UNIVERSITÀ DEGLI STUDI DI GENOVA

FACOLTÀ DI MEDICINA E CHIRURGIA

PREDICTING DISABILITY ACCRUAL AND COGNITIVE DECLINE IN THE ERA OF HIGH EFFICACY DISEASE MODIFYING TREATMENTS IN MULTIPLE SCLEROSIS

Tesi di Specializzazione

Scuola di Specialità in Neurologia

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1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS). Its manifestations, driven by both immune and neurodegenerative mechanisms, make MS the leading cause of non-traumatic disability in young adults (1). MS natural history can present with different manifestations and disease courses. Most patients experience reversible episodes of transient neurological deficits, known as relapses, lasting days or weeks. These episodes define the early stages of the disease, such as clinically isolated syndrome (CIS) and relapsing–remitting MS (RRMS). Over time, many patients no longer experience relapses but develop permanent neurological deficits and progressive disability, transitioning to secondary progressive MS (SPMS). A smaller subset of patients exhibits a progressive course from the onset, referred to as primary progressive MS (PPMS). (2) The occurrence of disability progression independent from relapse activity (PIRA) since the earliest stages of the disease (3), has challenged the traditional phenotypes classification. To date, based the presence of inflammatory activity, MS subtypes can be classified as either active or inactive (4). Active patients exhibit relapses or inflammatory activity (i.e. new lesions), while inactive patients, free from overt inflammation, may exhibiting ongoing disability accrual.

Recent advancements in high-efficacy disease-modifying treatments (HETs) have revolutionized MS management, by markedly reducing inflammatory activity and effectively delaying disability accrual (5). The persistent progression of motor and cognitive disability despite effective control of acute inflammation highlights that disease progression is driven by different pathophysiological mechanisms on which currently available drugs have limited effect. The persistent disability accrual in patients with suppression of inflammatory activity emphasizes the need to identify new biomarkers to study and target neurodegeneration in MS (6).

The first section of this experimental thesis provides an overview of MS, with a particular focus on the clinical implications of HETs. This section also discusses the

pathophysiological mechanisms underlying the silent progression of the disease and how these mechanisms support motor and cognitive disability accrual.

The second section offers a general overview of the main neuroimaging biomarkers applied in the experimental part, current knowledge about their application, and some technical clarifications regarding their acquisition.

The third section, which constitutes the experimental part, presents two studies conducted during my research activity. These studies specifically focus on identifying the drivers of motor and cognitive disability in patients undergoing treatment with HETs.

2. Multiple Sclerosis

2.1 Epidemiology of MS

About 2.3 million people worldwide are affected by MS, with the highest prevalence among individuals of European descent. RRMS onset typically occurs between 20 and 35 years of age, while PPMS start around 40 years. Prevalence ranges from 2 per 100,000 in Asia to as high as 1 per 1,000 in Western countries, with some high-latitude areas reporting 1 affected per 400 patients (1). The disease is more common at higher latitudes, likely due to genetic factors (e.g., HLA-DRB1 haplotype) and environmental risks such as low vitamin D from reduced sun exposure (7). The prevalence of MS has recently increased, particularly in women, with the female-to-male ratio rising from 2:1 to ~3:1 in recent decades (1,8). This shift may be influenced by environmental factors like smoking, obesity, and hormonal influences (9).

2.2 Etiology and Pathophysiology

MS is an autoimmune disease characterized by the accumulation of demyelinating lesions in the white and grey matter of the brain and spinal cord. Acute inflammatory activity (i.e. new lesions, relapses) and disability accrual in absence of inflammatory activity (i.e. PIRA) are driven by different pathological mechanisms (10).

Blood-brain barrier (BBB) disruption is the first event of MS new lesion development, as testified by MRI-gadolinium leakage on newly formed lesions. After the BBB permeabilization, inflammatory cells derived from the peripheral blood trigger inflammation and focal demyelination (11). Both helper (CD4+) and cytotoxic (CD8+) T cells populate active and chronic MS lesions, thus leading to the initial hypothesis of a prevalently T mediated pathophysiology for MS lesions (12). The success of B-cell depleting therapies in reducing overt inflammatory events, has deepened our understanding of the disease, highlighting the pivotal role of B cells and their antibody production (e.g. oligoclonal bands) (13,14). New MS lesions arise from the complex interactions between T cells, B cells, myeloid cells, and CNS-resident cells and

resolve with the BBB reparation (11). Figure 1 shows the mechanism of acute inflammation in MS.

Along with BBB permeabilization and immune cells coming from the peripheral blood, other pathological mechanisms have been identified in MS patients. CNS resident microglia and astrocytes produce neurotoxic substances like cytokines, chemokines, and reactive oxygen species that sustain chronic demyelination, neuro-axonal damage and neurodegeneration (15,16). Furthermore, while some lesions lose their inflammatory cellular phenotypes and become inactive following the reconstitution of the blood-brain barrier, others are characterized by the persistence of inflammatory cellular phenotypes (particularly activated microglia) even many years after the barrier has been restored. These lesions, known as chronic active lesions, participate in maintaining a chronic inflammatory state within the CNS, also referred to as “smouldering” or compartmentalized CNS inflammation (17).

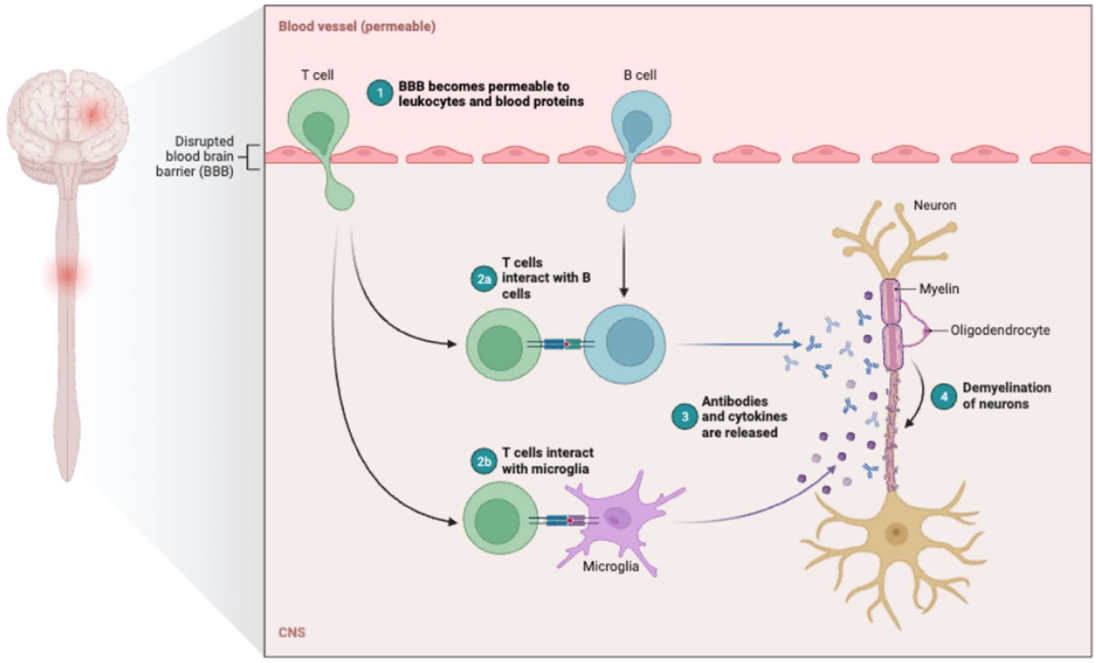


Fig.1 *This figure illustrates the disruption of the blood-brain barrier (BBB) in the central nervous system (CNS), leading to increased permeability to leukocytes and blood proteins (Step 1). T cells infiltrate the CNS, interacting with B cells (Step 2a) and microglia (Step 2b). These interactions result in the release of antibodies and*

inflammatory cytokines (Step 3). This immune activity causes damage to the myelin sheath of neurons by targeting oligodendrocytes, leading to neuronal demyelination (Step 4).

2.3 Clinical presentation and diagnosis

MS typically affects young adults between 20 and 40 years of age, with a higher prevalence in women (8). However, some patients may experience their first demyelinating event during childhood or adolescence (18). The disease presents with a wide range of neurological symptoms, such as muscle weakness, visual disturbances, and cognitive deficits. These symptoms are driven by the interplay of focal inflammation, diffuse axonal damage, and neurodegenerative mechanisms. Parallel to the motor disability, MS patients present progressive reduction in their cognitive abilities. These alterations mainly manifest in the domain of attention and information processing speed with a significant impact on their quality of life (19). When MS follows its natural course, patients quickly progress to a severely disabling condition with significant limitations to their autonomy (e.g., walking with a cane or rapidly becoming wheelchair-bound) (4). Thanks to HETs, as will be discussed later, the symptomatic landscape of the disease is changing, as the reduction in the number of acute inflammatory events significantly delays the time to disability accrual and improves patients' quality of life (5).

Diagnosis mainly relies on demonstrating the dissemination of demyelinating lesions across different CNS regions (dissemination in space, DIS) and over time (dissemination in time, DIT) through clinical evaluation and imaging findings (20). MRI plays a crucial role in identifying demyelinating lesions, monitoring disease activity, and assessing the response to disease-modifying treatments (DMTs). Recent advancements in imaging and understanding of MS pathology have significantly improved diagnosis and treatment options, allowing for better disease management and a delay in progression for many patients (21,22).

2.3 Smouldering Inflammation and the Inside-Out Hypothesis

Smouldering inflammation refers to a chronic, subtle inflammatory process within the CNS that drives MS progression independently of relapses. Unlike overt inflammatory activity, observed as clinical relapses or new lesions on MRI, smouldering inflammation operates persistently and sub-clinically (6). Pathological and imaging studies have shown that smouldering inflammation begins very early in the disease course and persists throughout its progression. Key mechanisms involved include the chronic activation of microglia and macrophages, mitochondrial damage, oxidative stress, and energy deficits (23).

Chronic active lesions are considered an hallmark of smouldering inflammation (17,23). They are a subset (5-10%) of white matter lesions that exhibit a necrotic core surrounded by a rim of activated microglia that remains unchanged over time. Their presence has been associated with worse clinical outcomes and progressive forms of the disease, further supporting their potential role as a biomarker of smouldering inflammation (23–25).

