCT evaluation

At baseline, pwPPMS exhibited thinner RNFL (r.e., 90.4 ± 12.7; l.e. 90.2 ± 13.5) compared with pwRRMS (r.e. 94.6 ± 13.1; l.e. 94.3 ± 14.8) ($p<0.01$). Similarly, reduced GCL thickness was detected in PPMS (r.e., 80.1 ± 11.2; l.e. 80.3 ± 12.6) compared with RRMS (r.e., 85.1 ± 9.5; l.e. 84.9 ± 9.3) ($p<0.05$) (Table 4; Fig. 1). RNFL thickness decreased bilaterally in both pwRRMS and pwPPMS at 3-year follow-up ($p<0.01$) (Table 5; Fig. 2). Similarly, a thinner GCL was detected in both eyes when OCT was performed at follow-up compared with baseline values in both

pwRRMS ($p<0.01$) and pwPPMS ($p<0.001$) (Table 5; Fig. 3).

Table 4. OCT, motor function and neuropsychological assessments performed at baseline in RRMS and PPMS patients.

	RRMS (419)	**PPMS** (68)	**p**
RNFL r.e., µ mean ± SD	94.6 ± 13.1	90.4 ± 12.7	**<0.01**
RNFL l.e., µ mean ± SD	94.3 ± 14.8	90.2 ± 13.5	**<0.01**
GCL r.e., µ mean ± SD	85.1 ± 9.5	80.1 ± 11.2	**<0.05**
GCL l.e., µ mean ± SD	84.9 ± 9.3	80.3 ± 12.6	**<0.05**
9HPT d, sec mean ± SD	22.7 ± 11.9	29.5 ± 13.9	**<0.05**
9HPT nd, sec mean ± SD	24.5 ± 12.0	33.6 ± 15.8	**<0.05**
T25FWT, sec mean ± SD	7.6 ± 4.6	10.1 ± 4.1	**<0.05**
SDMT mean ± SD	36.1 ± 12.2	24.9 ± 7.7	**<0.01**
CVLT tot mean ± SD	9.9 ± 3.1	7.3 ± 3.9	**<0.01**
BVMT I mean ± SD	9.0 ± 3.0	8.0 ± 2.9	n.s.
BVMT II mean ± SD	9.8 ± 2.6	8.0 ± 3.0	**<0.01**
BVMT III mean ± SD	10.7 ± 2.9	9.0 ± 4.1	**<0.01**
CoI N (%)	152 (36.3)	31 (45.7)	**<0.05**

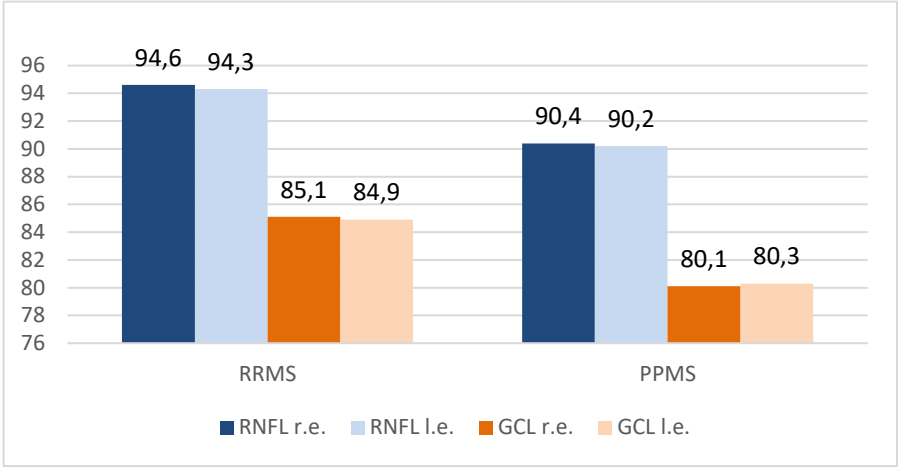
OCT: optical coherence tomography; RRMS: relapsing-remitting multiple sclerosis; PPMS: primary progressive multiple sclerosis; RNFL: retinal nerve fiber layer; GCL: ganglion cell layer; r.e.: right eye; l.e.: left eye; µ: micron; 9HPT: 9-hole peg test; d: dominant; nd: non-dominant; sec: seconds; T25FWT: timed 25-foot walk test; SDMT: symbol digit modalities test; CVLT: California verbal learning test; BVMT: brief visuospatial memory test; CoI: cognitive impairment; SD: standard deviation.

Table 5. Results from OCT assessment at baseline in the study population (487 patients).

	RRMS			**PPMS**		
	Baseline	**Follow-up**	**p**	**Baseline**	**Follow-up**	**p**
RNFL r.e., µ mean ± SD	94.6 ± 13.1	91.9 ± 14.6	**<0.01**	90.4 ± 12.7	86.9 ± 16.9	**<0.01**
RNFL l.e., µ mean ± SD	94.3 ± 14.8	91.6 ± 16.0	**<0.01**	90.2 ± 13.5	86.7 ± 15.3	**<0.01**
GCL r.e., µ mean ± SD	85.1 ± 9.5	82.0 ± 11.1	**<0.01**	80.1 ± 11.2	72.8 ± 13.8	**<0.001**
GCL l.e., µ mean ± SD	84.9 ± 9.3	81.8 ± 11.9	**<0.01**	80.3 ± 12.6	72.5 ± 15.2	**<0.001**

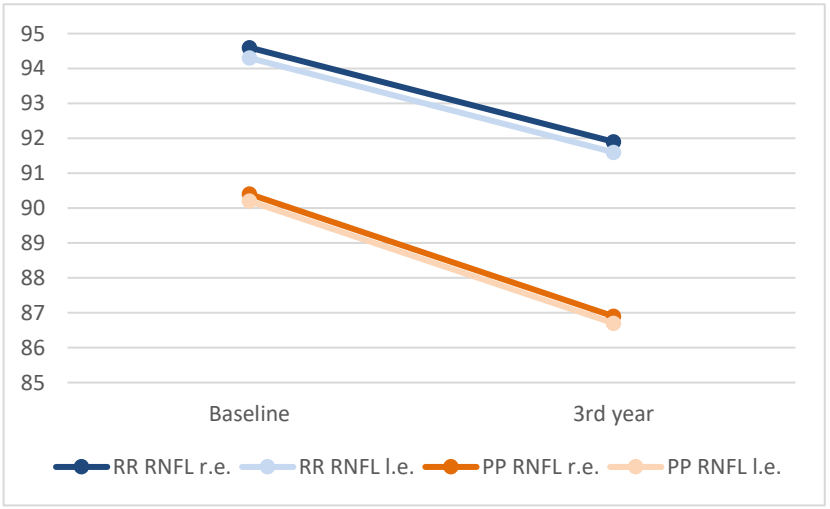
RRMS: relapsing-remitting multiple sclerosis; PPMS: primary progressive multiple sclerosis; RNFL: retinal nerve fiber layer; GCL: ganglion cell layer; r.e.: right eye; l.e.: left eye; µ: micron; SD: standard deviation.

Fig. 1. OCT parameters detected at baseline in RRMS and PPMS patients.



