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RELEVANCE OF MRI BIOMARKERS OF MACROSCOPIC AND MICROSCOPIC STRUCTURAL DAMAGE TO COGNITIVE IMPAIRMENT IN MULTIPLE SCLEROSIS.

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Chapter 1. Multiple Sclerosis

1.1 Introduction

Multiple sclerosis (MS) is the commonest non-traumatic disabling disease to affect young adults (Kobelt G et al, 2017). Although its pathogenesis is not entirely known, MS is considered an autoimmune pathology in which a triggered event, still unknown, is able to produce an abnormal response of the immune system against myelin antigens.

The disease is characterized by the presence of multiple areas of demyelination scattered throughout the central nervous system (CNS), with a preference for long and periventricular white matter, spinal cord, optic nerves, brainstem and cerebellum. (Bitsch A et al, 2000). From the neuropathological point of view, these multple lesions appear as circumscribed areas, with perivenular site, with variable degree of demyelination, inflammation, edema, damage and axonal loss, and gliosis. (Lassman, 2018). A certain degree of remyelination is visible in acute lesions, more rare in chronic lesions, thus suggesting that oligodendrocytes lose this ability over time. In fact, over the years neurodegenerative phenomena prevail, especially axonal loss, whose clinical correlation is represented by the accumulation of neurological disability (Lucchinetti et l, 2000). Although it is described as a disease that classically affects the myelin, the involvement of white matter (WM) has long been known as well as for the gray matter (GM). The course is variable and unpredictable but more frequently myelin loss sheath and axonal degeneration determine the appearance of recurrent episodes of focal CNS pain, which initially tend to spontaneous regression,

but which cause deficits over time permanent neurological. Moreover not rarely after years from the beginning, the disease takes a progressive course with or without exacerbations.

Among the various diagnostic methods, magnetic resonance imaging (MRI) has made it possible to diagnose MS at an early stage, thanks to the possibility of identifying lesions even in clinically "silent" brain areas. (Frohman et al, 2003).

1.2 Epidemiology and aetiology

MS occurs mainly in young adults between 20 and 40 years, but can rarely begin under the age of 16 or in adulthood (50-60 years). In general, the female sex is more affected than the male (average ratio of 3: 1), except for the primarily progressive form (PP), which affects both sexes with similar frequency (Duquette et al, 1992). The prevalence of MS has a characteristic geographical distribution, with a gradient that increases with increasing latitude in both hemispheres.

In Italy the prevalence is around 100 cases per 100,000 inhabitants, except for Sardinia, where there is a higher frequency of illness (Ebers CG et a, 2008; Baranzini et al, 2009).

Many genes modestly increase disease susceptibility in addition to several well defined environmental factors, in particular vitamin D deficiency, ultraviolet B light (UVB) exposure, Epstein–Barr virus (EBV) infection, obesity and smoking (Ascherio A, 2013). There is a genetic influence on MS susceptibility; about one in eight patients have a family history of MS (Hewer S et al, 2013; Harichian MH et al 2017).

There are several genes that could determine an increase in susceptibility to MS. These are mostly genes involved in the regulation of the immune response or of components of the HLA histocompatibility complex. The first studies had shown association with the HLA haplotype DRB1 * 1501 and with the DQB1 * 0602 while, more recently, large-scale studies have found the presence of about 14 genomic regions connected to the predisposition to the disease, among which the CD58 regions, IL2RA, IL7RA and CD6. (Hollenbach JA et al 2015; Moutsianas L et al 2015).

1.3 Pathology and immunology

The most accredited pathogenetic hypothesis is the autoimmune one, according to which an autoimmune inflammatory process is responsible for the demyelination typical of MS.

The concept that MS is a disease mediated by CD4 helper T lymphocytes derives from studies based on EAE (experimental autoimmune encephalomyelitis), the animal model of MS, according to which the disease can be induced by the injection of T lymphocytes directed against antigens of myelin such as "myelin basic protein" (MBP) and "myelin oligodendrocyte glycoprotein" (MOG) (Huseby ES et al, 2001). More recent studies have suggested that the inflammatory process in MS caused by an imbalance between the two subpopulations of CD4 T lymphocytes, the Th1 implicated in the production of proinflammatory interleukins (IL) such as INF-ϒ, and Th2, deputies to secretion of IL anti-inflammatory (Trapp BD et al 1998).

Furthermore, the Th17 subtype of CD4 + T lymphocytes appears to play a leading role in the pathogenesis of MS, as demonstrated by the finding of elevated levels of messenger RNA encoding IL17, a proinflammatory cytokine produced by them, in the blood, cerebral spinal fluid (CSF) and brain parenchyma of MS patients. In addition to CD4 + T lymphocytes, cytotoxic CD8 T cells are also involved in the pathogenesis of MS. In fact, they have been shown to induce EAE and are present in large quantities in MS lesions, correlating with neuroaxonal damage. The involvement of B-lymphocytes in the disease is demonstrated by the almost constant presence of an intrathecal production of oligoclonal immunoglobulins (oligoclonal bands [OB]) in patients with MS. A clonal expansion of B-lymphocytes was found both in the lesions and in the CSF of patients with MS (Lassman H, 2013). Furthermore, in patients with SMSP lymphatic follicles formed by B-lymphocytes have been demonstrated at the level of the meninges and correlate with damage to the cerebral cortex (Prineas JW et al, 2001).