The traditional view of MS pathogenesis has been termed the **"outside-in" hypothesis**, which views MS as a primarily autoimmune condition caused by peripheral immune system dysregulation and subsequent CNS inflammation. This inflammatory activity is supposed to trigger neurodegenerative and smouldering processes causing ongoing chronic inflammation and persistent damage within the CNS. Pathological studies and in-vivo biomarkers have revealed that chronic inflammation and neurodegeneration arise since the beginning of the disease challenging the traditional view of MS pathology and leading to the **"inside-out" hypothesis** (6). It postulates that MS originates within the CNS itself, with primary neurodegenerative processes such as axonal damage or mitochondrial dysfunction triggering the release of CNS antigens. The inside-out hypothesis finds support in the persistence of smouldering inflammation even in the absence of peripheral immune activation suggesting that neurodegeneration is not merely a consequence of

inflammation but may instead be a primary driver of the disease, setting off a cascade of inflammatory responses that exacerbate damage.

2.5 High Efficacy Disease-Modifying Treatment

Advances in HETs have significantly changed the natural history of MS, particularly when used early in the disease course (5). They offer robust suppression of neuroinflammation, reduction in relapse rates, and prevention of long-term disability. These therapies include monoclonal antibodies (e.g., natalizumab, ocrelizumab, alemtuzumab), sphingosine-1-phosphate (S1P) receptor modulators (e.g., fingolimod), and immunomodulators like cladribine. Figure 2 shows each currently available therapy for MS and their mechanism of action. Early intervention with HETs capitalizes on a critical window of therapeutic opportunity, minimizing irreversible CNS damage, brain atrophy and long-term disability (26). This proactive approach also mitigates progression independent of relapses, which often drives long-term disability. By targeting underlying neurodegenerative processes early, HETs preserve CNS integrity and enhance the patient's quality of life.

Traditionally, treatment algorithms followed an escalation approach, starting with low or medium efficacy DMTs and transitioning to HETs after treatment failure. However, this strategy often results in suboptimal disease control, delaying access to the most effective therapies. Emerging evidence advocates for an "induction therapy" model, where HETs are introduced as the first-line therapy for patients with aggressive disease or unfavourable prognostic markers (27,28).

Despite their proven benefits, barriers to early HETs use persist. Concerns about long-term safety, logistical challenges in monitoring, patient hesitancy, and regulatory restrictions limit widespread adoption. However, recent data suggest acceptable safety profiles, with manageable risks and fewer long-term side effects than anticipated (5).

Despite being revolutionary for MS management, HETs primarily target inflammatory mechanisms and are most effective in reducing relapse associated worsening (RAW). Their impact on PIRA, however, appears more limited (29,30). In other words, the introduction of HE-DMTs has changed MS management, shifting the therapeutic paradigm from managing relapses to preventing silent disability progression.

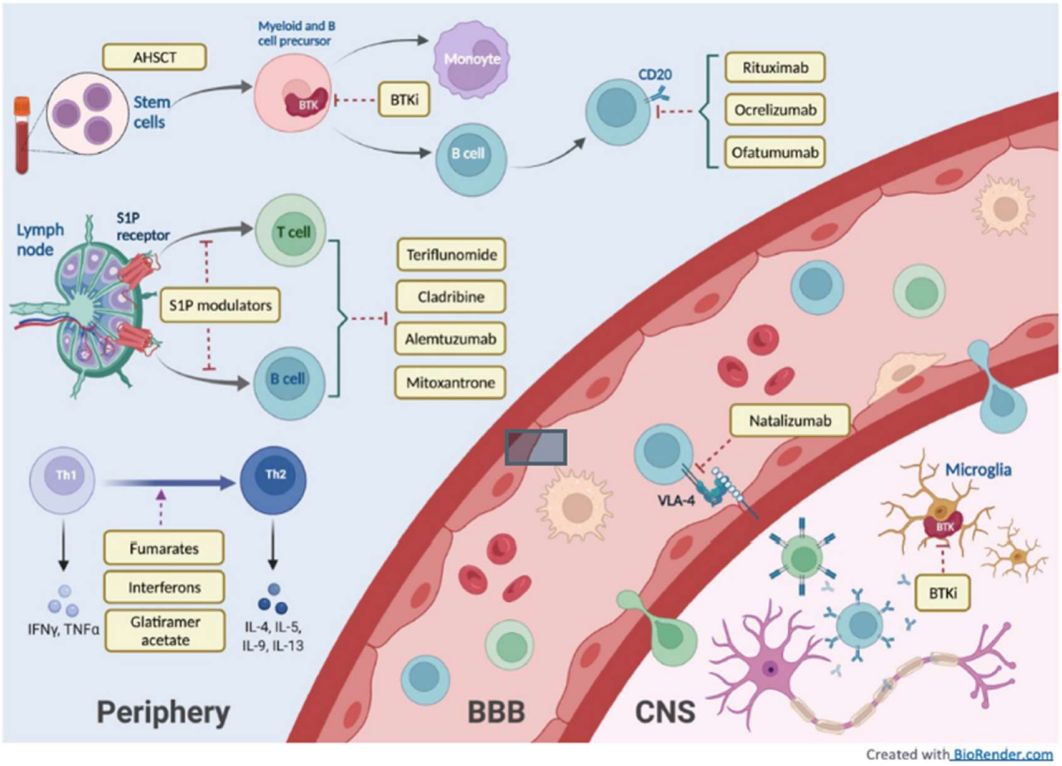


Fig 2. *This figure depicts the mechanisms of immune cell modulation in the treatment of multiple sclerosis. In the periphery, therapies target immune cells at various stages: S1P modulators inhibit T and B cell egress from lymph nodes by targeting the S1P receptor.*

Fumarates, interferons, and glatiramer acetate regulate Th1/Th2 balance and cytokine release.

Stem cell therapies (AHSCT) aim to reset the immune system by replacing myeloid and B cell precursors.

Anti-CD20 monoclonal antibodies, such as rituximab, ocrelizumab, and ofatumumab, deplete B cells.

At the blood-brain barrier (BBB), natalizumab prevents immune cell infiltration into the central nervous system (CNS) by blocking VLA-4. Within the CNS, immune cell-

mediated inflammation damages myelin and contributes to neurodegeneration. These therapeutic interventions aim to modulate immune activity and protect neuronal integrity.

2.6 How Patients with MS Acquire Disability

Disability in MS develops through distinct but overlapping pathways. RAW occurs when relapses cause incomplete recovery, leaving residual neurological deficits. This process is most prominent during the early stages of RRMS and is characterized by acute inflammation and damage. However, PIRA represents a more insidious driver of disability that operates independently of clinical relapses. PIRA begins early in the disease course (30,31), even in patients with paediatric and RRMS, and becomes the dominant mechanism in progressive forms of the disease (29,32).

PIRA reflects underlying disease processes including chronic inflammation, microglial activation, and neurodegeneration, leading to gradual and irreversible CNS damage. Large cohort studies point out that PIRA is the main driver of disability progression in MS underscoring the need for specific DMTs targeting smouldering and neurodegenerative processes.

Recent MRI studies have shown that PIRA is associated with higher brain volume loss, cortical atrophy, presence of chronic active lesions and microstructural changes in the CNS (33). These findings highlight the possibility to predict the occurrence of PIRA favouring future interventions for its treatment.

Figure 3 summarizes the two main mechanisms of disability accrual in MS.

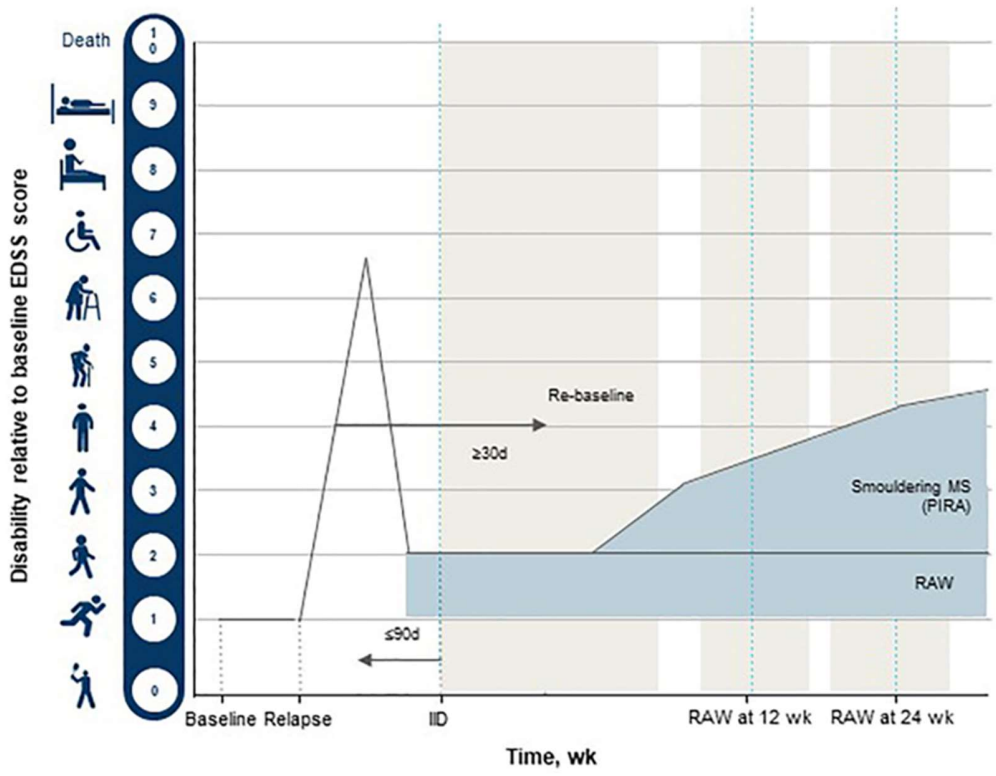


Fig 3. *This figure illustrates the dynamics of disability progression in multiple sclerosis (MS) over time, measured using the Expanded Disability Status Scale (EDSS). The graph differentiates between two mechanisms of disability worsening:.*

- ***RAW****: Represents an acute increase in disability following a relapse. It is typically evaluated within 90 days (≤90d) after a relapse onset and can partially improve over time, leading to a re-baseline of disability (≥30d).*
- ***PIRA****: Refers to the gradual and steady increase in disability unrelated to relapses, also called "smouldering MS." It occurs independently of inflammatory relapses and reflects chronic, underlying neurodegeneration. This process is assessed over longer periods, such as 12 and 24 weeks.*

2.7 Cognitive Disability Progression in Multiple Sclerosis

In addition to physical disability progression, cognitive decline in MS has emerged as a significant concern, particularly through mechanisms independent of relapse activity. Cognitive deficits are often under-recognized, as they may occur silently and independently of physical symptoms (19). Cognitive progression independent of

relapse activity (cognitive PIRA) mirrors the concept of motor PIRA but focuses on insidious cognitive deterioration without concurrent clinical relapses or physical disability worsening (34). The mechanisms underlying cognitive PIRA are thought to be overlapping with motor PIRA, including chronic neuroinflammation, microstructural changes, and cortical atrophy. As for motor disability accrual, cognitive decline independent from relapses accounts for the vast majority of cognitive decline in MS and represent and intriguing target for cognitive rehabilitation therapies.