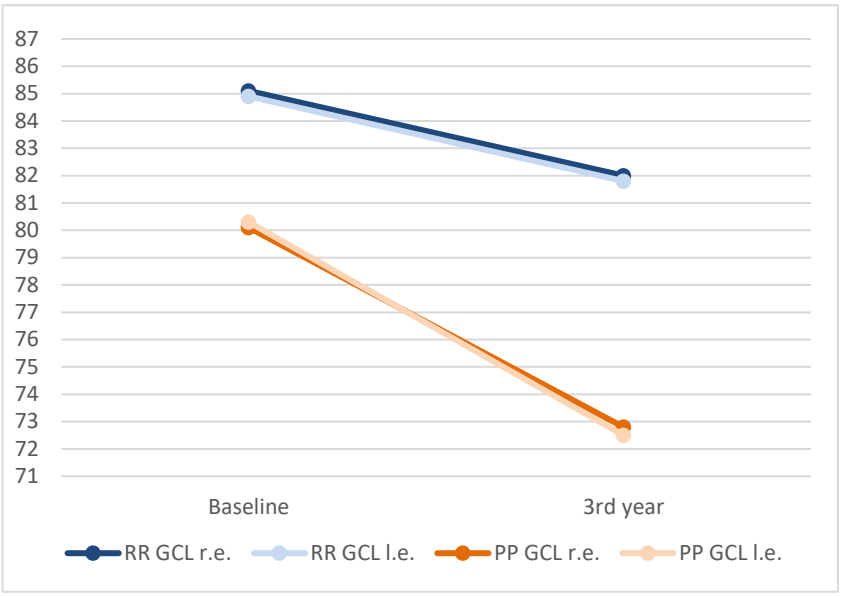
RRMS: relapsing-remitting multiple sclerosis; PPMS: primary progressive multiple sclerosis; RNFL: retinal nerve fiber layer; GCL: ganglion cell layer; r.e.: right eye; l.e.: left eye

Fig. 2. RNFL thickness at baseline and at 3-year follow-up in RRMS and PPMS patients.



RRMS: relapsing-remitting multiple sclerosis; PPMS: primary progressive multiple sclerosis; RNFL: retinal nerve fiber layer; r.e.: right eye; l.e.: left eye

Fig. 3. GCL thickness at baseline and at 3-year follow-up in RRMS and PPMS patients



RRMS: relapsing-remitting multiple sclerosis; PPMS: primary progressive multiple sclerosis; GCL: ganglion cell layer; r.e.: right eye; l.e.: left eye

Baseline and follow-up NPS evaluation

Comprehensively, 152 of 419 RRMS (36.3%) and 31 of 68 PPMS (45.6%) subjects were cognitively impaired at baseline evaluation (Table 4). PwRRMS exhibited significantly lower scores at 3-year SDMT (33.8 ± 16.6) compared with baseline values (36.1 ± 12.2; $p<0.05$), while no differences were detected in other tests (Table 6).

Table 6. Neuropsychological examination at baseline and at 3-year follow-up in the study population (487 patients).

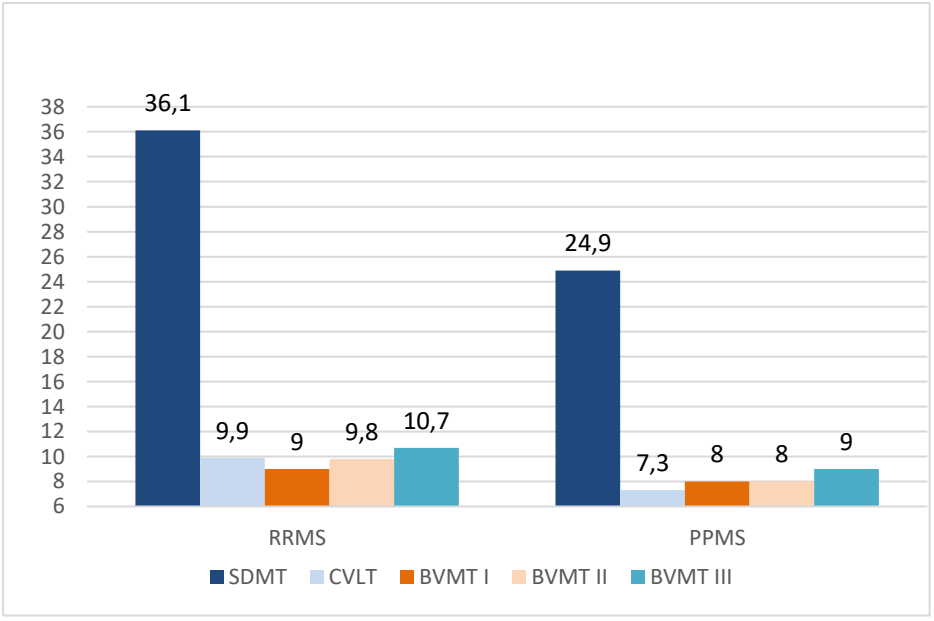
	RRMS			PPMS		
	Baseline	**Follow-up**	**p**	**Baseline**	**Follow-up**	**p**
SDMT	36.1 ± 12.2	33.8 ± 16.6	**<0.05**	24.9 ± 7.7	19.8 ± 8.2	**<0.001**
CVLT	9.9 ± 3.1	9.4 ± 2.9	n.s.	7.3 ± 3.9	7.1 ± 3.1	**<0.01**
BVMT I	9.0 ± 3.0	8.8 ± 3.7	n.s.	8.0 ± 2.9	6.4 ± 3.5	**<0.01**
BVMT II	9.8 ± 2.6	9.3 ± 2.7	n.s.	8.0 ± 3.0	6.5 ± 2.1	**<0.01**
BVMT III	10.7 ± 2.9	9.6 ± 4.3	n.s.	9.0 ± 4.1	6.9 ± 4.2	**<0.01**
CoI	152 (36.3)	184 (43.9)	**<0.05**	31 (45.7)	37 (54.4)	**<0.05**

RRMS: relapsing-remitting multiple sclerosis; PPMS: primary progressive multiple sclerosis; SDMT: symbol digit modalities test; CVLT: California verbal learning test; BVMT: brief visuospatial memory test; CoI: cognitive impairment; SD: standard deviation.

PwPPMS performed worse than pwRRMS at baseline SDMT (respectively, 24.9 ± 7.7 vs 36.1 ± 12.2; $p<0.01$), CVLT (7.3 ± 3.9 vs 9.9 ± 3.1; $p<0.01$), BVMT II (8.0 ± 3.0 vs 9.8 ± 2.6; $p<0.01$) and BVMT III (9.0 ± 4.1 vs 10.7 ± 2.9; $p<0.01$) (Fig. 4). Further, they exhibited a significant worsening in cognitive performance at all tests at 3-year follow-up ($p<0.01$) (Table 6). The prevalence of CoI at 3-year follow-up was

significantly higher than at baseline for both RRMS (respectively, 43.9% vs 36.3%; $p<0.05$) and PPMS patients (respectively, 54.4% vs 45.7%; $p<0.05$).

Fig. 4. Cognitive performance of RRMS and PPMS patients at baseline and at 3-year follow-up at different neuropsychological tests (average scores).



RRMS: relapsing-remitting multiple sclerosis; PPMS: primary progressive multiple sclerosis; SDMT: symbol digit modalities test; CVLT: California verbal learning test; BVMT: brief visuospatial memory test.