Despite the progresses made in recent years, we are still far from fully understanding the pathogenetic mechanism of MS. Probably all the cells of the immune system are involved in different ways depending on the stage of the disease, clinical form or type of damage (myelin and / or axonal). One hypothesis is that the process would start by means of the self-reactive Th17 lymphocytes that, in the context of reduced immune regulation, cross the blood-brain barrier (BEE) through specific adhesion molecules and chemokines and migrate into the ventricles, to then penetrate in the periventricular cerebral parenchyma. The production by the Th17 lymphocytes of IL

proinflammatory (IL17 and IL22) would lead to an increase in the permeability of the BEE favoring the passage of other cells such as the Th1 lymphocytes, the CD8 + T lymphocytes and the B lymphocytes that are found in the perivascular infiltrate.

From the neuropathological point of view, the presence of four different types of demyelinating lesions of the white substance has been demonstrated. Type 1 and type 2 are characterized by a marked perivascular demyelination associated with an inflammatory infiltrate dominated by T lymphocytes and macrophages to which antibodies and complement fractions are added in type 2. In both types of lesion, an apoptotic process affecting the oligodendrocytes cannot be detected. In type 3 and type 4, on the other hand, there is a picture of demyelination not exclusively perivascular characterized by apoptosis (type 3) or dysfunction (type 4) of oligodendrocytes in the absence of inflammatory cells. Numerous factors, such as oxidative damage, glutamate toxicity, cytokines, mitochondrial dysfunction or autoantibodies, could induce the death of oligodendrocytes which, in turn, could trigger a secondary inflammatory process whose function is to remove myelinic debris and other products of cellular degradation (Lassman 2013).

Moreover, in the last decade, neuropathology and MRI studies have shown that in MS the lesions are also present in the gray cerebral substance, especially at the cortical level (cortical lesions [CLs]). The histopathological features of CLs differ substantially from lesions of the white matter, which suggests the presence of a different immunopathological process. A new MRI technique such as the DIR (Double Inversion Recovery) has allowed the in vivo identification of these lesions

(Calabrese et al, 2010).

1.4 Clinical features

MS can affect any area of the CNS and therefore the clinical manifestations are very variable, both in terms of onset and evolution. In the early stages of the disease, there is almost complete remission of the symptoms while over time the remissions may be incomplete, with the consequent accumulation of neurological disability. Onset symptoms are very variable, with signs of mono- or multifocal involvement of the CNS, in acute, subacute or progressive form. MS is tipically suspected when a person presents with a clinically isolated syndrome (CIS). This can be mono-or polysymptomatic depending on the location of the eloquent lesions (Dobson R et al, 2019)

The most common symptoms in MS are:

Visual disorders: Retrobulbar optic neuritis (NORB) is one of the most frequent clinical manifestations of MS, a symptom of onset in about 25% of cases. Generally it manifests with a decrease in visual acuity, usually unilateral, often associated with pain during eye movements and alteration of color vision (in particular red and green). The risk of evolution of NORB in MS is about 50% after 10 years, especially in the presence of brain MR lesions at the time of onset. Other visual disturbances are diplopia (isolated or in the context of an internuclear ophthalmoplegia due to lesion of the medial longitudinal fasciculus), campimetric defects (unilateral centrocecal scotoma, concentric reduction of the visual field, hemianopsia) and, less frequently, oscilloscopy, linked to the presence of nystagmus (Toosy AT et al 2014, Galetta SL

et al 2015).

Motor disorders: hyposthenia of varying degree, involving one or more limbs, and concomitant presence of signs of involvement of the pyramidal system (spastic hypertonicity, hyperreflexia, clonus of the foot, Babinski sign, reduction / disappearance of abdominal reflexes); more rarely, facial nerve palsy and facial hemispasm (McAlpine D. et al, 1972).

Sensitivity disorders: "tingling"-type paresthesia, "numbness" sensation, tactile, thermo-pain and vibratory hypoesthesia with variable localization. (Rai-Grant, A-D et al, 1999).

Cerebellar disorders: ataxia of the trunk and / or limbs, dysmetria, nystagmus, tremors and adiadocokinesia. These symptoms are quite disabling to the patient. The atasso-spastic gait is frequent, due to the simultaneous involvement of the pyramidal and cerebellar system. It is possible to have dysarthria, intentional tremor and nystagmus (Charcot triad) at the same time. (McAlpine D. et al, 1972)

Fatigue: difficulty in carrying out and supporting common everyday activities. This symptom is one of the most frequent in patients with MS and perhaps one of the most disabling (Lerdal et al, 2007)

Other symptoms: bladder disorders (urinary urgency, incontinence, difficulty in 'starting urination with incomplete bladder emptying) and intestinal problems (especially constipation), sexual disorders (reduction / loss of libido, erectile dysfunction, impotence), trigeminal neuralgia, sign of Lhermitte (from lesion of the

posterior columns of the cervical medulla), painful spasms of the limbs, alteration of the tone of the humor (above all depression, sometimes euphoria and disinhibition). Some disturbances, even transient, may be precipitated by physical exercise or exposure to heat (Dillon BE et al 2014, Kanchandani R et al 1982).

The clinical course of the disease can take different forms (Lublin et al 1996):

- *Relapsing-Remitting (RR)*: represents the most frequent variant (85% of cases). It is characterized by the repetition of acute clinical episodes of disease (relapses) destined to regress completely or in part in a variable time. The clinical stability that follows (remission) can be interrupted by the onset of new clinical episodes.

-*Secondary progressive (SP):* it develops as an evolution of the RR form. Over time, the clinical picture is characterized by a progressive and irreversible neurological disability. In 50% of cases this occurs after about 10 years from the onset of the disease. Relapses superimposed on the progressive course may be present or not.

- *Primay Progressive* (PP): is characterized by a progressive disability from the beginning of the illness, in the absence of clinical relapses. It occurs in about 10% of cases.