3. MRI Biomarkers

MRI biomarkers offer invaluable insights into the mechanisms driving disability progression (35,36). Quantitative MRI provide biomarkers for different pathological processes in MS (myelin content, microstructural brain tissue damage, integrity of grey matter) allowing for the in-vivo assessment of inflammatory and neurodegenerative processes. The availability of powerful MRI scanners and the numerous scientific evidences are currently reducing the gap between their application in research and clinical settings (37). In this manuscript we evaluated two MRI biomarkers: brain atrophy (38) and paramagnetic rim lesions (PRLs) (17).

3.1 Brain Atrophy

Brain atrophy, defined as the loss of brain tissue volume occurs throughout the disease course, since the beginning of the disease. T1-weighted contrast enables the distinction between grey matter (hypointense), white matter (hyperintense) and cerebrospinal fluid (hypointense). Through segmentation of different brain tissues (i.e. grey matter, white matter), T1-weighted images enable to quantify brain loss across different timepoints thus allowing for quantification of brain loss in time. Brain atrophy reflects the ongoing damage to both grey and white matter in MS exhibiting typical patterns including widespread cortical and subcortical thinning (particularly in the periventricular regions) and enlargement of the lateral ventricles (Fig 4). Unlike acute inflammatory lesions, brain atrophy provides a cumulative measure of tissue damage, reflecting the neurodegenerative processes, and is closely associated with long-term disability progression (38). Scientific evidence consistently demonstrate that brain volume loss in MS exceeds that of normal aging, with annual rates ranging from 0.5% to 1.35% in MS patients compared to 0.1% to 0.3% in healthy individuals and a recently proposed cut-off of 0.4% annual rate proposed as a cut off between physiological and pathological brain loss in the disease (39). Brain atrophy in MS is driven by different mechanism including focal lesions, subtle widespread pathology in the normal-appearing white matter, gray matter loss (particularly in thalamus and

subcortical areas). Despite its established relevance, the integration of brain atrophy measurements into routine clinical practice remains limited. Factors such as variability in MRI acquisition protocols, lack of standardized thresholds, and the need for sophisticated post-processing tools hinder widespread implementation (40). Furthermore, translating group-based atrophy data to actionable insights at the individual patient level poses significant challenges.

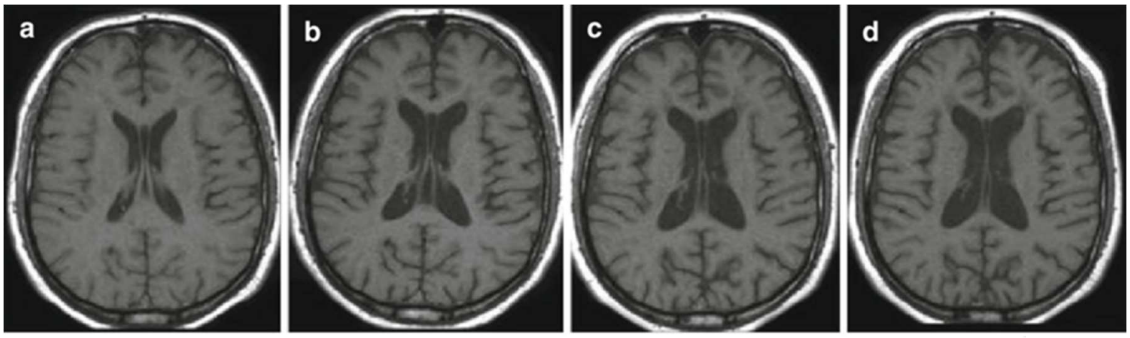


Fig 4. *This series of MRI images illustrates the progressive brain atrophy observed in multiple sclerosis (MS), characterized by the enlargement of the ventricles over time. Panel a show the baseline image with normal ventricular size while panels b, c, and d reveal the gradual widening of the lateral ventricles, a hallmark of brain atrophy in MS.*

3.2 NEDA-3 and the Emergence of NEDA-4

In recent years, the concept of "No Evidence of Disease Activity" (NEDA) has been widely adopted to assess the efficacy of disease-modifying therapies (DMTs) in MS. NEDA-3, the traditional standard, evaluates three domains: absence of relapses, no new or enlarging T2 lesions or gadolinium-enhancing (Gd+) lesions on MRI, and no confirmed disability progression (41). While NEDA-3 has been a pivotal tool for monitoring inflammatory activity, it fails to fully capture the neurodegenerative processes that drive long-term disability (42). NEDA-3 primarily focuses on inflammatory markers and short-term disease control. However, growing evidence suggests that neurodegeneration—manifested as brain volume loss (BVL)—occurs early in the disease course and progresses independently of inflammation. This

limitation means that patients meeting NEDA-3 criteria may still accumulate irreversible tissue damage and disability over time. For example, studies show that patients with seemingly stable MRI findings on NEDA-3 may exhibit significant BVL, a hallmark of underlying neurodegeneration (39).

To address the gaps in NEDA-3, the concept of NEDA-4 has emerged. This expanded framework incorporates brain volume loss as a fourth parameter, reflecting the neurodegenerative aspect of MS. By adding BVL thresholds (less than 0.4% annualized brain volume loss), NEDA-4 provides a more holistic view of disease activity and therapy response (39). By encompassing both inflammatory and neurodegenerative dimensions of MS, NEDA-4 represents a significant advancement over NEDA-3. It underscores the importance of targeting all facets of the disease to optimize patient outcomes and provides a promising framework for future therapeutic evaluation.

3.3 Paramagnetic Rim Lesions: A Biomarker of Chronic Active Lesions

Paramagnetic rim lesions (PRLs) have emerged as a powerful MRI biomarker for chronic active or "smoldering" lesions in MS (17,43). These lesions, identified by a hyperintense rim on advanced imaging techniques such as quantitative susceptibility mapping (QSM), are linked to persistent inflammation, iron deposition, and neurodegeneration. Their unique pathophysiology and clinical implications make PRLs a critical focus in understanding disease progression and tailoring therapeutic strategies.

QSM is an advanced MRI technique that measures the magnetic susceptibility of tissues, which reflects their composition, including iron and myelin content. Unlike conventional imaging methods, QSM provides quantitative and spatially resolved data on tissue properties, making it particularly effective for detecting PRLs (44).

PRLs are defined by a QSM-hyperintense rim, indicative of chronic active inflammation. This rim reflects iron-laden macrophages and microglia, which create

a distinct susceptibility signal. Compared to other lesion types, PRLs exhibit higher magnetic susceptibility due to iron deposition, persistent inflammatory activity at the lesion border, visible even in the absence of gadolinium enhancement, limited remyelination and extensive axonal damage, as shown by reductions in myelin water fraction and neurite density index (43,45).

QSM-based detection of PRLs allows to use them as an in vivo biomarker for ongoing chronic inflammation and prognosis evaluation in MS patients. Their presence correlates strongly with higher EDSS scores, faster progression to secondary progressive MS (SPMS), and greater brain volume loss. Importantly, PRLs provide a window into smoldering inflammatory activity that may not be captured by traditional MRI markers such as gadolinium-enhancing lesions or T2 hyperintensities (17,25).

4. Experimental applications

Based on the current scientific literature, we can conclude that a new era in the treatment of MS has started. HETs developed in recent years have transformed the disease paradigm by nearly-eliminating the inflammatory component, relapses, and new lesion occurrence. These advancements have unveiled new pathogenic mechanisms of the disease that continue to subtly impair patients' quality of life, leading more insidiously to motor and cognitive disability.
My research has focused on identifying prognostic factors associated with silent worsening, with the aim of recognizing patients at higher risk of disease progression who may, in the future, be eligible for treatment with drugs targeting these newly discovered disease mechanisms.

4a. Real-World Application of NEDA-4 as a Predictor of Long-Term Disability Progression in Multiple Sclerosis Patients on High-Efficacy Treatments

ABSTRACT

Introduction

No Evidence of Disease Activity-4 (NEDA-4) is a proposed composite outcome for treatment efficacy in Multiple Sclerosis (MS) that addresses brain volume loss (BVL) as a biomarker of neurodegeneration. With high efficacy treatments (HETs) significantly reducing inflammatory activity, NEDA-4 could serve as a valuable biomarker for their efficacy in preventing disability accrual.

Methods

A real-world cohort of 80 MS patients initiating HETs underwent clinical assessments, re-baseline and one-year follow-up brain 3T-MRI for the evaluation of NEDA-4. The occurrence of confirmed disability progression (CDP) was assessed during a median follow-up of 5 years. Cox regression analyses were performed to assess the impact of 12-months NEDA-4 loss on long-term CDP.

Results

23 (28.8%) patients achieved NEDA-4 during the first year and 21 patients (26.3%) experienced CDP after 5.3(4.0-6.2) years follow-up. Multivariable analyses showed NEDA-4 loss [HR (95%CI): 6.69 (1.50-29.77); p:0.01] and higher baseline EDSS [HR (95%CI): 1.58 (1.21-2.08); p<0.01] as independent predictors of long-term CDP.

Discussion

NEDA-4 loss is associated with long-term disability progression in MS patients undergoing HETs. HETs seem efficient in reducing BVL and preventing disability accrual in a subset of MS patients but show limited efficacy in highly disabled individuals.

INTRODUCTION

In Multiple Sclerosis (MS), high efficacy treatments (HETs) allow near-complete resolution of inflammatory disease activity, leading to slower disability progression (1). Nevertheless, disability accumulation is also driven by neurodegenerative and smouldering processes (2) with older age and high EDSS as key predictors of disability accrual (3,4). No Evidence of Disease Activity – 3 (NEDA-3) is a composite measure to evaluate treatment response in MS (3) . While it provides a valuable framework for short-term monitoring, NEDA-3 status primarily reflects absence of inflammatory disease activity (5) and does not fully predict long-term disability progression (3). To address the neurodegenerative components of the disease, brain volume loss (BVL) – an established predictor of long-term disability (6) - was included in an expanded composite outcome, NEDA-4 that showed association with lower risk of disability accrual (7). The feasibility of NEDA-4 in a clinical setting remains highly debated. Several factors, including the pseudoatrophy effect, limit its applicability (8) and a recent meta-analysis showed no advantage over NEDA-3 in predicting disability accumulation in placebo, platform and fingolimod treated patients (9,10) . With markedly reduced inflammatory events, the efficacy of HETs in slowing neurodegenerative processes warrants evaluation, potentially enhancing the relevance of NEDA-4 in this context.