Predictors of Cognitive Impairment and EDSS worsening

Patients with RNFL thickness less or equal to 88 µm at baseline exhibited

a significalty increased odds of being cognitively impaired at baseline and after 3 years from diagnosis (OR=5.32, 95% CI=1.84-9.12, p=0.02). Other independent risk factors for the presence of CoI at baseline were a higher EDSS at diagnosis (OR=4.22, 95% CI=1.32-15.98, p=0.003), a progressive course (OR=4.54, 95% CI=1.65-6.98, p=0.001). EDSS at diagnosis, progressive phenotype, RNFL ≤88 μm confirmed their role as independent risk factors for the detection of CoI at 3-year follow-up, along with higher disease duration (OR=4.32, 95% CI=1.87-18.12, p=0.02) and annualized RNFL loss (OR=3.31, 95% CI=1.87-9.54, p=0.001). Conversely, a higher educational level was a protective factor for the outcome at baseline (OR=0.66, 95% CI=0.16-0.96, p=0.01) and after 3 years from diagnosis (OR=0.81, 95% CI=0.16-0.96, p=0.01).

The odds of EDSS worsening at 3 years was independently predicted by EDSS at diagnosis (OR=1.76, 95% CI=1.11-8.12, p=0.01), disease duration (OR=5.14, 95% CI=2.01-7.23, p=0.001), progressive course (OR=5.78, 95% CI=1.13-11.33, p=0.01). Additionally, RNFL thickness lower or equal to 88 μm (OR=3.18, 95% CI=1.21-10.33, p=0.05) and a higher annualized RNFL loss (OR=4.13, 95% CI=1.84-8.21, p=0.01) were respectively associated to a 3-fold and a 4-fold increased risk of EDSS

worsening at 3-year follow-up.

Discussion

Results from our study supported the reliability of RNFL and GCL as potential predictive biomarkers for the development of physical and cognitive deterioration during follow-up in pwMS. Both pwRRMS and pwPPMS exhibited significantly lower values of RNFL and GCL thickness, higher EDSS scores and worse cognitive performance over a 3-year follow-up compared with baseline, although the latter performed worse at all assessments already at baseline.

Several studies reported a significant difference in RNFL and GCL thickness in pwMS compared with controls, regardless of a previous history of ON (6,12,13,35). Particularly, the measurement of GCL proved to be even more sensitive in the early phases of the disease and strongly associated with brain atrophy (13–15). Further, several studies reported evidence of RNFL thickness reduction over time (14,37–39), including a recent 2-year prospective study involving 135 pwMS and 16 controls (14). However, when comparing these parameters among MS phenotypes,

results were controversial.

In a study involving 326 pwMS and 94 controls, progressive MS patients (PMS) were characterized by decreased RNFL thickness compared with RRMS, and both groups exhibited a smaller thickness compared with controls, regardless of previous ON (12). Another study found a significant thinning of RNFL, GCIPL and outer plexiform layer in PMS compared to RRMS (7). Differently, no distinction emerged between pwPPMS and pwRRMS without ON in some studies (8,35), while others detected differences in RNFL only or particularly in SPMS rather than PPMS compared with controls (13,36). Still, a few studies compared only progressive phenotypes with controls (36) or considered them as a single group, without distinction between SPMS and PPMS (35).

As expected according to the natural disease course, in our study pwPPMS were older and more physically and cognitively impaired at the time of diagnosis, compared with pwRRMS. Therefore, it was not unforeseeable that pwPPMS exhibited greater disability and worse cognitive performances at 3-year follow-up. It is interesting that RNFL and GCL thickness reflected this trend, with a more pronounced reduction in pwPPMS both at baseline and at follow-up.

We used a threshold of 88.0 μm, which corresponded to the lowest tertile of data distribution in our sample, to define a significant reduction in RNFL thickness. Irrespective of previous ON or DMT, pwMS with RNFL thickness lower than the chosen cut-off exhibited a 5-fold risk to develop CoI in our population. This is in line with results from a cross-sectional study involving 217 pwMS and evaluating possible associations between inner retinal layer atrophy and CoI (20). Not only cognitively impaired patients exhibited significantly lower mean RNFL and GCIPL than cognitively preserved ones, but RNFL lower than 85.0 μm and GCIPL below 88.1 μm, which were the median values in the data distribution, were respectively associated to 4-fold and 3-fold increased odds of subsequent CoI (20). As in our study, SDMT was used to assess the presence of CoI. It was the only test in our study population, within BICAMS battery, which was sensitive to cognitive worsening of both pwRRMS and pwPPMS over time, thus confirming its clinical usability as a first-line screening test for CoI (40). Additionally, previous results indicated a good correlation between visual test performance and processing speed, more than memory function (41). As expected, pwPPMS achieved lower scores at all cognitive tests and exhibited a

significantly higher prevalence of CoI both at baseline (45.7%) and at follow-up (54.3%) compared with RRMS (36.3% and 43.8%, respectively). Indeed, as reported by previous studies (42), EDSS at diagnosis and progressive phenotype were known independent risk factors for the detection of CoI, both at baseline and follow-up, while a higher educational level was a protective factor for the investigated outcome. Other studies reported the association between CoI and the detection of smaller RNFL and GCIPL thickness (6,16,21,43,44). A large multicenter prospective one reported a significant association between worse cognitive performance and a thinner RNFL at baseline (44). Particularly, patients with RNFL thickness values in the lowest quintile were 11% more likely to fail at least one neuropsychological test and those in the two thinnest quintiles exhibited a double risk of perfoming worse at follow-up cognitive assessments.

In our study, a RNFL thickness lower or equal than 88.0 μm was also an independent predictor of disability worsening (OR=3.18; 95% CI=1.21-10.33; p=0.05), regardless of previous history of ON and use of DMT. Further, a higher annualized RNFL loss was associated with 4-fold increased odds of EDSS worsening at 3 years. As expected, EDSS at

diagnosis, disease duration and progressive course were also predicting factors for EDSS worsening at follow-up. In another study, a reduced RNFL thickness was associated with a worse cognitive performance at SDMT, as well as with higher physical disability, confirmed by higher EDSS scores (45).

In a 3-year prospective study involving 141 RRMS patients, an annual RNFL thinning rate higher than 1.5 μm distinguished between stable and progressing patients with a sensitivity of 76.1% and a specificity of 90.0%, and such a threshold was associated with a 15-fold increased risk of clinically progressing MS (46). In another study, faster rates of annualized GCL thinning were associated with clinical and radiological disease-activity and disability progression during follow-up (47). Additionally, a multicentre cohort study found an association between a RNFL below 88 μm and a double risk of disability worsening during a 3-year follow-up (33).

Comprehensively, our results supported the use of RNFL and GCL as a biomarker of axonal damage since the early phases of the disease and, more notably, even in the absence of previous ON. A threshold of 88 μm, further, could be helpful to distinguish pwMS at high-risk of developing

physical and cognitive disability over a short-term follow-up. This further supports preliminary evidence about the association between RNFL thinning and progression independent from relapse activity (PIRA) (46), which accumulates since the very early phases of MS course in all phenotypes. Indeed, despite pwPPMS exhibited worse physical and cognitive performances than pwRRMS at all time-points in our study, RNFL and GCL thinning significantly predicted the development of physical and cognitive disability over years in both groups of patients, corroborating the recently proposed "one-MS hypothesis", characterized by a unique underlying smouldering process, reflected by RNFL and GCL thinning, and by a superimposed focal inflammatory activity which differs among clinical phenotypes.

Our study has several limitations. First, we did not investigate the presence of cortical atrophy or the location of demyelinating lesions in specific brain regions, which are known to be relevant for cognitive functions (19). Assessing the association with radiological characteristics could have further enhanced the reliability of OCT in predicting CoI and disability worsening, with the advantage of providing easier, shorter, and less expensive evaluations compared with MRI.