- *Clinically isolated syndrome* (CIS): it is characterized by a single neurological episode suggestive of MS, lasting at least 24 hours.

- *Other forms*: benign MS (about 10% of cases) and characterized by a paucisintomatico course, with functionality preserved after at least 15 years from the onset of the disease and minimal neurological disability. In about 5% of patients, the course can be rapidly progressive, with the achievement of a significant degree of

disability within the first 5 years after the onset of the disease.

Lublin et al (Lublin et al 2013) propose refined descriptors that include consideration of disease activity (based on clinical relapse rate and imaging findings) and disease progression, redefining the clinicl phenotype (see figure1).

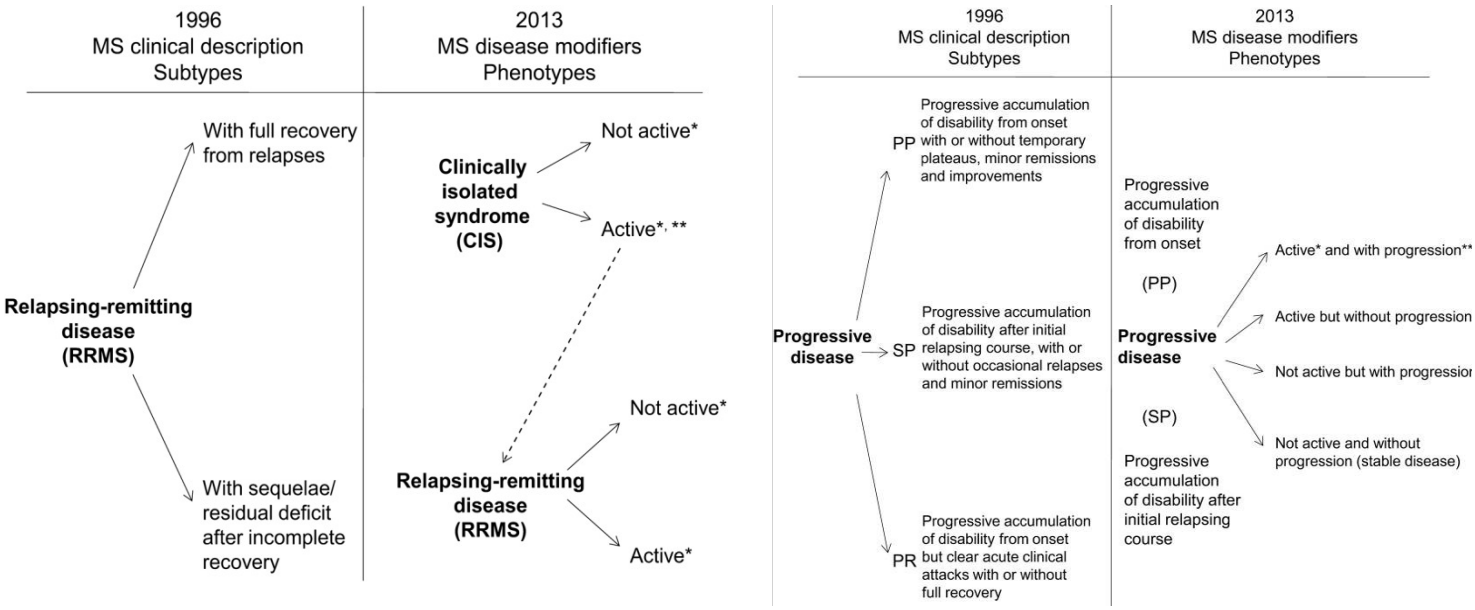


Figure 1: the 1996 vs 2013 multiple sclerosis phenotype descriptions for progressive disease
*Activity determined by clinical relapses assessed at least annually and/or MRI activity (contrast-enhancing lesions; new and unequivocally enlarging T2 lesions). **Progression measured by clinical evaluation, assessed at least annually. If assessments are not available, activity and progression are "indeterminate." MS = multiple sclerosis; PP = primary progressive; PR = progressive relapsing; SP = secondary progressive.

1.5 Cognitive Impairment in MS

Cognitive Impairment (CI) has long been recognized as one of the prominent disabling sequelae of MS because severely impacts on quality of life, employment status and compliance to therapy (Rao SM et al 1991; Julian LJ et al 2011). The

prevalence of CI ranges from 40% to 70% and can be present even in the early stages of diseases (Chiaravalloti ND et al, 2008). MS people experience cognitive dysfunction in a number of domains, mostly in attention, information processing speed (IPS), executive functions, attention (the divided more than the sustained one) , immediate and delayed recall or memory and verbal fluency (Chiaravalloti et al 2008).

These deficiencies start early in the disease course, generally worsen over time and is not strictly linked to physical disability (Benedict RH et al 2011). IPS impairment is the central cognitive dysfunction in MS, more frequent than memory problems, expecially in the early stages and can be detected with the Symbol digit Modalitie Test (SDMT), the most reliable, sensitive and clinically meaningful test of Rao-Battery .

Cognitive test scored have shown moderate-to strong correlations with a series of brain MRI markers, such as T2LV and brain atrophy. Thus, recently, in order to better explain the pathophysiology of CI in MS in association to brain tissue changes, new advanced MRI tecniques have been proposed and implemented.

1.6 Diagnostic Criteria

Neuropathology and MRI studies have shown that axonal damage occurs early in the CNS of MS patients, playing a key role in the subsequent accumulation of disability.

An early diagnosis of the disease is therefore necessary, based on objective and reproducible criteria, which do not compromise its diagnostic accuracy.