In this study we evaluated NEDA-4 status in the first 12 months of treatment as a predictor of confirmed disability progression (CDP) after 5 years in a real-world cohort of MS patients starting an HET.

METHODS

Study population and outcomes definition

Consecutive MS patients starting a HET were prospectively enrolled at the MS centre of the University of Genova. MS diagnosis was formulated according to (11). Clinical evaluation and MRI re-baseline assessment were performed between 3 and 6 months from HET start (12). Follow-up MRI assessments were carried out 12 months after re-baseline, while clinical evaluations were performed every six months. Clinical relapses were defined according to (11). MRI activity was defined as new/enlarging T2 lesions on MRI scans. CDP was defined as: increase of ≥ 1.5 EDSS steps from baseline score of 0, 1 step from baseline scores 1-5.0 or 0.5 step from baseline score ≥5.5 sustained at two or more consecutive visits separated by ≥6 months. NEDA-3 was defined as no clinical relapses, no CDP and no MRI activity (3). NEDA-4 was defined as NEDA-3 plus AR(Annualized-rate)-BVL < 0.4% (7).

MRI protocol and processing

Each re-baseline and 12-months follow up MRI were performed on a 3T scanner (Prisma, Siemens Helthineers). The MRI protocol included:

(i) 3D sagittal T2-FLAIR (TR/TI/TE 5000ms/1800ms/393ms; original resolution: 0.8x0.8x1 mm^3; resolution of reconstructed images: 0.4x0.4x1 mm^3);

(ii) 3D sagittal T1-MPRAGE (TR/TI/TE 2300ms/919ms/2.96ms; resolution 1x1x1 mm^3);

FLAIR-hyperintense lesions were segmented on FLAIR images using a semiautomated segmentation technique (SinLab; Siena Imaging; https://sinlab-rhb.sienaimaging.com/) and lesion volume was computed. T1 lesion filling was performed using FSL v6.0.5. Based on T1-filled images FreeSurfer v6.0 was used to extract total brain volume (TBV) and total intracranial volume (TIV) at baseline, while SIENA (Structural Image Evaluation using Normalization, of Atrophy) (13) was used to assess percentage brain volume change (PBVC). Two investigators (VDB, GB) visually inspected each segmentation. AR-BVL was computed using the subsequent formula: ((PBVC/100+1)^(365.25/days)-1)*100 (7) .

Statistical analysis

Total brain volume (TBV) was corrected saving unstandardized residuals from linear regressions run with age, sex and TIV as the independent variables. Fisher's exact test was used for the comparison of categorical variables. A survival analysis employing both the log-rank test and multivariable Cox regression was performed to evaluate the impact of NEDA-4 on CDP prediction.

RESULTS

Demographics, clinical and MRI characteristics

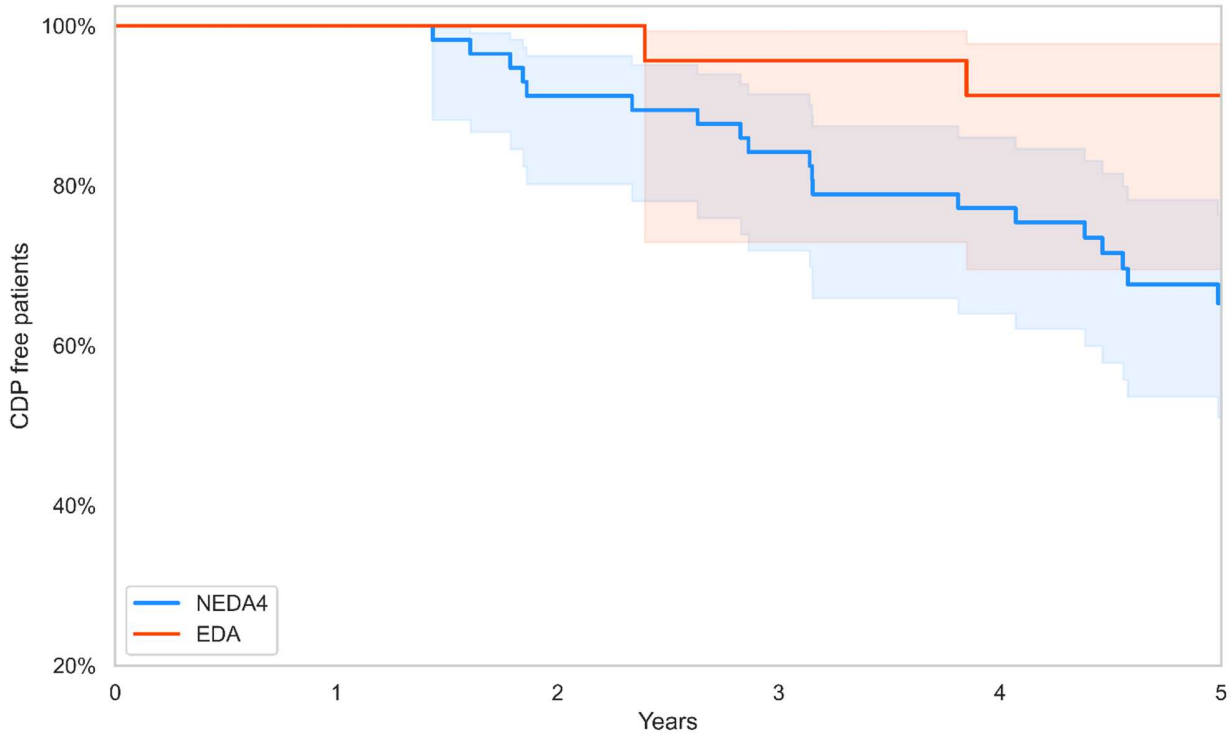
80 MS patients [47 (58.8%) females, mean (SD) age 38.3 (11.5) years, mean (SD) disease duration 9.5 (9.6) years, median (range) EDSS 2 (1-7)] were enrolled (Table 1). Median (range) time from HET start to re-baseline MRI was 3.5 (3.0-6.3) months. During the first year [follow-up MRI: mean (SD) 12.8 (2.1) months], 5 patients (6.3%) lost NEDA-3 [0 relapse, 3 MRI activity (0/3 exhibited 5-years CDP) and 2 CDP (1/2 exhibited 5-years CDP)], 55 (68.8%) patients experienced pathological AR-BVL and 23 (28.8%) patients achieved NEDA-4 status. During follow-up, 15/80 (18.8%) discontinued therapy: 7 showed inflammatory activity, 5 showed adverse drug reactions, 2 exhibited JCV-positivity, and 1 patient underwent de-escalation.

Predictors of confirmed disability progression

After median (range) 5.3 (4.1-6.3) years follow-up, 21 patients (26.3%) experienced CDP. Among those 19/21 (90.5%) lost NEDA-4 status compared to 38/59 (64.4%) among CDP-free patients (χ^2:5.25; p:0.03). The log-rank test showed association between CDP and NEDA-4 ($\chi^2 = 4.72$; $p = 0.03$) (Fig1) but no association with NEDA-3 ($\chi^2 = 0.05$; $p = 0.81$). Univariate Cox regression analysis revealed association between older age [HR (95%CI): 1.05 (1.01-1.09); p:0.02], higher EDSS [HR (95%CI): 1.50 (1.20-1.87); p<0.01] and NEDA-4 loss [HR (95%CI): 4.38 (1.02-18.82); p:0.05]

(supplementary table 1). Multivariable Cox regression analysis showed higher EDSS [HR (95%CI): 1.58 (1.21-2.08); p<0.01] and NEDA-4 loss [HR (95%CI): 6.69 (1.50-29.77); p:0.01] as independent predictors of CDP (table 2).

Fig 1 Confirmed disability progression over 5 years according to NEDA-4 status in the first 12 months after treatment start



The figure shows the Kaplan-Meier survival curves for patients with and without NEDA-4 status after the first year MRI assessment. The x-axis indicates time while the y-axis indicates the estimated survival probability. Cox regression analysis showed NEDA-4 loss [HR (95%CI): 3.53(1.16–10.77); p:0.03] to be associated with higher risk of CDP.

CDP: Confirmed disability progression

NEDA-4: No Evidence of Disease Activity (no clinical relapses, no asymptomatic MRI activity, no confirmed disease progression, AR-BVL < 0.4%)

EDA: Evidence of Disease Activity

Table 1 Clinical and MRI characteristics of the sample

N	80
Female, number (%)	47 (58.8)
Phenotype, number (%) **RR (%)** **SP (%)** **PP (%)**	 68 (85.0) 6 (7.5) (7.5)
Treatment naïve, number (%)	25 (31.3)
DMT, number (%) **antiCD20** **S1P-inhibitors** **Cladribine** **Natalizumab** **Alemtuzumab** **Bone marrow transplantation**	 48 (60.0) 11 (13.8) 7 (8.8) 5 (6.3) 4 (5.0) 5 (6.3)
Age, years, mean (SD)	38.3 (11.5)
Disease Duration, years, mean (SD)	9.5 (9.6)
Baseline EDSS, median (range)	2 (1-7)
Lesion Volume, mL, mean (SD)	8.9 (10.2)
Total brain volume, mL, mean (SD)	1112.3 (42.7)
No Evidence of Disease Activity-3 1 year loss, number (%) **Relapses** **MRI activity** **PIRA**	5 (6.3) 0 (0) 3 (3.8) 2 (2.5)
Percentage Brain Volume Change, mean (SD)	-0.89 (1.1)
Annualized Rate – Brain Volume Loss < -0.4%, number (%)	55 (68.8)
No Evidence of Disease Activity-4 1 year loss, number (%)	57 (71.3)
5 years EDSS, median (range)	2 (1-7.5)
5 years Confirmed Disease Progression, number (%)	21 (26.3)

RR: relapsing-remitting; SP: Secondary Progressive; PP: Primary Progressive; DMT: Disease Modifying Treatment; S1P: Sphingosine 1 phosphate; EDSS: Expanded Disability Status Scale.