Still, we did not deepen the impact of different DMT on OCT parameters over time, as well as on cognitive performance and physical disability. Further, patients diagnosed with SPMS were not present in our study population since newly diagnosed patients were enrolled. However, this could be considered an advantage in order to strictly compare pwPPMS and pwRRMS. Indeed, progressive patients have often been considered as a single group in some studies exploring the predictive role of OCT, despite some authors reported differences in RNFL thickness between SPMS and PPMS (13,35,36). Additionally, we disposed of a large amount of data from 487 MS patients with a recent diagnosis and naïve to any DMT, analyzed at two different timepoints.

In this view, we believe that the use of OCT, already implemented in the diagnostic work up, should definitely be considered as a valuable resource to monitor disease course in pwMS, providing relevant information by performing a rapid, non-invasive and quantitative evaluation.

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2.2.3 Molecular biomarkers

Among molecular biomarkers, the term “prognostic” is usually related to pathological findings of axonal damage, astrocyte activation and remyelination, prevailing in the progressive phases of the disease (15). However, molecular biomarkers predicting high disease-activity are often considered as prognostic too, since it is well known that at least part of disease worsening, the so-called relapse-associated worsening (RAW), is dependent on the presence of high disease-activity, including frequent and severe clinical and radiological exacerbations (80,82). In this regard, CSF IgM OCB, glial fibrillary acidic protein (GFAP) and neurofilament light chains (NfL) are some of the most widely investigated molecular biomarkers.

CSF IgM OCB have been associated with severe disease course in RRMS, with earlier conversion to SPMS. Moreover, in patients with CIS, the presence of IgM OCB correlated with greater brain atrophy rates and MRI lesion load (123–127).

An increasing number of studies explored the role of glial fibrillary acidic protein (GFAP), which is expressed in the cytoskeleton of astrocytes, as a

prognostic biomarker for MS. Higher concentrations of this protein have been detected in models of experimental autoimmune encephalomyelitis, in MS lesions and in the CSF and serum of MS patients with greater EDSS scores, progressive phenotype and longer disease duration (128–132).

A recent study explored the role of serum GFAP and NfL in prognosticating disease progression in patients with MS (133). Higher serum GFAP levels at baseline predicted accelerated grey matter brain volume loss and confirmed disability worsening independently from the occurrence of relapses. When used together with serum NfL, their combined increase was associated to a nearly 5-fold increased risk of confirmed disability worsening and PIRA.

Neurofilament Light Chains

Neurofilaments (Nf) are protein polymers which are part of the neuronal cytoskeleton and provide structure and support to axons allowing the physiologic nerve conduction (134). Nf consist of heavy (NfH), medium (NfM) and light (NfL) chains, whose concentration in CSF increases when axonal damage occurs (135). On this assumption, Nf have been explored

as a marker of neurodegeneration in several neurological diseases, including amyotrophic lateral sclerosis (136,137), Alzheimer's disease (138), frontotemporal dementia (139), stroke (140), MS (135), Huntington disease (141), atypical parkinsonian syndromes and neurocognitive impairment in HIV-positive individuals (142). Particularly, Nf have been investigated in MS as a potential diagnostic (54,132,143), disease activity (144,145), prognostic (132,146,147) and treatment-response biomarker (148–152).

While higher CSF NfH concentrations have been reported in patients with SPMS, suggesting a correlation with age and chronic axonal damage (153,154), increased

CSF NfL values have been found in RRMS patients during exacerbations and up to 5 months before relapses, suggesting a relation with inflammation and acute axonal damage (144,152).

The correlation between NfL levels and EDSS changes is more controversial. In a recent study, NfL correlated with EDSS in PMS, but not with EDSS worsening in the previous year and up to 27-months in both RRMS and PMS (145). Differently, other studies reported associations between NfL and long-term cognitive and physical disability, brain and

spinal atrophy, NEDA-3 independently from MRI activity, and disease progression in both relapsing and stable patients (146,155–162). Additionally, CSF NfL proved to be an independent risk factor for conversion to CIS and MS in patients with RIS (54) and for conversion to MS in patients with CIS (132,143,153). In some prognostic studies, serum NfL also correlated with MRI outcomes, including brain volume loss and the increase of lesion load and decreased within 6 months from drug administration in those patients who achieved NEDA-3 (152,159,163,164).

As in most of the aforementioned studies exploring prognostic biomarkers in MS, serum NfL, and not CSF NfL, are often employed (165–168). Indeed, the use of single molecule array (Simoa) allowed to measure NfL levels in serum, where concentrations are nearly 42-fold lower, with the advantage of a less invasive procedure and the possibility of repeated measurements (165–167,169). However, despite a good correlation between serum and CSF NfL has been reported, some recent studies reported a 40-60% variance in serum NfL compared with CSF ones, maybe due to a possible peripheral release of serum NfL, in patients with clinical and radiological evidence of spinal cord injury (170). Reference values

have not been established for CSF and serum NfL so far, although serum concentrations between 16-20 pg/mL have been reported in a heterogeneous group of healthy controls recruited from different studies, tending to increase along with age-related physiological axonal damage (135,169).

In a recent study, reference values corrected for age and body-mass index (BMI) at a group level were reported, and serum NfL Z score ≥1.5 was associated with a 3-fold increased risk of future clinical or radiological activity in all patients with MS, including patients who reached NEDA-3 (171).

Article 4

Cerebrospinal fluid neurofilament light chains predicts early disease-activity in Multiple Sclerosis

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Abstract

Background: Among biomarkers of axonal damage, neurofilament light chains (NFL) seem to play a major role, representing a promising and interesting tool in Multiple Sclerosis (MS).

Our aim was to explore the predictive role of cerebrospinal fluid (CSF) NFL in patients with a recent diagnosis of MS, naïve to any MS therapy.

Methods: We retrospectively collected data of patients diagnosed with MS, referred to the Neurology Clinic of the University-Hospital G. Rodolico of Catania between January 1st 2005 and December 31st 2015. All patients underwent CSF collection at the time of MS diagnosis and were followed-up for at least three years afterwards. NFL levels were measured in CSF samples with Simoa NFLight advantage kit at the CRESM (University Hospital San Luigi Gonzaga, Orbassano, Torino). Symbol Digit Modalities test (SDMT) was performed at baseline, at 1-year and at 3-year follow-up. Multivariate logistic regression analysis was performed to investigate LogNFL as a potential risk factor of different clinical outcomes.

Results: 244 MS patients (230 relapsing-remitting, RRMS; 94.3%), with

a mean age at diagnosis of 37.0±11.1 years, were recruited. LogNFL levels did not correlate neither with EDSS score at diagnosis and at subsequent follow-up up to 12 years, nor with SDMT performed at diagnosis, at 1 year and at 3 years. LogNFL were an independent factor for the occurrence of at least one relapse during the first two years after MS diagnosis (OR=2.75; 95% CI 1.19-6.31; p=0.02) and for the occurrence of gadolinium-enhanced (Gd+) lesions during the first 2 years from diagnosis at brain and spine MRI scans (OR=3.45, 95% CI 1.81-6.57; p<0.001).

Conclusion: The detection of CSF NFL at the time of MS diagnosis can be a useful support to predict the two-year risk of clinical and radiological relapses, thus affecting therapeutic choices in the very early phases of the disease.