McDonald's diagnostic criteria, introduced in 2001, differ from Poser's (Poser et al 1983) previous criteria for the inclusion of MRI in the diagnostic procedure, thus favoring a more early diagnosis. The general principles to which these last criteria have been inspired, since their first version, are aimed at demonstrating, through anamnestic, clinical and instrumental evidence, the dissemination in space (DIS) and in time (DIT) of CNS lesions (Polman CH et al 2011). The criteria of McDonald's have been revised over the years and the latest revision of 2017 is summarized in Tables 1, 2 and 3 (Thompson AJ et al 2018).

The new criteria reiterate the concept that a correct interpretation of symptoms and signs is a fundamental requirement for the diagnosis of MS. The definition of attack (relapse or exacerbation) has also been reconsidered in order to indicate symptoms reported by the patient or objectable signs, typical of an acute or ongoing CNS inflammatory / demyelinating event, lasting at least 24 hours, in absence of infections or fever. However, the exclusion of other CNS diseases of known aetiology (infectious, neoplastic, inflammatory, etc.) is necessary, which can cause symptoms similar to those of MS.

In general, the laboratory and instrumental diagnostic process includes:

• hematological examinations, in the context of a correct differential diagnosis (vasculitis, rheumatological diseases, infectious diseases, etc.);

• MRI of the brain and spinal cord, necessary for the differential diagnosis with other pathologies of the CNS, and the demonstration of DIS and DIT of the lesions;

• CSF examination (to document the intrathecal synthesis of immunoglobulins (seen

with the increase in the Link index which expresses the ratio between CSF and serum Ig, and the presence of oligoclonal bands (OBs) in the alkaline region and not in serum) is not mandatory in some cases (eg, a patient with a typical clinically isolated syndrome supported by characteristic MRI findings demonstration of DIS and DIT, and an absence of atypical clinical or imaging features) but strongly recommended in the following situations: when clinical and MRI evidence is insufficient to support ; when there is a presentation other than a typical clinically isolated syndrome, including a progressive course at onset (primary progressive multiple sclerosis); when clinical, imaging, or laboratory features are atypical of multiple sclerosis; and in populations in which multiple sclerosis is less common (eg, children, older individuals, or non-white populations). the Panel recommended that with a typical clinically isolated syndrome, fulfilment of clinical or MRI criteria for DIS, and no better explanation for the clinical presentation, demonstration of CSF oligoclonal bands in the absence of atypical CSF findings allows a diagnosis of multiple sclerosis to be made, even if the MRI findings on the baseline scan do not meet the criteria for DIT and in the absence of either a second attack or MRI evidence of a new or active lesion on serial imaging (table; Nevertheless, the absence of CSF oligoclonal bands does not rule out multiple sclerosis (Andersson M et al 1994; Stangel M et al, 2013.).

• evoked potentials, especially visual ones (VEP) that may show an increase in P100 wave latency regardless of the occurrence of a retrobulbar optic neuritis.

The diagnosis of RRMS is based in particular on the finding of at least two signs of CNS involvement in distinct anatomical sites and the presence of at least two clinical

episodes, lasting> 24 hours and separated by at least 1 month from each other.

Diagnostic criteria for multiple sclerosis combining clinical, imaging, and laboratory evidence have evolved over time, with the most recent being the 2017 revision of McDonald criteria from the International Panel on Diagnosis of Multiple Sclerosis (Poser et al 1983; Mc Donald WI et al 2001; Polman CH et al 2005; Polman CH et al 2011; Thompson AJ et al 2018) (table 1, 2, 3).

Studies in the past year have shown that inclusion of symptomatic lesions in the MRI determination of DIS or DIT increases diagnostic sensitivity with little or no reduction in specificity and was proposed in the 2016 MAGNIMS criteria (Filippi M et al 2016). On the basis of these data, the Panel recommended including symptomatic and asymptomatic MRI lesions in the determination of DIS and DIT.

The Panel recommended that, in addition to juxtacortical lesions, cortical lesions can be used to fulfil MRI criteria for DIS, although it recognised that standard MRI currently has limited ability to detect cortical lesions or distinguish cortical lesions in multiple sclerosis from those with other causes. The diagnostic criteria for primary progressive multiple sclerosis in the 2017 McDonald criteria remain the same as those outlined in the 2010 McDonald criteria, aside from removal of the distinction between symptomatic and asymptomatic MRI lesions and that cortical lesions can be used (Brownlee WJ et al 2016; Tintore M et al 2016).

At the time of diagnosis, a provisional disease course should be specified (relapsing-remitting, primary progressive, or secondary progressive) and whether the course is active or not, and progressive or not based on the previous year's history. The

phenotype should be periodically re-evaluated based on accumulated information. This recommendation is an addition to the 2010 McDonald criteria (Thompson AJ 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¶

If the 2017 McDonald Criteria are fulfilled and there is no better explanation for the clinical presentation, the diagnosis is multiple sclerosis. If multiple sclerosis is suspected by virtue of a clinically isolated syndrome but the 2017 McDonald Criteria are not completely met, the diagnosis is possible multiple sclerosis. If another diagnosis arises during the evaluation that better explains the clinical presentation, the diagnosis is not multiple sclerosis. An attack is defined in panel 1. *No additional tests are required to demonstrate dissemination in space and time. However, unless MRI is not possible, brain MRI should be obtained in all patients in whom the diagnosis of multiple sclerosis is being considered. In addition, spinal cord MRI or CSF examination should be considered in patients with insufficient clinical and MRI evidence supporting multiple sclerosis, with a presentation other than a typical clinically isolated syndrome, or with atypical features. If imaging or other tests (eg, CSF) are undertaken and are negative, caution needs to be taken before making a diagnosis of multiple sclerosis, and alternative diagnoses should be considered. †Clinical diagnosis based on objective clinical findings for two attacks is most secure. Reasonable historical evidence for one past attack, in the absence of documented objective neurological findings, can include historical events with symptoms and evolution characteristic for a previous inflammatory demyelinating attack; at least one attack, however, must be supported by objective findings. In the absence of residual objective evidence, caution is needed. ‡The MRI criteria for dissemination in space are described in panel 5. §The MRI criteria for dissemination in time are described in panel 5. ¶The presence of CSF-specific oligoclonal bands does not demonstrate dissemination in time per se but can substitute for the requirement for demonstration of this measure.