Table 2 Multivariable Cox Regression Analysis for 5 years Confirmed Disease Progression

Variable	HR (95% CI)	p-value
Age	1.02 (0.98-1.07)	0.23
Baseline EDSS	1.60 (1.22-2.09)	<0.01
NEDA-4 loss	6.91 (1.55-30.89)	0.01

EDSS: Expanded Disability Status Scale; NEDA-4: No Evidence of Disease Activity-4

Multivariable Cox Regression Analysis showed higher EDSS [HR (95%CI): 1.58 (1.21-2.08); p<0.01] and NEDA-4 loss [HR (95%CI): 6.69 (1.50-29.77); p:0.01] as independent predictors of 5-years confirmed disability progression in a cohort of 80 MS patients undergoing HETs.

Supplementary table 1

Univariate analyses for the prediction of Confirmed Disability Progression (CDP)

Variable	HR (95% CI)	p-value
Age	1.05 (1.01-1.09)	0.02
Disease Duration	1.01 (0.97-1.06)	0.57
Baseline EDSS	1.50 (1.20-1.87)	<0.01
Lesion Volume	1.02 (0.99-1.06)	0.22
Baseline Total Brain Volume	0.99 (0.99-1.00)	0.07
NEDA-3 loss	0.79 (0.11-5.87)	0.82
NEDA-4 loss	4.38 (1.02-18.82)	0.05
DMT suspension	0.69 (0.20-2.34)	0.55

In a cohort of 80 Multiple Sclerosis patients undergoing high efficacy treatments, univariate Cox regression analyses show that higher age [HR (95%CI): 1.05 (1.01-1.09); p:0.02], higher baseline EDSS [HR (95%CI): 1.50 (1.20-1.87); p<0.01] and NEDA-4 loss [HR (95%CI): 4.38 (1.02-18.82); p:0.05] predicted 5 years confirmed disability progression. NEDA-3 loss [HR (95%CI): 0.79 (0.11-5.87); p:0.82] showed no effect on predicting confirmed disability progression.

DISCUSSION

In a real-world cohort of MS patients treated with HETs, 85/90 (93.7%) and 23/90 (28.8%) achieved NEDA-3 and NEDA-4 statuses during the first year while 21 (26.3%) showed long-term CDP. In this population, NEDA-4 independently predicted CDP and no patient exhibiting early MRI activity showed long-term disability accrual. These observations align with the evolving understanding of disability progression in MS: currently available HETs effectively suppress inflammatory events and limit their role in disability progression (1,2). Consequently, composite measures that heavily rely on inflammatory activity show limited sensitivity in tracking disability accrual (3), highlighting the need for novel outcomes with a particular focus on neurodegeneration. Neurodegeneration in MS is thought to be initially triggered by inflammation and later sustained by chronic compartmentalized inflammation or axonal degeneration (14). By suppressing inflammatory activity, HET might halt or delay neurodegeneration leading to slower brain volume and grey matter loss (1). Although limited by methodological factors (i.e. hydration status, segmentation error, age and sex-dependence) (15) BVL serves as a valid biomarker for neurodegeneration and guidelines for its use in clinical practice as well as pathological cut-offs have been provided (10,12). In a setting of near-complete suppression of inflammatory events, evaluating HETs efficacy through a composite measure that accounts for neurodegenerative processes, as NEDA-4, seems more appropriate.

NEDA-4 loss was an independent predictor of 5-years CDP [HR (95%CI): 6.69 (1.50-29.77); p:0.01] suggesting that patients exhibiting limited response to HETs exhibit higher risk of disability accrual. Conversely, when HETs effectively halt BVL, CDP events are prevented in a subset of MS patients. This finding supports the use of NEDA-4 as an early biomarker of therapeutic efficacy for HETs and as a valuable tool to assess their efficacy in preventing neurodegenerative processes leading to disability.

As confirmed in other reports (3,4), our analysis shows that higher EDSS [HR (95%CI): 1.58 (1.21-2.08); p<0.01] is associated to higher risk of CDP, suggesting that HETs fail in halting disability accrual in high-disabled patients. HETs effectively prevent inflammatory events that trigger neurodegenerative processes in the earliest stages of the disease but fail to halt these processes once they are already established (1,14). Higher baseline disability likely represents established and ongoing neurodegenerative phenomena that are poorly targeted by current HETs.

In conclusion, NEDA-4 emerges as the sole “treatment-related” predictor of long-term CDP in patients undergoing HETs. The significant impact of baseline disability highlights the critical importance of early high-efficacy treatment to achieve better clinical outcomes in MS.

4b. Cognitive changes in relapse free MS patients treated with high efficacy therapies: the predictive value of paramagnetic rim lesions

INTRODUCTION

In multiple sclerosis (MS), high-efficacy disease-modifying therapies (HETs) have radically changed disease management by achieving near-complete control over acute inflammatory episodes and by altering the disease trajectory for most patients (5). Nevertheless, progressive motor and cognitive deterioration without overt inflammatory activity remains a major concern (32,34). Along with motor progression independent from relapse activity (PIRA), MS patients without acute inflammatory events may also experience progressive cognitive decline, referred to as "cognitive PIRA"(34). The subtle disability accrual in MS (“smouldering MS”) is thought to be driven by chronic CNS compartmentalized inflammation leading to neurodegenerative processes over years (6). Paramagnetic rim lesions (PRLs), assessed using quantitative susceptibility mapping (QSM) MRI, have emerged as a potential biomarker for chronic compartmentalized inflammation (24,43,53). Both PRLs and structural biomarkers of grey matter (GM) damage have been associated with cognitive impairment (54–56) with earlier research reporting higher cognitive impairment in patients exhibiting more than 3 PRLs (25). Nonetheless, the role of PRLs and other MS lesion phenotypes, such as QSM isointense lesions, characterized by lower microstructural damage and higher myelin content (43,57) in predicting cognitive decline over time remains to be elucidated.

Based on their effectiveness on motor disability and radiological disease activity, natalizumab, sphingosine-1-phosphate inhibitors, alemtuzumab, cladribine, anti-CD20 therapies, and immunosuppressive therapies such as cyclophosphamide, used in hematopoietic stem cell transplantation (aHSCT), are considered HETs (5,58). Earlier initiation of HETs has been linked to reduced risk of cognitive decline and the potential for cognitive improvement (59,60).

The Symbol Digit Modalities Test (SDMT) is a reliable tool for the assessment of cognitive function in MS, particularly evaluating information processing speed (19). A consensus first established a 4-point cut off for SDMT as reliable measure of decline for MS patients (19) but a subsequent study proposed a more stringent 8 points cut off (61). Determining significant changes in neuropsychological test performance for MS patients is challenging, since changes may merely reflect not statistically-relevant fluctuations and practice effects from repeated testing. Portaccio and colleagues recently proposed regression-based reliable change index (RB-RCI), a novel individualized measure of change in a patient's neuropsychological test performance that takes into account sex, age, education and baseline score (62). This measure holds promise for overcoming the statistical challenges associated with longitudinal changes in test scores, thereby providing a more accurate assessment of cognitive changes over time. Additionally, test performance in MS may also be influenced by disease-related events such as clinical relapses or radiological reactivations (63).

In this study we aimed to:

- Assess SDMT changes in relapsing-remitting (RR) MS patients treated with HETs with no evidence of acute inflammatory episodes, by applying the RB-RCI methodology for both decline and improvement.
- Evaluate the potential role of PRLs and QSM-isointense lesions in predicting cognitive changes over time by developing both univariable and multivariable binomial regression models.

METHODS

Study design

One hundred consecutive (i.e. included in chronological order without additional selective criteria) RRMS patients fulfilling the 2017 McDonald's criteria (20) aged 18-65 years undergoing HETs (5) were prospectively enrolled from June 2020 to June 2021

at the MS centre of the University of Genoa, Italy. Exclusion criteria at baseline were MRI intolerance, evidence of clinical relapses or MRI activity (defined as new T2 and/or gadolinium enhancing lesions) in the previous 3 months, the presence of gadolinium enhancing lesions at baseline MRI (since relapses could affect cognitive performance at baseline (63)) and progression independent from relapse activity (PIRA) in the previous 2 years (in order to exclude patients transitioning into the progressive phase of the disease) (31). All patients underwent MRI assessment at baseline while clinical and neuropsychological evaluation were performed at baseline and after 24 months follow up. To ensure the inclusion of only patients without acute inflammatory disease activity and maintained on stable HETs treatment throughout the entire follow up, those exhibiting clinical relapses, asymptomatic MRI activity or any disease-modifying treatment (DMT) change during the observation period were excluded.

Written informed consent was obtained from all patients included in the study, in accordance with the approval from the local ethical standards committee (CER Liguria: 07/04/2020 - ID10346).

Neuropsychological assessment and definition of statistically significant cognitive changes

The validated alternate SDMT oral forms (form 1 and 2) were administered at baseline and after 24 months in a random order to minimize possible learning effects (64). The number of correct responses in 90 seconds was considered as the raw SDMT score (SDMT r). Based on Italian normative data (65) raw scores were converted to t-scores and z-scores, which are normalized values adjusted for age, sex and education for each individual. Patients with an SDMT z-score below -1.5 at baseline were classified as SDMT impaired (19). Using the data from the multivariable model provided by Portaccio et al. (62), predicted follow-up scaled scores were evaluated for each patient and compared to the actual follow-up raw and scaled scores to obtain a regression-based reliable change index (RB-RCI). Z-values with an absolute value

greater than 1.65 were considered indicative of an improvement or deterioration in SDMT performance, thus considering a conservative 90% confidence interval. To account for possible comorbidities that could influence neurocognitive performance, each patient completed an assessment using the Hospital Anxiety and Depression scale (HADS) scale at baseline, a brief self-reporting two-dimensional questionnaire for the screening of mood disorder in MS (66).

MRI protocol and processing

Each patient underwent MRI examination at baseline on a 3T-MRI scanner (Prisma, Siemens Helthineers). The MRI protocol included:

(i) 3D sagittal T2-FLAIR (TR/TI/TE 5000ms/1800ms/393ms; original resolution: 0.8x0.8x1 mm^3; resolution of reconstructed images: 0.4x0.4x1 mm^3);

(ii) 3D sagittal T1-MPRAGE (TR/TI/TE 2300ms/919ms/2.96ms; resolution 1x1x1 mm^3);

(iii) 3D sagittal-segmented echo-planar-imaging (EPI) (TR/TE 64ms/35ms; Flip Angle=10°; resolution 0.65x0.65x0.65 mm^3) providing magnitude and phase images.