Keywords: multiple sclerosis, neurofilaments, biomarkers, cerebrospinal fluid, prognosis, axonal damage, disease-activity

Introduction

In recent years, neurofilament (NF) have been thoroughly investigated as a prognostic, disease-activity and drug-response biomarker for Multiple Sclerosis (MS). Being components of neuronal cytoskeleton, their increase in cerebrospinal fluid (CSF) and serum occurs in case of axonal damage, leading to speculations about the use of this biomarker as a predictor of the neurodegeneration rate in several diseases(1,2). In MS, NF light chains (NFL) levels proved to correlate well with acute inflammation, being higher in relapsing remitting MS (RRMS) than in progressive phenotypes and during clinical relapses compared with periods of remission(3,4).

Nevertheless, evidence on the long-term prognostic value of NFL is not univocal. On the one hand, increased levels of NFL were an independent risk factor for conversion to MS in subjects with radiologically isolated syndrome (RIS) and clinically isolated syndrome (CIS) and also predicted long-term cognitive and physical disability up to 19 years(5–7). Further, several studies reported CSF NFL (cNFL) as a prognostic factor associated with No Evidence of Disease Activity (NEDA-3), as well as with brain atrophy and spinal cord volume loss even in absence of MRI activity, and with disease progression in both relapsing and clinically stable

patients(4,8–14). On the other, in some studies serum NFL levels (sNFL) were not predictive of EDSS progression in the previous year and during a median follow-up of 27 months, neither in RRMS nor in progressive MS (PMS)(15).

Certainly, the use of NFL in clinical practice is restricted by the lack of a precise cut-off, which precludes the chance to distinguish pathological values from those reported in healthy people and to stratify the individual risk of disease activity, also considering that this biomarker is not specific for MS. In previous studies, sNFL values between 16-20 pg/mL were reported as a normal range in healthy controls(16), with a trend to increase along with age-related physiological axonal damage(17). Recently, the use of reference values corrected for age and body-mass index (BMI) at a group level allowed to identify an association between sNFL Z score above 1.5 and a three-fold increased risk of future clinical or radiological activity in all patients with MS, including patients who reached NEDA-3(18).

Since the monitoring of cNFL levels is limited by the invasiveness of performing serial lumbar punctures, sNFL levels have been often reported in recent studies(19–22). Despite sNFL correlates with cNFL levels, 40% to 60% of variance remains unexplained and higher levels of NFL have been recently found in serum but not in CSF of patients with clinical and radiological evidence of spinal cord injury, probably due to a NFL release

from peripheral axons directly into blood, bypassing the CSF(23).

Reflecting axonal damage, which contributes to the development of cognitive disability, NFL have been investigated in several studies as a potential marker of cognitive impairment in MS(24). Some studies reported significant associations between NFL and non-motor symptoms as cognition and fatigue(11,24,25), while others did not(26–28). Comprehensively, studies evaluating the association between NFL and cognition in patients with MS are not only controversial, but also limited in number and often include patients with long disease duration or progressive phenotypes(29).

In this context, we aimed to evaluate the association between cNFL levels at the time of diagnosis and the clinical course at follow-up of patients with a recent diagnosis of MS, naïve to any disease-modifying drugs (DMDs). As a secondary aim, we assessed correlations between cNFL levels and cognitive performance in the same cohort of patients over a follow-up of 3 years.

Materials and methods

Study population

In this retrospective study, we included patients with a confirmed

diagnosis of MS, among those admitted to the Neurology Clinic of the University-Hospital G. Rodolico of Catania between January 1st 2005 and December 31st 2015. Inclusion criteria were: age older than 18 years at the time of diagnosis; a diagnosis of MS according to the current revision of the McDonald criteria (31); availability of CSF samples collected via lumbar puncture (LP) at the time of MS diagnosis; a follow-up of at least 3 years at the MS Center of the Neurology Clinic of the University-Hospital G. Rodolico of Catania. The study was approved by our local ethical committee. All patients signed a written informed consent before the execution of LP to authorize the procedure and to allow data collection and use for study purpose.

Data collection

Demographic and clinical data prospectively collected at every clinical examination were retrospectively accessed via electronic medical records using the iMed© software (6.5.6, Merck Serono SA, Geneva, Switzerland). The Expanded Disability Status Scale (EDSS) score (32) was computed at diagnosis (baseline) and at least yearly. As a baseline EDSS, we considered the score attributed at the first neurological evaluation performed within 6 months after diagnosis and at least 30 days after a clinical relapse. EDSS worsening was attributed by comparing the

EDSS score at 3 years from diagnosis with the EDSS score at diagnosis. Particularly, “worsening” was identified by ≥ 1.5-point increase from a baseline EDSS score of 0.0, or by ≥ 1-point increase from a baseline EDSS score of ≥ 1.0 and ≤ 5.5, or by ≥ 0.5-point increase from a baseline EDSS score of ≥ 6.0 (33).

Radiological data were collected at every annual brain and spine MRI. NEDA-3 was defined as the absence of clinical relapses, new T2 lesions or Gd^+ lesions at MRI scans, and confirmed EDSS worsening at 2 years from MS diagnosis.

All participants underwent a neuropsychological assessment with Symbol Digit Modalities Test (SDMT) at the time of diagnosis, and after 1 year and 3 years. SDMT scores were corrected according to age, sex and education level (34).

Cerebrospinal fluid samples collection and neurofilament light chains detection

At the time of MS diagnosis, patients underwent CSF collection through diagnostic LP, obtaining a total volume of 10 ml of CSF. CSF samples were stored at -80°C until use according to international consensus guidelines[35]. CSF samples were sent to the Clinical Neurobiology Laboratory within the Multiple Sclerosis Regional Referral Centre

(CRESM) at the University Hospital San Luigi Gonzaga (Orbassano, Torino). A digital immunoassay based on the single molecule array technology (Simoa) was used for the quantitative determination of NFL in CSF on SR-X detection System (Simoa NFLight advantage kit, Quanterix)[36]. All CSF analyses were performed by trained technicians or biotechnologists blinded to clinical information.

Statistical analysis

Data were analyzed with SPSS© (IBM Corp. IBM SPSS Statistics for Windows, Version 26.0). After assessed for normality with the Kolmogorov-Smirnov test, continuous variables were reported as means (μ) ± standard deviation (SD) or medians and quartiles, as appropriate. Categorical variables were reported as frequencies and percentages (%). Student's t-test and one-way ANOVA were used to compare normally distributed continuous variables among groups. Friedman test was used to assess differences between SDMT scores repeated at baseline, at 1 and at 3 years. Spearman's correlation coefficient (r) was used to assess correlations between continuous variables. cNFL values were plotted on a logarithm scale with base 10 as LogNFL.

We estimated the risk of reaching different clinical outcomes by calculating the odds ratio (OR) and 95% confidence intervals (CI) in

binary logistic regression models, including LogNFL as the independent variable and sex, age at NFL collection and treatment with moderate-efficacy treatment (MET) or high-efficacy treatment (HET) as covariates and potential confounders. A p value of <0.05 was considered significant for all tests.

Results

Study population characteristics

We recruited 244 MS patients (152 female, 62.3%), with an age at diagnosis of 37.0±11.1 years. Among them, 230 patients (94.3%) were affected by RRMS at diagnosis, of which 12 (5.2%) turned to SPMS after 115.7±68.3 months (median=123; IQR=45.0-169.3). The median diagnostic delay from disease onset was 27.0 months (IQR=6.0-67.8).

After MS diagnosis, 7 patients (2.9%) did not start any DMDs, 106 (43.4%) of patients were only treated with moderate-efficacy treatment (MET) during follow-up, 39 (16.0%) with high-efficacy therapies (HET), 70 (28.7%) underwent an escalation therapy, and 22 (9.0%) an induction strategy.