Table 1: the 2017 McDonald criteria for diagnosis of multiple sclerosis

• dissemination in space can be demonstrated by one or more T2-hyperintense lesions that are characteristic of multiple sclerosis in two or more of four areas of the cns: periventricular, cortical or juxtacortical, and infratentorial brain regions, and the spinal cord*

• dissemination in time can be demonstrated by the simultaneous presence of gadolinium-enhancing and non-enhancing lesions at any time or by a new t2-hyperintense or gadolinium-enhancing lesion on follow-up mri, with reference to a baseline scan, irrespective of the timing of the baseline mri*

**unlike the 2010 mcdonald criteria, no distinction between symptomatic and asymptomatic mri lesions is required. †for some patients—eg, individuals older than 50 years or those with vascular risk factors—it might be prudent for the clinician to seek a higher number of periventricular lesions.*

Table 2: 2017 McDonald criteria for demonstration of dissemination in space and time by MRI in a patient with a clinically isolated syndrome (Thompson et al 2018)

• in a patient with a typical clinically isolated syndrome and fulfilment of clinical or mri criteria for dissemination in space and no better explanation for the clinical presentation, demonstration of csf-specific oligoclonal bands in the absence of other csf findings atypical of multiple sclerosis allows a diagnosis of this disease to be made. this recommendation is an addition to the 2010 mcdonald criteria.

• symptomatic and asymptomatic mri lesions can be considered in the determination of dissemination in space or time. mri lesions in the optic nerve in a patient presenting with optic neuritis remain an exception and, owing to insufficient evidence, cannot be used in fulfilling the mcdonald criteria. in the 2010 mcdonald criteria, the symptomatic lesion in a patient presenting with brainstem or spinal cord syndrome could not be ncluded as mri evidence of dissemination in space or time.

• cortical and juxtacortical lesions can be used in fulfilling mri criteria for dissemination in space. cortical lesions could not be used in fulfilling mri criteria for dissemination in space in the 2010 mcdonald criteria.

• the diagnostic criteria for primary progressive multiple sclerosis in the 2017 mcdonald criteria remain the same as those outlined in the 2010 mcdonald criteria, aside from removal of the distinction between symptomatic and asymptomatic mri lesions and that cortical lesions can be used.

• at the time of diagnosis, a provisional disease course should be specified (relapsing-remitting, primary progressive, or secondary progressive) and whether the course is active or not, and progressive or not based on the previous year's history. the phenotype should be periodically re-evaluated based on accumulated information. this recommendation is an addition to the 2010 mcdonald criteria.

Table 3: 2017 revision to McDonald diagnostic criteria for Multiple Sclerosis (Thompson et al 2018).

Chapter 2: Magnetic Resonance Imaging (MRI) and cognitive dysfunctions in Multiple Sclerosis

Magnetic Resonance Imaging (MRI) in MS represents a fundamental tool for the diagnostic workup, disease monitoring over time and also for treatment response. On the one hand, conventional MRI showed high sensitivity to identify macroscopic focal white matter (WM) lesions as well as infraclinical disease activity; on the other hand and more recently, non-conventional MRI techniques have allowed to investigate in vivo the pathophysiology of MS, showing the presence of diffuse microscopic damage outside focal WM lesions, including normal-appearing WM (NAWM) and gray matter (GM), so in normal-appearing brain tissue (NABT). (Giorgio et al 2016).

Finally, functional MRI (FMRI), a more advanced non-conventional MRI technique, is able to investigate the mechanism of brain neuroplasticity in MS, showing very promising results (Faivre et al 2012).

2.1 Focal tissue damage and conventional MRI

Focal WM lesions, detected by MRI, are the pathological hallmark of MS showing some relation to clinical disability, expecially in the long run. MS lesions typically have an oval or elliptical shape and in terms of MRI sequences, fluid-attenuated inversion recovery (FLAIR) and T2-weighted sequences are able to show inflammation, edema, demyelination, gliosis and axonal loss whereas postcontrast

T1-weighted images indicate the presence of acute inflammation (Lucchinetti et al 2000).

Although T2 lesions are a simple expression of general and macroscopic tissue damage, reflecting disease activity, a greater specificity is provided by the lesions appearing dark on T1-weighted images, defined as "black holes". Most of these lesions originate from areas of Gd enhancement (acute black holes), resolve over a period of approximately 6 months. The remaining ones (persistent black holes), account only for 36 % of all T1-enhancing lesion, and represent severe demyelination and irreversible axonal loss (Barkhof F et al 1998; van Waesberghe JH et al 1998).

The presence of cerebral WM lesions may lead to a functional disconnection of cortical and deep GM regions, and this may be responsible for development of Cognitive Impairment (CI).