(iv) 3D turbo spin-echo T1-weighted after injection of a single dose of Gadoteridol (0.2 mL/kg) (TR/TE 700ms/12ms; resolution: 1x1x1 mm^3);

FLAIR-hyperintense lesions were segmented on FLAIR images using a semiautomated segmentation technique based on user-supervised local thresholding (SinLab; Siena Imaging; https://sinlab-rhb.sienaimaging.com/) and visually checked by two experienced raters (V.D.B. and G.B.); lesions <0.03 mL were excluded from the analysis. FLAIR images were then rigidly registered to the T1 images using ANTs (https://stnava.github.io/ANTs/) and the derived transformations were used to register the FLAIR-hyperintense lesions to the T1 space with nearest-neighbour interpolation. Lesion filling was performed on T1 images using FSL (the

FMRIB Software Library, v6.0.5 Oxford, UK). Based on the T1-filled images, FreeSurfer image analysis suite, version 6.0 (http:// surfer.nmr.mgh.harvard.edu/) was used to extract total intracranial volumes, whole brain volumes and cortical volumes. Thalamic volumes were extracted using FIRST, part of FSL, as it provides more robust segmentation in the presence of MS lesions (67). Each volume segmentation obtained from FreeSurfer and FIRST was reviewed and manually corrected where necessary. Quantitative susceptibility mapping (QSM) and susceptibility map weighted imaging (SMWI) images (68) were generated from 3D EPI using a custom set of codes in MATLAB (MathWorks) with STI Suite routines (https://people.eecs.berkeley.edu/~chunlei.liu/software.html) for image phase-unwrapping, background phase removal, and dipole deconvolution as in (45). FLAIR images and FLAIR lesions were then registered to the EPI magnitude images using ANTs and, respectively, linear registration and nearest neighbour interpolation. Two independently trained neurologists with over five years of experience in MS neuroimaging (V.D.B. and G.B.) visually classified each lesion following the recently published guidelines (24) reaching consensus in cases of uncertain classification. Lesions exhibiting an hyperintense rim compared to the lesion centre were classified as PRLs and lesions with equal QSM intensity compared to the surrounding normal appearing tissue were classified as isointense lesions (ISO). Fig1 shows examples of lesions classified as PRLs and ISO.

Statistical analysis

Frequency tables were used to describe categorical and ordinal variables, mean and standard deviation were used for continuous variables unless otherwise specified. Differences in demographic, MRI and neuropsychological variables among study subgroups were evaluated using the Fisher's exact test, Student's t-test, ANOVA, Mann-Whitney test and Kruskal-Wallis as appropriate. PRLs occurrence was evaluated both as a categorical variable [presence of at least one PRL (PRLs+), absence of PRLs (PRLs-)] and as an ordinal variable [absence of PRLs (PRLs-),

presence of 1-3 PRLs (PRLs 1-3), presence of 4 or more PRLs (>3PRLs)] at baseline, as the cutoff of 4 PRLs seems to effectively distinguish patients with a greater clinical impact of PRLs presence (25,53). Whole brain volumes, cortical volumes and thalamic volumes were corrected for total intracranial volume (TIV), saving unstandardized residuals from linear regressions run with the TIV as the independent variable and whole brain volume, cortical volume and thalamic volume as the dependent variables. Spearman correlation was used to investigate the association between baseline demographic, MRI and neuropsychological variables and a significance threshold of $p < 0.01$ was applied for the interpretation of results, considering the high number of coefficients in the correlation matrix.

Univariate binomial regressions were performed to evaluate predictors of statistically significant SDMT decline and improvement. Variables accounting for demographic characteristics (age, sex), clinical features [(total HADS score and expanded disability status scale (EDSS)], volumetric MRI measures [total lesion volume (TLV), corrected whole brain volume, corrected cortical volume, corrected thalamic volume], and lesion heterogeneity metrics (categorical PRLs, number of PRLs, percentage of PRLs lesion volume, percentage of ISO lesion number, and percentage of ISO lesion volume) were evaluated as independent predictors. Predictors showing at least a trend toward significance (p-values <0.10) in the univariate analyses were included in multivariable binomial regression models for SDMT decline and improvement. For each regression model, McFadden's R-squared (R^2McF) is reported, along with the odds ratio (OR) and 95% confidence interval (CI) for each predictor. Throughout the study, p-values below 0.05 were considered statistically significant, while those between 0.05-0.10 were interpreted as indicative of a trend; statistical analyses were performed using Jamovi v2.3.28.

RESULTS

Baseline Demographics, MRI and Neuropsychological Assessments

During the follow up period, 4 patients had a clinical relapse and were switched to another DMT and 6 patients experienced asymptomatic MRI activity. The final sample consisted of ninety patients [females 54 (60.0%), mean (SD) age 40.30 (10.77), disease duration 8.83 (9.06), median (range) EDSS 2 (0-6)]. 47 (52.2%) patients were receiving anti-CD20 therapies, 17 (18.9%) sphingosine-1 receptor inhibitors, 11 (12.2%) cladribine, 10 (11.1%) natalizumab, 3 (3.3%) patients underwent aHSCT, and 2 (2.2%) patients underwent alemtuzumab therapy. Baseline demographical, MRI and neuropsychological characteristics are displayed for the whole sample in table 1. 44 (48.9%) patients did not have PRLs (PRLs-), 35 (38.9%) patients had 1-3 PRLs (1-3PRLs), and 11 (12.2%) patients had 4 PRLs or more (>3PRLs). Mean and percentage number and volumes for different lesion subtypes are shown in Table 1. No differences were noted in demographical, clinical and MRI characteristics between PRLs- and PRLs+ patients and between 0/1-3/>3 PRLs patients except for higher TLV in PRLs+ patients (U: 534; p<0.01) and lower thalamic volumes in >3PRLs patients (H:11.77; p<0.01) (Supplementary eTable 1). Nine (10%) patients were classified as SDMT impaired at baseline and exhibited higher EDSS (U = 199.5; p = 0.03), lower thalamic volume (U = 143.0; p < 0.01), lower percentage volume of ISO lesions (U = 213; p = 0.04) and a trend towards higher TLV (U:229.0; p:0.07) and lower whole brain volumes (t:1.94; p:0.05), as shown in supplementary eTable 2. A correlation heatmap for baseline characteristics is reported in Fig 2. Baseline SDMT r showed positive correlations with cortical (rho:0.33; p<0.01) and whole brain volumes (rho: 0.34; p<0.01), and negative correlation with age (rho: -0.30; p<0.01). SDMT t only showed weak positive correlation with whole brain volumes (rho:0.22; p:0.04) not deemed significant after correction for multiple coefficients (supplementary eFigure 1).

Statistically meaningful SDMT changes over follow up

13 patients (14.4%) showed SDMT decline at 24 months, while 8 patients (8.9%) showed SDMT improvement. 12/13 SDMT-declined patients (χ2:10.32; p<0.01) and 2/8 SDMT-improved patients (χ2:2.40; p:0.15) were PRLs+ (Table 2, Supplementary

eTable 3 and eFigure2). At follow up, 6 patients (6.7%) exhibited PIRA events, with 3 of them being PRLs+ (χ2:0.001; p:0.96). No association was found between patients experiencing PIRA events and SDMT decline (χ2:1.09; p:0.59). A comparison of the differences between the assessment of SDMT changes using RB-RCI and the 8 points cut off is provided in Supplementary Material, e Table 4.

Univariate analyses for the prediction of SDMT decline and SDMT improvement are provided in Table 3. Higher TLV [p:0.03; OR 1.00 (1.00-1.01)], PRLs occurrence [y/n: p:0.01; OR: 15.17 (1.88-122.57)], PRLs number [p:0.04; OR: 1.25 (1.01-1.56)], percentage of PRLs volume [p<0.01; OR: 1.04 (1.01-1.06)] and lower percentages of ISO lesion volumes [p:0.01; OR: 0.96 (0.93-0.99)], significantly predicted SDMT decline while male sex showed a trend [p = 0.09; OR: 2.8 (0.83-9.39)]. Higher thalamic volumes [p:0.04; OR 1.01 (1.01-1.02)] and lower percentages of ISO lesion volume [p<0.01; OR: 1.06 (1.02-1.09)] were predictors of SDMT improvement.

Multivariable models for SDMT decline were built including TLV, ISO percentage volume, sex and the different PRLs metrics. Each model significantly predicted SDMT decline (χ^2 ranging from 12.45 to 17.59; R^2McF ranging from 0.17 to 0.23; p ≤0.01) as shown in Supplementary eTable 5. The multivariable model including TLV, PRLs occurrence (y/n), ISO percentage volume and sex significantly predicted SDMT decline (χ^2: 17.59, p < 0.01, R^2McF: 0.23) with PRLs occurrence being an independent predictor (β: 2.39; p:0.04; OR:10.90 (1.14-104.26)) (Table 4). The multivariable model including thalamic volumes and ISO percentage volume significantly predicted SDMT improvement (χ^2: 16.62, p < 0.001, R^2McF: 0.31) with higher ISO percentage volume as an independent predictor (β:0.10; p<0.01; OR 1.05 (1.02-1.09)) (Fig3) and higher thalamic volumes showing a trend (β:0.01; p:0.08; OR: 1.01 (0.99-1.01) (table 4).