Median EDSS score at diagnosis was 1.5 (IQR=1.0-3.0) and 58 (23.8%) patients exhibited EDSS worsening at 2 years of follow-up. The mean

number of relapses occurred respectively during the first and second year after diagnosis was 1.5±0.7 and 1.3±0.7. Brain and spine MRI data are reported in Table 1. The mean cNFL concentration was 1819.1±3455.6 pg/ml (median 917.6 pg/ml; IQR=468.1-1956.3). LogNFL concentration was 3.0±0.5.

Table 1. MRI characteristics and SDMT scores of the study population (244 patients).

	Year 1	**Year 2**	**Year 3**
Brain T2 lesions N (mean± SD)	23.9±22.7	27.8±25.5	28.6±26.0
Brain T1 lesions N (mean± SD)	7.7±9.1	8.6±10.2	9.1±10.1
Brain Gd+-enhanced lesions (N, %)	51 (20.9)	19 (7.8)	9 (4.1)
Spine T2 lesions N (mean± SD)	2.7±2.4	2.9±2.1	2.9±1.9
Spine Gd+-enhanced lesions (N, %)	27 (11.1)	6 (2.5)	6 (2.5)
SDMT score N (mean± SD)	44.8±10.1	43.4±10.2	41.5±10.2

Neurofilament light chains and clinical characteristics

No differences in NFL levels were detected between male (1829.4±2442.0 pg/ml; LogNFL 3.1±0.4) and female (1813.0±3952.6 pg/ml; LogNFL 2.9±0.5; p=0.09) patients. LogNFL did not correlate with age at disease onset (r=0.002, p=0.98) and age at diagnosis (r=-0.09, p=0.18), while a weak inverse correlation emerged between LogNFL and disease duration at diagnosis (r =-0.20; p = 0.001).

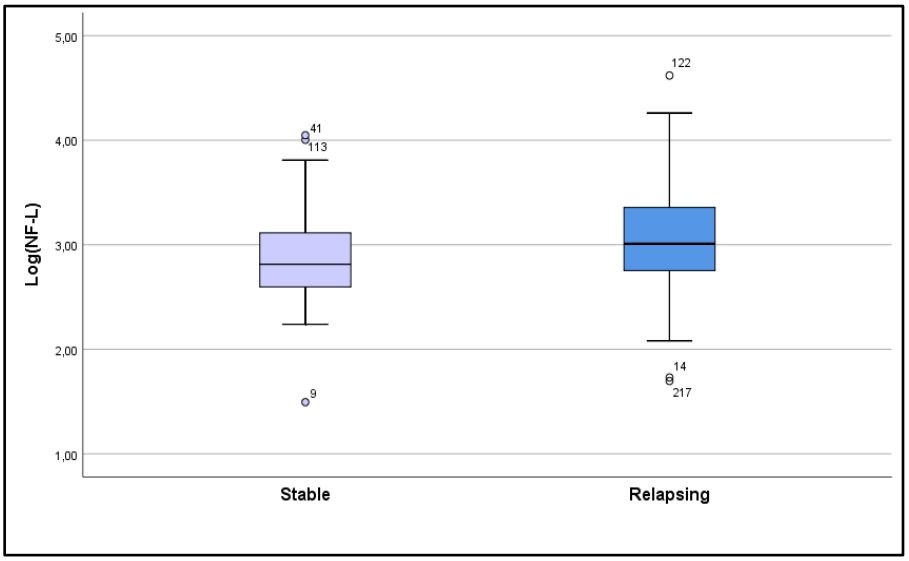
An upward trend in NFL levels emerged in RRMS (1882.1±3542.9 pg/ml; LogNFL 3.0±0.5) compared with PMS patients (784.5±938.5 pg/ml; LogNFL 2.8±0.3), though not reaching statistical significance (p=0.06).

LogNFL levels did not correlate with EDSS score at diagnosis (r=0.07, p=0.30), nor at subsequent follow-up at 3 (r=0.04, p=0.55), 6 (r=-0.02, p=0.76), 9 (r=-0.11, p=0.31) and 12 years (r=-0.16, p=0.33). Further, the binary logistic regression analysis did not reveal a statistically significant impact of LogNFL levels on the achievement of EDSS worsening at 3 years from diagnosis (OR=1.8, 95% CI 0.7-4.8; p=0.22).

In the total sample, 156 patients (63.9%) experienced at least one relapse within 3 months before diagnosis. Relapsing patients (2139.5±4067.4 pg/ml; LogNFL 3.1±0.5) exhibited significantly higher values of NFL compared with stable ones (1251.3±1835.5 pg/ml; 2.9 ± 0.4, respectively;

p=0.002) (Fig. 1).

Figure 1. Box plot showing NFL distribution in stable and relapsing patients.



Neurofilament light chains and cognitive performance

SDMT scores obtained at baseline and during follow-up are reported in Table 2. PMS patients performed worse than RRMS at SDMT at baseline (respectively, 37.6±8.5 vs 45.0±10.0, p=0.01), at 1 year (35.7±8.7 vs 43.4±10.1, p=0.01) and at 3 years (35.3±8.1 vs 41.5±10.1, p=0.03). No differences in cognitive performances were observed comparing male and female patients (respectively, 45.3±10.1 vs 44.1±9.9, p=0.58), nor patients treated with MET or HET (44.5±9.8 vs 45.0±10.8, p=0.72).

Lower SDMT scores were recorded from patients who exhibited EDSS

worsening at 3 years compared with those who did not, at baseline (respectively, 41.3±10.8 vs 47.5±11.0, p=0.02), at 1-year (respectively, 39.8±10.9 vs 46.6±10.8, p=0.01) and at 3-year follow-up (respectively, 36.5±9.6 vs 44.8±10.4, p=0.001). LogNFL levels did not correlate with SDMT scores at baseline (r=0.1, p=0.10) and at subsequent 1-year (r=0.06, p=0.39) and 3-year follow-up (r=0.06, p=0.43).

Neurofilament light chains and magnetic resonance imaging

LogNFL were an independent risk factor for the occurrence of Gd+ lesions during the first 2 years from diagnosis at brain and spine MRI scans (OR=3.45, 95% CI 1.81-6.57; p<0.001). Furthermore, LogNFL levels predicted the number of Gd+ lesions at 1-year brain (F=13.9, p<0.001) and spine MRI (F=4.33, p=0.04), but not at subsequent follow-up scans.

Further, LogNFL levels predicted the number of T1 black holes at brain MRI scan at 1 year (F=6.35, p=0.01), but not at further radiological evaluations. There was no association between the occurrence of new T2 lesions at brain and spine MRI scans and LogNFL, and only a weak correlation emerged with the brain T2 lesion load at 1 year (r=0.17, p=0.02) and at 3 years (r=0.16, p=0.03). Similarly, LogNFL levels were not a significant risk factor for the achieving of NEDA-3 (OR=0.45, 95% CI 0.2-1.3, p=0.15).

Discussion

According to our results, cNFL detected at the time of MS diagnosis seem to be a good predictor of short-term disease activity, being associated with the occurrence of clinical relapses and Gd+ lesions at MRI brain and spine scans within two years from MS diagnosis. Conversely, cNFL were not associated with EDSS scores neither at the time of diagnosis nor during 12-year follow-up.