Early MRI studies reported a higher frequency of CI in RRMS with higher lesion load, not only for the classical WM lesions but also for lesions located in the cortical/subctortical brain regions (Swirsky-Sacchetti T et al, 1992). Total T2-weighted lesions and their location in the cerebral WM are linked to specific patterns of CI in MS, targeting specific domains such as memory, abstract, conceptual reasoning, language and visuospatial problem solving (Rao SM et al 1989b, Swirsky-Sacchetti T et al, 1992). In detail, higher WM lesions load in the frontal lobe was associated with worse performance on a conceptual reasoning task. In addition, lesions in the left frontal lobe proved to be a good predictor for impaired abstract problem solving, memory and word fluency whereas lesions in the left

parietooccipital lobe predicted deficits in verbal learning and complex visual-integrative abilities (Swirsky-Sacchetti et al, 1992).

Lesions in juxcortical regions and posterior fossa have proven to be associated respectively with lower performance in memory and executive functions (Miki et al, 1998, Archibald et al, 2004).

As regards the monitoring of the response to therapy, the role of MRI seems to be less defined. Therefore a score was recently created, the the so-called Rio score (Rio J et al 2009), based on clinical and MRI characteristics, with the aim to guide the neurologist in determining a non-responder patient to IFNβ therapy, then eventually modifying the therapeutic regimen. Recently the Rio score has been modified (table 4 and figure 2), leading to increased sensitivity, in particular based on the number of new T2 lesions accumulated by the patient over one year (Sormani MP et al 2014.).

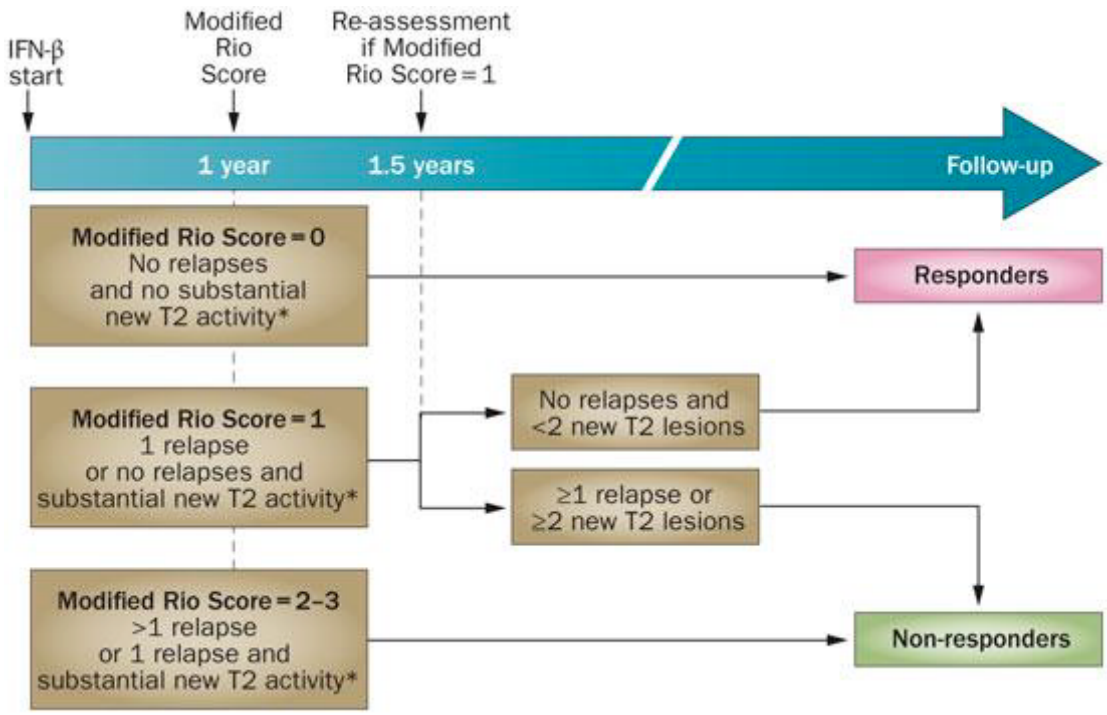
Table 4. Comparison between Rio score and modified Rio score (Sormani MP et al 2014).

Table 2 | The Rio and Modified Rio Scores

Rio Score		Modified Rio Score	
Criterion	**Change over the first year**	**Criterion**	**Change over the first year**
MRI criterion = 0 MRI criterion = 1	≤2 active* T2 lesions >2 active T2 lesions	MRI criterion = 0 MRI criterion = 1	≤4 (5)‡ new T2 lesions >4 (5)‡ new T2 lesions
Relapse criterion = 0 Relapse criterion = 1	No relapses ≥1 relapse	Relapse criterion = 0 Relapse criterion = 1 Relapse criterion = 2	No relapses 1 relapse ≥2 relapses
EDSS criterion = 0 EDSS criterion = 1	Increase in EDSS score of <1 point Increase in EDSS score of ≥1 point, sustained over at least 6 months	Not included	Not included
Rio Score = MRI criterion + relapse criterion + EDSS criterion		Modified Rio Score = MRI criterion + relapse criterion	

*Active lesions defined as new or enlarging T2-weighted lesions plus gadolinium-enhancing lesions over the first year. ‡The cut-off of four lesions was in the validation set; the cut-off of five lesions was in the training set. Abbreviation: EDSS, Expanded Disability Status Scale.