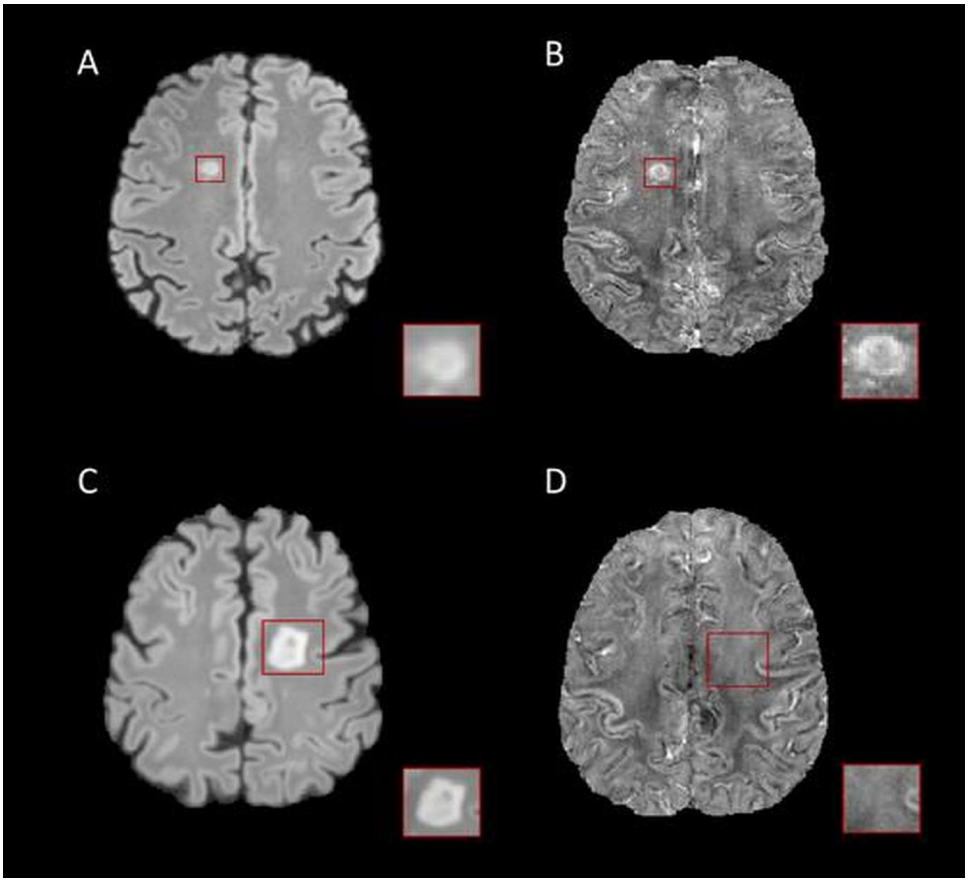


Figure 1 Paramagnetic rim and QSM-isointense white matter lesions

The figure displays two different lesion phenotypes in FLAIR and quantitative susceptibility mapping (QSM) images.

The first row displays a right periventricular lesion classified as paramagnetic rim lesion (PRL). In the FLAIR image (panel A), the lesion is characterized by a homogeneously hyperintense signal. In QSM image (panel B), the lesion's centre appears isointense relative to the surrounding normal appearing tissue, while the border exhibits a hyperintense signal, thereby resulting in a hyperintense rim.

The second row shows a left periventricular isointense (ISO) lesion. Although the lesion demonstrates a hyperintense FLAIR signal, it does not show any corresponding signal and appears isointense to the surrounding normal appearing tissue on QSM.

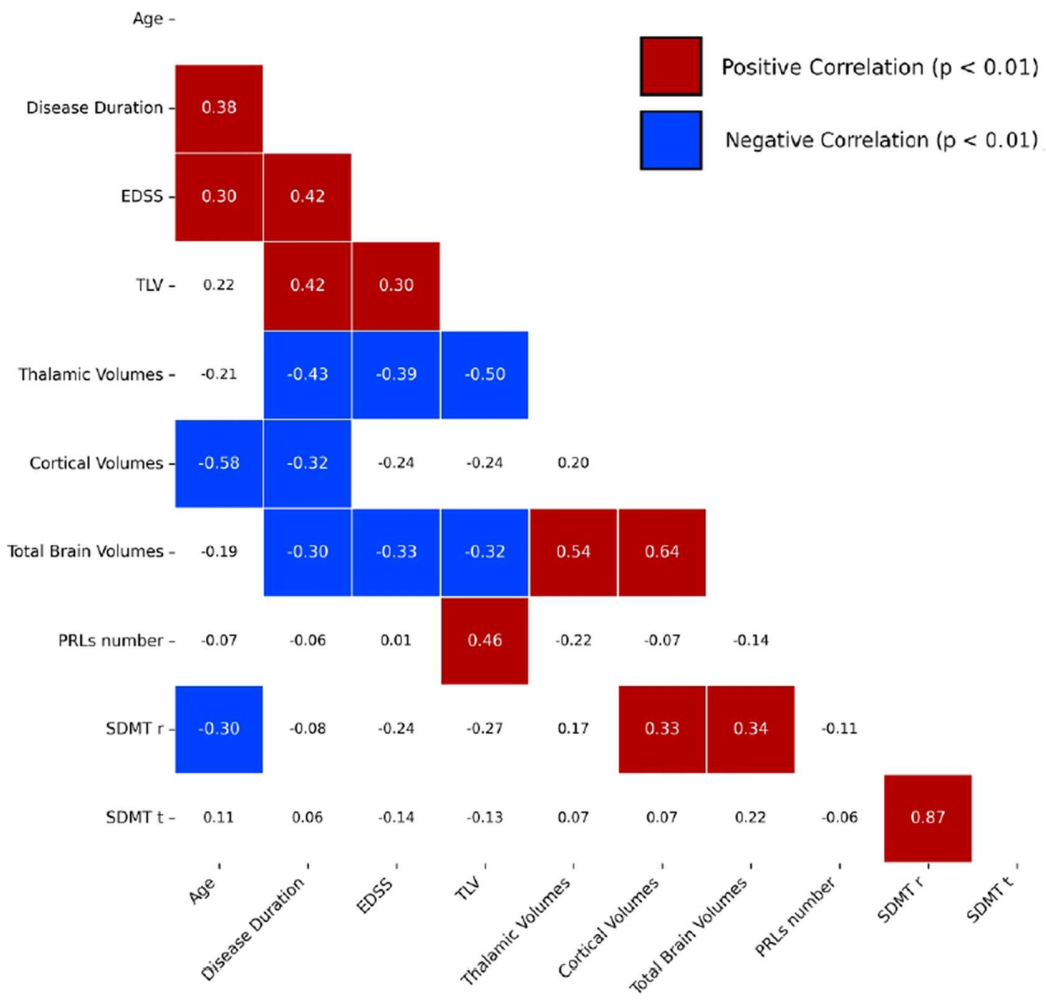


Figure 2 Correlation heatmap for baseline demographic, clinical, MRI and neuropsychological characteristics

The heatmap shows the Spearman correlation of demographic, clinical, MRI, and Symbol Digit Modalities Test (SDMT) variables. The Spearman correlation coefficient is indicated within each box. To account for the numerous coefficients introduced in the correlation matrix, a significance threshold for p-value < 0.01 was applied. Significant positive correlations are displayed in red, while significant negative correlations are displayed in blue. Uncoloured boxes indicate associations that were not deemed significant after applying a significance threshold of p < 0.01.

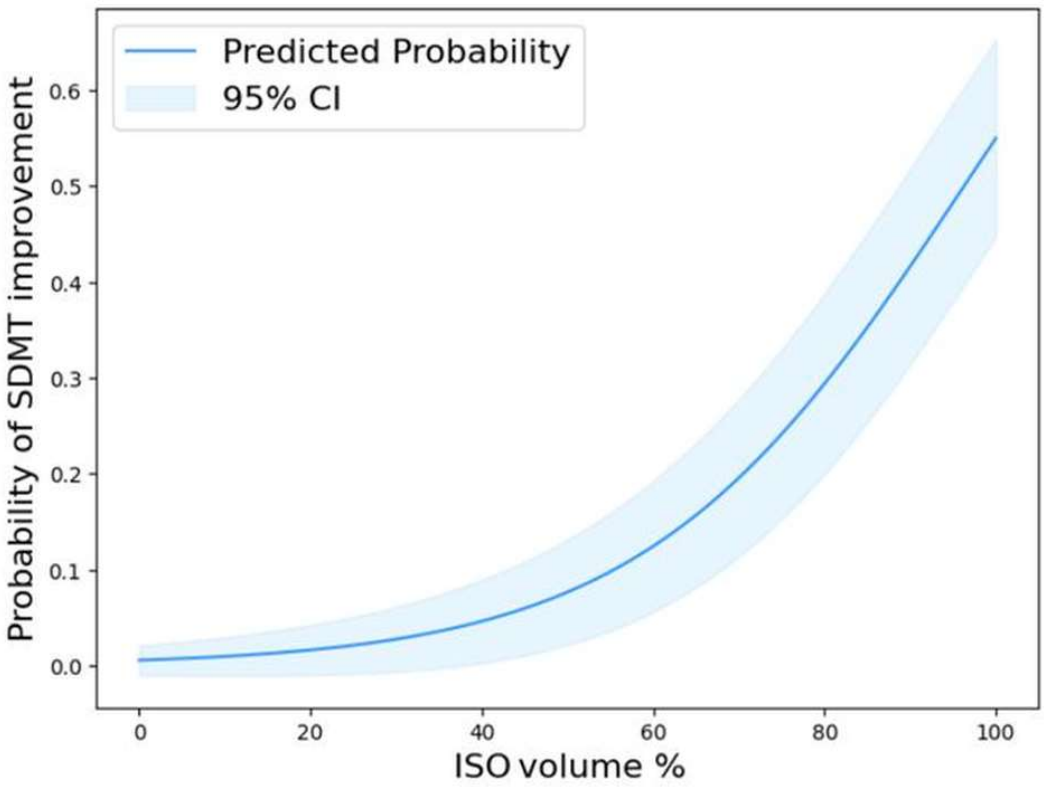


Figure 3: Predicted Probability of SDMT Improvement in relation with QSM isointense lesion volume percentage

The plot shows the predicted probability of SDMT improvement as a function of ISO volume percentage. The plot is derived from a binomial logistic regression model, which estimates the likelihood that a given patient will experience improvement in their SDMT score based on their ISO volume percentage. Model's prediction of the probability of SDMT improvement across different values of ISO volume percentage (solid blue line) and 95% confidence intervals (light blue shaded area) are displayed.