In the latest years, NFL have been extensively investigated as a potential prognostic biomarker for several neurological diseases, including MS, amyotrophic lateral sclerosis (1,36), Alzheimer's disease (37), frontotemporal dementia (38), stroke (39), MS (16), Huntington disease (40), atypical parkinsonian syndromes (41). However, their prognostic role in MS has not been fully clarified, since controversial results emerged from studies evaluating the correlation between NFL and EDSS scores (7,42–44). Several studies reported associations between cNFL and long-term cognitive and physical disability, brain and spinal atrophy, NEDA-3, and disease progression in both relapsing and stable patients (4,8–14,45). In other prognostic studies, sNFL correlated with MRI outcomes, including brain volume loss and the increase of lesion load, and decreased within 6 months from drug administration in those patients who achieved NEDA-3

(11,46–48).

However, a more recent study explored the role of serum glial fibrillary acidic protein (GFAP) and NFL in prognosticating disease progression in patients with MS (49). While serum GFAP levels at baseline predicted accelerated grey matter brain volume loss and confirmed disability worsening independently from the occurrence of relapses, NFL only correlated with clinical and radiological disease-activity, and the combined use of serum NFL and GFAP proved to be associated to a nearly 5-fold increased risk of confirmed disability worsening and progression independent from relapse activity (PIRA). Additionally, another recent study did not report significant associations between cNFL and EDSS worsening in the previous year and up to 27-months in both RRMS and PMS (15). Actually, cNFL reflect underlying pathological processes that are not necessarily captured by disability scores, especially since slowly progressive patients rarely present a disease course with acute clinical worsening or dynamic changes in the EDSS score(50).

On the other hand, cNFL proved to be a reliable short-term disease-activity biomarker in our study, predicting the occurrence of both clinical and radiological relapses, but only in the first 2 years after the time of diagnosis, independently from DMT. cNFL also inversely correlated with disease duration at diagnosis, albeit weakly, probably due to a more

prominent release of NFL in the early inflammatory phase of the disease and temporally closer to the occurrence of relapses(4). Previous studies reported higher concentrations of cNFL in patients with clinical and radiological activity compared with stable ones (12,20,44,51–55). Further, cNFL values have been found in RRMS patients during exacerbations and up to 5 months before relapses, suggesting a relation with inflammation and acute axonal damage (3,46). In a recent study, serum NFL Z score ≥1.5 was associated with a 3-fold increased risk of future clinical or radiological activity in all patients with MS, including patients who reached NEDA-3 (18).

While some studies reported an association between cNFL levels and new MRI brain and spine lesions at follow-up compared with patients with no radiological activity (12,20,51–55), in our study cNFL levels did not predict the occurrence of new T2 lesions at brain and spine MRI scans and only a weak correlation emerged with brain T2 lesion load during follow-up. According to clinical practice, most of our patients perform MRI scans yearly, so the detection of new lesions in a subsequent scan not necessarily reflects very recent inflammatory exacerbations.

Despite cNFL physiologically tend to increase with age(41), we did not find higher concentrations in older patients. However, it should be noted that younger individuals often exhibit higher disease-activity, which

is related to greater NFL values, and that several factors other than MS can impact cNFL concentrations, as BMI or other comorbidities(42,43). This inter-subject variability explains the difficulty in identifying a single cut-off value or a range of normal values in healthy controls.

cNFL levels were not a predictor of cognitive performance in our study, assessed with the administration of SDMT, nor at baseline neither at successive evaluations. Previous studies assessing the association between cNFL and cognition provided controversial results. In a recent prospective study enrolling 45 MS patients with stable disease and EDSS ≤5.0, sNFL were not associated with cognitive performance assessed with a comprehensive neuropsychological battery, though 40% of them were diagnosed with cognitive impairment defined at the SDMT(28). Differently, other studies supported the association between elevated sNFL and a more severe and faster cognitive decline (56–58). One of the main limitations is often represented by the small number of patients examined and the inclusion of those with progressive phenotypes and long disease duration, both associated with worse cognitive performance (29). Indeed, patients with progressive phenotypes performed worse than those with RRMS at the neuropsychological assessment in our study, as well as those who reached EDSS worsening compared with stable disease.

Our study has some limitations, including the retrospective nature and the

timing of CSF collection and MS diagnosis, which was made after a variable amount of time from disease onset. However, clinical data have been collected prospectively in a quite large sample of patients and the diagnostic delay detected in our population was in line with the one reported in previous studies(59,60).

Further, cNFL levels were only measured at the time of MS diagnosis, since the collection of CSF samples is not suitable to repeated measurements due to the invasiveness of the procedure. This does not allow us to monitor patients over time and to test the effect of DMDs on cNFL concentrations. However, despite the collection of sNFL is less invasive and thus more replicable, making it suitable as a potential treatment-response biomarker, recent studies have pointed out that sNFL can be influenced by serum creatinine and renal function(61), as well as by a peripheral release by damaged axons(23), leaving some doubts about the correspondence between CSF and serum measurements. Furthermore, CSF collection is routinely performed during the diagnostic process of patients with suspected MS, and CSF itself, due to anatomic and physiological reasons, can be still considered the most appropriate source of biomarkers among body fluids for several neurological diseases(62,63). According to our results, NFL can be considered a good short-term disease-activity biomarker, but are not suitable to be used for

prognostication in MS. It can be supposed that NFL are able to reflect only the relapse-associated worsening (RAW) and not PIRA, both concurring to the accrual of disability in MS. The combined use of different molecular biomarkers, able to reflect both mechanisms involved in MS disease progression, could be a valuable support for prognostication in MS and deserves further evaluations.

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3. General discussion

The opportunity to use objective and quantifiable indicators, for both diagnostic and prognostic purposes, has radically changed the decision-making processes in the management of neurological diseases. Indeed, for years, only probable diagnoses have been formulated for most of neurological diseases, purely relying on clinical aspects. In this respect, MS represented a pioneer, since the introduction of MRI in the 2001 revision of McDonald's diagnostic criteria has radically changed the disease course, allowing earlier diagnosis and treatment. Additionally, IgG OCB were recognized, first among molecular biomarkers, as a fundamental diagnostic support for MS, despite they have been not included in the diagnostic criteria as a substitute for DIT until the 2017 latest revision. IgG OCB and MRI currently represent the only diagnostic biomarkers for MS which have been implemented in clinical practice worldwide, and whose clinical usefulness relies on high values of diagnostic accuracy. As part of my PhD project, I investigated the potential of another molecular biomarker, κFLC Index, which has been known for years in the scientific scene, but never really exploited (*Article 1*). Despite the main limitation to its use in clinical routine relies on the lack of a

consensus about the cut-off that should be adopted, our study suggested that this is not an unsolvable problem. Indeed, most of previous studies focused on values in the range 4.25-6.6, which did not significantly differ in terms of diagnostic accuracy, but exhibited higher sensitivity (80.0-86.8%) than IgG OCB (72.2%) with only slightly lower values of specificity (88.1-92.9% versus 95.2%, respectively). Additionally, κFLC Index could identify patients with MS who are OCB-negative, a substantial group in our sample (21.2%). Not only κFLC Index proved to be equally or more accurate than IgG OCB for the diagnosis of MS in our study, but the use of this biomarker would take countless benefits, including the possibility of assessing the probability of MS diagnosis for each unit increment, which is an absolute novelty. We therefore strongly believe that it is worth performing the analysis of κFLC Index during the routine diagnostic workout, that already includes the performance of lumbar puncture for the physico-chemical analysis of CSF.

Whilst some diagnostic biomarkers are already used in clinical practice, none has been implemented for prognostic purpose so far. In my PhD project, I explored different types of potential prognostic markers, including demographic and clinical factors (*Article 2*), imaging (*Article 3*) and molecular ones (*Article 4*).