Figure 2. Proposed algorithm based on the modified Rio score for the evaluation of 4-year risk of progression in MS patients treated for 1.5 years with IFNβ. *substantial new T2 activity is defined as> of 4-5 new lesions in 1 year of treatment or> 1-2 new lesions if the follow-up MRI was performed at least 6 months after the start of therapy



2.2 Cortical Lesions

Cortical involvement in MS may result from several pathological processes including demyelination, Wallerian degeneration, neuroaxonal loss and meningeal inflammation (Stadelmann C et al 2008) and is present since the early stage of disease (Lucchinetti CF et al, 2011). Special MRI sequences sucheas double-inverion recovery (DIR) and phase-sensitive inversion recovery (PSIR) have greatly

contributed to detect Cortical Lesions (CLs). Pathological and MRI studies indicate that some cortical brain regions are particularly vulnerable to tissue damage, responsible for CI in MS, in a wide spectrum of MS phenotypes (Audoin B et al, 2005). In RRMS, an increase CLs in the hippocampus correlated with low scores of visuospatial memory (Roosendaal SD, et al 2009) and in another study (Calabrese M, et al 2009) RRMS patients with CI had a higher volume of CLs compared to CP ones. These results have been explained by a relationship between CL load and global CI detected with BRB (Rao B et al, 1990), demonstrating that CLs can be an independente predictor of CI in MS.

2.3 Atrophy Measures

Although the involvement of WM represents the main hallmark of MS, also the focal and widespread damage of GM play an important role in the pathological definition of the disease, particularly evident in the cortical GM.

Unfortunately, detection of GM damage in vivo by conventional methods is not always possible, being often limited by a series of reasons such as small size of GM abnormalities, low contrast between affected and unaffected GM, poor inflammatory activity with a low number of contrast enhancing lesions, partial volume effects due to CSF, unavailability in routine clinical practice.

The recent introduction of non-conventional MRI techiniques allowed quantifying GM damage and its correlate with clinical measures (physical and cognitive impairment).

The most used tools for segmentation and measurement of volume/thickness of cortical GM are: 1) Structural Image Evaluation using Normalization of Atrophy for longitudinal (SIENA) and cross-sectional (SIENAX) volumetric studies; 2) Voxel-Based Morphometry (VBM), which is implemented in Statistical Parametric Mapping (SPM software package) and allows to investigate regionl GM atrophy, and 3) FreeSurfer, which accurately measures GM volumes as well as whole and regional cortical GM thickness.

Brain atrophy is present from the earliest stage of MS (De Stefano N et al 2003) and seems to be largely independent from the disease subtype (De Stefano N et al , 2010). Some authors first reported (Amato MP et al, 2010) cortical GM atrophy in MS patients with CI and successively a greater cortical GM loss in those with cognitive scores worsened during a 2.5-year follow-up. These results have been subsequently confirmed and expanded by other research groups.

Several studies found that cognitive performance scores were lower in MS people with larger third ventricle, lateral ventricles, widening of cortical sulci and atrophy of corpus callosum. The latter in particular seems to predict worse performance in information processing speed and problem solving (Rao et al, 1989a) whereas atrophy of the anterior part of callosum was associated with low scores on verbal fluency tasks. Moreover, atrophy of different parts of callosum was associated to worse performance on task exploring interhemispheric information treansfer (motor, sensory and motor), suggesting MS as a model of disconnection syndrome (Dineen RA et a, 2009). Several studies confirmed the role of third ventricle in predicting CI

in MS, particularly in distinguishing secondary progressive from RR course (Benedict RH et al, 2006).

Neocortical atrophy was also found in CI MS patitents, including the benign forms (Amato MP et al 2004, Amato MP et al 2008) and resulted correlated with worse performance in attention/concentrations and verbal fluency. Thus, in patients with mild disability, performance on PASAT was correlated with volume of the whole GM and of cortical regions involved in executive functions and working memory, including prefrontal cortex, precentral gyrus, superior parietal cortex bilaterally and cerebellum (Morgen K et al 2006). More recently, in addition, atrophy of deep GM structures such as thalamus, caudate and hippocampus has also been recognized as a relevant factor in CI. Thalamic volume proved to be a reliable predictor of CI in MS, showing a moderate-to-strong correlation with cognitive performance in different cognitive domains (Houtchens MK et al, 2007). On the other hand, volume loss of the hippocampus and its regions seems to be related to worse performance on verbal fluency but not on information processing speed (Sicotte NL et al , 2008).

Moving to regional GM atrophy, it is worthy of mention the Voxel Based Morphometry (VBM), an MRI approach, which allows comparing average GM maps of different groups of subjects in order to extract areas/regions where GM density is reduced/increased respect to a control group. Using VBM, several studies showed a significant GM loss in different brain regions such as fronto-temporo-parietal ones, cerebellum and deep GM, also confirmed the front-parietal circuits involvement in

fatigued MS patients (Prinster A et al 2006; Middleton FA et al 2000; Mesaros S et al 2008; Weiker K et al, 2014).

2.4 Other advanced MRI techniques

Despite CI in MS depends on both lesion load and brain atrophy, to date, much of this substrate remains unclear. This because lesions represent a marker of focal macroscopic damage and that the lack of their pathological specificity makes them unable to differentiate the various processes of demyelination, inflammation, axonal damage, edema and gliosis. Moreover, tissue damage in MS results widespread across the brain, also involving areas of the so-called normal-appearing brain tissue, where the presence of microscopic disease is not fully explained by conventional MRI.

In this scenario, the development of more advanced MRI techniques, may help to overcome these issues. Lastly, functional MRI studies pointed out that brain cortical reorganization may play and adaptive role in limiting the clinical consequences of disease-related structural damge in the different disease phenotypes. (Cader S et al, 2006).