SDMT: Symbol Digit Modalities Test; ISO: isointense lesions

Table 1

Clinical and demographics of the study cohort

Number	90
Females, number (%)	54 (60.0%)
Age, years, mean (SD)	40.30 (10.77)
Disease Duration, years, mean (SD)	8.83 (9.06)
Baseline EDSS, median (range)	2 (0-6)
Follow up EDSS, median (range)	2 (0-6)
PIRA events, number (%)	6 (6.7%)
Therapies, number (%)	
Ocrelizumab	47 (52.2%)
S1P-inhibitors	17 (18.9%)
Cladribine	11 (12.2%)
Natalizumab	10 (11.1%)
aHSCT	3 (3.3%)
Alemtuzumab	2 (2.2%)
Education, years, mean (SD)	14.22 (3.20)
Baseline SDMT r, mean (SD)	55.22 (12.55)
Baseline SDMT t, mean (SD)	51.30 (11.37)
Total lesion volume, mL, mean (SD)	13.34 (19.28)
Whole brain volume, mL, mean (SD)	1095.68 (125.78)
Thalamic volume, mL, mean (SD)	14.32 (2.08)
Cortical volume, mL, mean (SD)	450.38 (51.06)
PRLs classification, number (%)	
PRLs- number	44 (48.9%)
1-3 PRLs, number	35 (38.9%)
>3 PRLs	11 (12.2%)
PRLs number, mean (range)	1.37 (0-12)
PRLs number %, mean (range)	5.53 (0-44.4)
PRLs volume, mL, mean (SD)	3.18 (11.20)

PRLs volume %, mean (range)	14.46 (0-86.2)
ISO number, mean (range)	15.04 (0-47)
ISO number %, mean (range)	47.02 (0-100)
ISO volume, mL, mean (SD)	3.75 (5.37)
ISO volume %, mean (range)	37.97 (0-100)
Follow up duration, years, median (range)	1.92 (1.10)

EDSS: Expanded Disability Status Scale; PIRA: Progression Independent from Relapse Activity; S1P-inhibitors: Sphingosine 1-phosphate inhibitors; aHSCT: Autologous haematopoietic stem cell transplantation; HADS: Hospital Anxiety and Depression scale; HADS-A: Hospital Anxiety and Depression scale – Anxiety; HADS-D: Hospital Anxiety and Depression scale – Depression; SDMT r: Symbol Digit Modalities test raw score; SDMT t: Symbol Digit Modalities test T score; PRLs: Paramagnetic Rim Lesions; PRLs-: Patients not exhibiting PRLs; 1-3 PRLs: Patients exhibiting 1-3 PRLs; >3 PRLs: Patients exhibiting 4 or more PRLs. ISO: QSM isointense lesions.

Table 2 Association between SDMT (Symbol Digit Modalities Test) decline obtained with the Regression-Based Reliable Change Index methodology and Paramagnetic Rim Lesions occurrence.

	PRLs+	PRLs-	Total
SDMT declined	12	1	13
SDMT not declined	34	43	77
Total	46	44	90

PRLs+: Patients exhibiting at least 1 PRL; PRLs-: Patients not exhibiting PRLs.

Table 4 Multivariable predictive models for SDMT decline and SDMT improvement

SDMT decline (χ^2: 17.59, p < 0.01, R^2McF: 0.23)			
Independent Variable	**Estimate**	**p**	**OR (95%CI)**
Intercept	-2.72	0.03	0.06 (0.01-0.74)
Sex	1.10	0.12	3.01 (0.75-12.10)
Total Lesion Volume	-0.02	0.30	0.98 (0.95-1.02)
PRLs (y/n)	2.39	0.04	10.90 (1.14-104.26)
%ISO volume	-0.03	0.15	0.97 (0.94-1.01)
SDMT improvement (χ^2: 16.62, p < 0.001, R^2McF: 0.31)			
Independent Variable	**Estimate**	**p**	**OR (95%CI)**
Intercept	-5.37	<0.01	0.01 (<0.01-0.06)
Thalamic volume	0.01	0.08	1.01 (1.00-1.01)
%ISO volume	0.10	<0.01	1.05 (1.02-1.09)

Multivariable models were built using independent variables that predicted SDMT decline and SDMT improvement, respectively. In the model for SDMT decline (χ^2: 17.59, p < 0.01, R^2McFadden: 0.23), PRLs occurrence was identified as an independent predictor (Estimate 2.39; p:0.04; OR:10.90 (1.14-104.26). In the model for SDMT improvement (χ^2: 16.62, p < 0.001, R^2McF: 0.31), %ISO volume was identified as an independent predictor (Estimate 0.10; p<0.01; OR:1.05 (1.02-1.09), while thalamic volume showed a trend (Estimate:0.01; p:0.08; OR:1.01(1.00-1.01).

DISCUSSION

Ongoing cognitive impairment independent from acute inflammatory activity is a prevalent and disabling condition in MS (34). Understanding the mechanisms underlying subtle cognitive progression in patients otherwise considered as “stable” represents the first step towards better care for MS (6). In addition to their impact on motor disability, HETs seem to have a beneficial effect on patients' cognition, either by improving test performance or halting cognitive decline (5,59,60). This study aimed to assess statistically meaningful SDMT changes using the RB-RCI methodology (62) in patients undergoing HETs with effective disease control, thereby minimizing potential biases from cognitive relapses (63) or therapy changes (69), and to evaluate baseline MRI biomarkers as potential predictors of these changes.

13/90 patients exhibited an SDMT decline, while 8/90 showed an SDMT improvement. Regression models revealed that PRLs occurrence was an independent predictor of SDMT decline, whereas the presence of QSM isointense lesions may play a role in predicting improvement.

By selecting MS patients free from acute inflammatory disease activity over two years and applying RB-RCI, we found that 13 (14.4%) patients showed statistically meaningful SDMT decline over 24 months. A previous study assessing cognitive decline in MS using RCIs showed a cognitive decline rate of approximately 28% in a population that included patients with progressive forms of the disease, higher EDSS scores and longer follow up (56). In our rigorously selected sample of RRMS patients effectively treated with HETs, a 14% rate of SDMT worsening underscores the importance of subtle cognitive deterioration in MS.

Interestingly, 12 out of 13 patients with a significant SDMT decline exhibited PRLs at baseline and PRLs occurrence emerged as an independent predictor of SDMT decline. PRLs are a validated biomarker for the identification of chronic compartmentalized inflammation and correspond to highly disrupted white matter

lesions as shown both by MRI and histopathological studies (43,45). Such lesions may cause greater network alterations, potentially worsening over time (70), and reduce physical and cognitive performance more markedly compared to less disruptive lesion phenotypes. In our sample, male sex showed a trend toward predicting SDMT decline in the univariate analysis but did not remain significant in the multivariable model. This finding aligns with previous studies reporting a higher incidence of cognitive decline in males (71). Moreover, male patients also seem to be at greater risk of developing PRLs (72), suggesting that these two factors might act synergistically to drive cognitive decline.

Of note, neither baseline cortical nor thalamic volumes significantly predicted SDMT decline in our study population. GM atrophy is a typical feature of disability accrual in MS with a close connection with cognitive abilities (55,56), as confirmed by the correlations we found at baseline between cortical volumes, whole brain volumes and SDMT scores. Our findings suggest that in "stable" MS patients, PRLs may play a more pivotal role in the deterioration of SDMT performance compared to GM damage. While HETs are believed to exert a long-term neuroprotective effect by mitigating brain volume loss over time and impacting the accumulation of clinical disability (73,74), their effect on PRLs appears more limited (75). It is possible to speculate that in those patients where chronic compartmentalized inflammation is not fully developed (namely PRLs-), the anti-inflammatory effect of HETs may allow for compensatory mechanism to occur thus resulting in pronounced increase in attention and processing speed. In contrast, in patients where PRLs have formed and chronic inflammation is already established, the efficacy of HETs in halting cognitive decline might be limited.

Eight (8.9%) patients showed meaningful improvement in SDMT over 24 months. Some evidence has already shown the possibility of cognitive improvement in patients undergoing HETs (59,76). By reducing structural damage (i.e. preventing new lesions occurrence) (73,74) HETs might slow progressive cognitive deterioration and

allow compensatory functional mechanisms to improve patient performance (77,78). In our regression analyses, we found that higher percentage of ISO lesion volumes is an independent predictor of meaningful SDMT improvement. ISO lesions evaluated with QSM overlap with lesions with extensive remyelination (covering at least 60% of the lesion surface) in pathological analyses and demonstrate lower microstructural impairment in advanced neuroimaging characterization (43,45). Higher volumes of low-damaged lesions with higher myelin content in some patients could align with findings from other MRI and Positron Emission Tomography studies, suggesting a potential distinction between "good remyelinators" and "bad remyelinators" (57,79). Patients with less aggressive disease course or a higher remyelination potential, possibly reflected by a higher volume of ISO lesions, could have higher chances of cognitive improvement.

This study is not without limitation. First, the reduced sample size limits the power of our predictive analyses. Relying solely on the SDMT, which is particularly sensitive to processing speed—a domain specifically affected by PRLs (54,80)—may restrict the ability to fully capture the diverse and heterogeneous nature of cognitive impairment in MS (81). Longitudinal observation of PRLs could offer a better understanding of their role in cognitive changes, but their slow dynamics and the limited short-term impact of high-efficacy therapies make such analyses challenging within feasible timelines (75). Finally, the RB-RCI methodology, does not account for the potential interference of common behavioural issues in MS which could influence cognition in MS (62). To partially address this issue, we conducted a baseline assessment using the HADS scale and incorporated it in our statistical analysis.

In conclusion, we found that despite the use of HETs and the suppression of acute inflammation, some patients experience a significant SDMT decline over time. The occurrence of PRLs seems to be an independent biomarker of statistically meaningful SDMT worsening and their presence could prompt strict monitoring of cognitive functions and the initiation of early neurocognitive training. Vice versa, we

found that SDMT improvement is detectable and seems to occur more often in patients with less damaged lesions with higher myelin content.

5. Conclusions

In this thesis work, we aimed to study the mechanisms underlying the continuous progression of disability independent of relapses in MS patients undergoing HETs

When relapses and inflammatory activity are suppressed by therapies, new pathophysiological mechanisms are unveiled, making it essential to identify neuroimaging biomarkers to study them.

In the first study, we demonstrated that the inclusion of brain atrophy in the composite NEDA4 measure allows for better classification of patients with a favourable response to HETs. Adding brain volume loss in the first year enhances the ability to predict future disability, highlighting the importance of including neurodegeneration measures in our evaluation of therapy effectiveness.

In the second study, we identified a biomarker that appears to have a highly significant impact on the progression of cognitive disability. PRLs represent lesions with high microstructural damage and may cause more significant network alterations than other lesion phenotypes, playing a key role in causing cognitive disability, even in patients treated with high-efficacy therapies.

The two experimental applications presented in this manuscript yield different results—one on motor disability and the other on cognitive disability—yet they converge in the same direction.

In the era of high-efficacy therapies, the clinical and neuroimaging biomarkers that proved effective in monitoring the disease until a few years ago are no longer adequate. As recent advancements have unveiled new pathophysiological mechanisms, novel prognostic biomarkers are needed to improve standard of care in MS.

The overarching goal of research in this field, in the near future, should be the subcategorization of patients using advanced neuroimaging biomarkers. Predicting

disease trajectories can assist clinicians in better defining a patient's prognosis and by identifying the primary mechanism driving their disability accrual. Advancement in this field would enable the development of more targeted therapies tailored to address specific disease mechanisms for each patient and facilitate the application of precision medicine in MS disease progression.

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