The role played by age and sex has been widely investigated and is not entirely univocal. Indeed, an older age at the time of diagnosis certainly is a negative prognostic factor for faster disability progression and conversion to SPMS within 5 years, but is also protective towards clinical and radiological activity, mainly driven by inflammation. Similarly, female patients proved to be more at risk of clinical relapses, but less likely to convert to SPMS.

This apparently contradictory evidence supports the novel concept of two distinct mechanisms driving MS course, RAW and PIRA, of which only the first is strictly related to inflammation. Particularly, only age, EDSS and disease duration from MS onset were predictors for the achievement of PIRA in our study.

The role played by DMT emerged unequivocally from our study as the most relevant protective factor towards the achievement of almost all explored outcomes. This confirms that the pharmacological management of MS, which has become much more complex in the latest years with the availability of a greater number of therapeutic options, has really changed the course and prognosis of MS for many patients. Two aspects are crucial in this regard: the use of high-efficacy treatments (HET) since the time of diagnosis and the achievement of an optimal therapeutic response during

the first year of treatment, both confirming the relevance of the correct timing for the introduction of HET.

If the monitoring of treatment-response during the first year seems to be so crucial, a further problem arises: how can we improve and optimize treatment-monitoring?

While we know how to impact the long-term prognosis, how can we detect subclinical disease worsening?

According to the results of our study (*Article 3*), the use of OCT could be helpful in monitoring disease progression, since the thickness of RNFL has proved to predict the physical and cognitive worsening at 3 years. We found that RNFL thickness lower than 88 μm was associated with a 3-fold and a 5-fold higher risk of EDSS worsening and cognitive deterioration, respectively, and that the annualized RNFL loss itself correlated with disability increase in patients with RRMS and PPMS. Despite patients with PPMS exhibited significantly lower absolute values of RNFL thickness at baseline and at all time-points during a 3-year follow-up, the RNFL loss observed during the 3-year follow-up exhibited a similar trend in both groups, confirming that a common accrual of axonal loss occurs in both phenotypes according to the recently proposed "one-MS hypothesis" (103). According to this concept, RNFL thinning would reflect the

underlying smouldering process leading to PIRA, independently from the focal inflammatory activity.

Among molecular biomarkers, several studies are currently ongoing to identify those predicting of PIRA. A recent study identified serum GFAP, whose levels were found to be higher in worsening progressive patients, with rapid grey matter brain volume loss, compared with stable ones, independently from clinical and radiological relapses (133). The study highlights how the combined use of serum GFAP and NfL increased the risk of confirmed disability worsening by 4 to 5 times, since they presumably reflect both mechanisms (PIRA and RAW) leading to the accrual of disability, which is only partly related to acute inflammation. Differently, NfL alone failed in predicting PIRA but exhibited higher concentrations in active RMS. The results of our study (*Article 4*) go in the same direction. Indeed, CSF NfL were an independent risk factor for the occurrence of clinical relapses and gadolinium-enhanced lesions at brain and spine MRI during the first 2 years from diagnosis, but did not correlate with EDSS scores, disability and cognitive worsening in the short-term follow-up. According to our results, measuring CSF NfL at the time of MS diagnosis could be helpful to predict the short-term disease-activity, but not to predict long-term disability accrual and PIRA.

A secondary aim of this PhD project was the identification of instruments and tools which can facilitate the implementation of the discussed biomarkers in clinical practice. In *Article 1,* we proposed a flowchart to guide the diagnostic workout for MS and to suggest how to restrict the demand for CSF IgG OCB analysis without lowering the diagnostic accuracy. In *Article 2,* prognostic nomograms, easy-to-use and highly customizable, were provided to calculate the personalized risk for different outcomes, based on the use of demographic and clinical characteristics that are widely available in each MS centre in out-patient settings.

4. Conclusions

The overall aim of this PhD project was to explore the real and practical usefulness of biomarkers in clinical practice. All biomarkers considered, which were of various typology and nature, are not currently used in clinical practice, although they have already provided preliminary evidence of accuracy and validity.

For diagnostic purpose, we strongly believe that the analysis of κFLC Index in all patients with suspected MS can be a useful support, providing high diagnostic accuracy with the further advantage of being measured with cost-effective, easy, rapid, operator-independent techniques.

For prognostic purpose, our proposal is to implement the use of OCT in clinical practice, since the measurement of RNFL thickness is predictive for the development of cognitive and physical disability and only requires a rapid and non-invasive examination. Differently, the analysis of CSF NfL, due to the high cost and complexity, to the lack of normative ranges and the failure in exhibiting a real prognostic value, does not seem to be suitable for clinical use at present.

Finally, the timely and early use of highly-effective DMDs and the achievement of an optimal treatment response turned out to be the most

relevant prognostic factors for most of the outcomes explored and should be carefully pursued in the management of RRMS. Nevertheless, with respect to the novel evidence of PIRA and to the concept of one-MS, other studies are required to identify new biomarkers and drugs which can impact the not-inflammation-related mechanisms of neurodegeneration.

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6. Statements

1. “Neuroscience is by far the most exciting branch of science because the brain is the most fascinating object in the universe. Every human brain is different - the brain makes each human unique and defines who he or she is” (Stanley Ben Prusiner, Nobel Prize in Physiology or Medicine 1997).

2. “I wanted to be a neurologist. That seemed to be the most difficult, most intriguing, and the most important aspect of medicine, which had links with psychology, aggression, behavior, and human affairs” (Sir Roger Bannister, neurologist).

3. “I don't make baseless claims like - I'll remove all your fears, I'll remove all your anxieties, I'll remove all your insecurities. I am a scientist, not an influencer - which means, I am dutybound to adhere to the truth, no matter how inconvenient they are, instead of peddling comforting lies for exposure” (Abhijit Naskar, neuroscientist).

4. “Research is to see what everyone else has seen, and to think what nobody else has thought” (Albert Szent-Gyorgyi, scientist).

5. “Rare are those people who use the mind, few use the heart and really unique are those who use both” (Rita Levi-Montalcini, neurologist and neuroscientist).

6. “But you look at science (or at least talk of it) as some sort of demoralising invention of man, something apart from real life, and which must be cautiously guarded and kept separate from everyday existence. But science and everyday life cannot and should not be separated. Science, for me, gives a partial explanation of life” (Rosalind Franklin, scientist).

7. “The good physician treats the disease; the great physician treats the patients who has the disease” (William Osler, physician).

8. “The terms can make patients feel compartmentalized. All of the patients with MS who I have met, regardless of what form of MS they had, seem so different from each other. So, part of me thinks that there is something different going on with each of us, but that it’s similar enough to be called MS. […] I like the idea of MS being described as a spectrum. Everybody is on it, just at different points. We’re all part of the same journey, just at different stages at a given moment” (Jeri Burtchell, patient with MS; from “Two Sides to Every Story: Perspectives from Four Patients and a Healthcare Professional on Multiple Sclerosis Disease Progression”, Neurol Ther 2019).

9. We have become increasingly skilled at formulating the diagnosis of MS, which affects more than 2 million people worldwide. However, we still know little about predicting its prognosis, which varies from patient to patient. Biomarkers could allow us to diagnose, treat and prognosticate,

realizing a more personalized medicine.

10. “Discovery follows discovery, each both raising and answering questions, each ending a long search, and each providing the new instruments for a new search” (J. Robert Oppenheimer, theoretical physicist).