2.4.1 Diffusion Tensor Imaging (DTI) and voxel-wise analyses.

This advanced MRI technique measures the Brownian motion of water molecules in the tissues, which is influenced by the presence of different barriers such as

cell membranes and other macromolecules (Inglese M et al 2010; Miller DH et al 1998). The water is normally free to move equally in all directions in an isotropic manner; when, otherwise, movement is limited within the cells or by the presence of tissue barriers, the movement becomes anisotropic. So, the movement of water molecules is markedly reduced in compact tissues as in WM, less in the GM and is completely free in the CSF (Moll MN et al, 2011). Pathological processes, altering the normal brain structure affect water movement by altering so-called diffusion parameters. Diffusion images can be obtained from a minimum of three gradient directions, giving rise to two different types of sequences: diffusion-weighted imaging (DWI) images and those with diffusion tensor imaging (DTI). The latter are obtained from a matrix acquired with at least 6 gradient directions, characterizing the water movement in a three-dimensional environment. The matrix can be represented as an ellipsoid with 3 main axes: the longest axis λ1 is the primary one and reflects the diffusion parallel to the fibers, the so-called axial diffusion (axial diffusivity-AD); on the other hand, the average of the two shorter axes λ2 and λ3 provides a measurement of the diffusion perpendicular to the fibers (radial diffusivity-RD). The above measures are able to discriminate axonal damage and myelin damage, specifically the first being more represented by the AD and the second from RD (Sbardella E et al 2013). In the WM of MS, RD is typically increased by indicating the myelin loss while the AD may be decreased or increased, indicating in the first case the axonal damage, and in the second the presence of mechanisms of compensation aiming at restoring functionality in the damage WM areas.

However, the most widely used DTI measurements are derived from a mathematical combination of the three vectors λ1, λ2 and λ3. Specifically, mean diffusion (MD) represents globally the movement of the water without a particular direction, the higher the MD value the greater the diffusion degree of water molecules; fractional anisotropy (fractional anisotropy-FA), instead, represents the diffusion mainly along one direction, the value of FA varies from 0 to 1 ed is greater in the compact WM areas, reduced in the GM and approaches 0 in the CSF.

Both MD and FA are mainly related to the content of myelin and partly to axonal density. Nevertheless, MD is mostly accepted as an index of free space, this meaning that processes such as vasogenic edema or myelin and axonal loss determine an increase of it (Horsfield MA et al 2002; Filippi M et al 2001).

On the other hand, FA is more sensitive to structural integrity of WM, unfortunately without pathological specificity, not being able to distinguish between edema, inflammation, demyelination or leukoaraiosis (Miller DH et al 2003; Inglese M et al 2010).

In WM the water molecules have a preferential movement along the direction of axons, this explains the high values of FA and AD in these regions (Inglese M et al 2010, Filippi M et al 2001). Numerous studies have highlighted an increase in MD and RD associated with the reduction of FA and AD in demyelinating WM lesions and similarly, although to a lower degree, in NAWM (Cercignani M et al 2000, Filippi M et al 2001). These alterations of the diffusion parameters vary with the severity, duration and phenotype of MS (Rovaris M et al 2007; Preziosa et al 2011).

Spatial variability in MD, RD, FA and AD measurements , as well as their change over time within the same lesion, suggests a considerable heterogeneity of the underlying pathological mechanisms and their severity (Inglese M et al 2010). It has also been demonstrated how alterations in diffusivity can be detected even before plaque formation (Rovaris M et al 2008). In conclusion then, in the course of MS, the increase in MD and RD and the reduction of AD and FA correlate respectively with demyelination and axonal loss (Miller DH et al 2003; Inglese M et al 2010; Horsfield et al 2002; Rovaris M et al 2007; Schmierer K et al 2007). It exists even today the possibility of generating FA-based maps representing the WM tracts ,able to assess in more detail how myelinated damage is heterogeneous along a single bundle of axons (Diffusion Tensor Tractography) and to correlate it with a particular type of clinical deficit. For example it was demonstrated how the damage of intercortical associative tracts is correlated with cognitive impairment and how the damage along the corticospinal pathways with walking disability (Inglese M et al 2010; Ceccarelli A et al 2012). As for the GM, both cortical and deep GM structures such as the thalamus and the caudate nucleus, several authors have identified in the course of MS a discrepancy between the alteration of DTI values compared to the WM, highlighting alongside a clear increase in MD values, minimal alteration of FA values, suggesting that demyelination prevails over axonal damage. This result could be explained by the type of pathology of the GM at the time of analysis; in the case of an activation the prevalence of microglia would show an increase in the values of FA, conversely, in the case of prevailing inflammation mechanisms we would have a reduction in

values (Sbardella E et al 2013; Calabrese M et al 2011; Hannoun S et al 2012). Furthermore, it has been shown that, clearly, cortical lesions tend to have more altered parameters of diffusion (greater increase in MD and FA) than in NAGM. Finally, as already discussed for the MTI, also in the course of DTI, NAGM and NAWM present pathological values that correlate with the clinical characteristics of the MS patients (Calabrese M et al 2011).

More recently, DTI was used in conjunction with tract-based and voxel-wise analyses to better understand the processes underlying the various clinical features of MS patients. Using tracted-based spatial statistics (TBSS) in mild RRMS, T2LV showed association with FA in lesions but also in NAWM, whereas a FA reduction in corpus callosum and internal capsule correlated with higher EDSS (Giorgio A et al 2010; Roosendal SD et al 2009).

Therefore, in patients with cognitive impairment, 50-76% of WM tracts were more damaged compared with healthy controls and cognitively preserved patients, expecially in corpus callosum, corticospinal tract, cingulum, fornix, forceps minor and inferior-superior longitudinal fasciculus (Hulst HE et al 2013).

These data prompt that NAWM damage of specific WM tracts, expecially those involved in interhemispheric communication, may lead a lower performance on clinical tasks (cognitive and motor), suggesting a possible mechanism of "disconnection" between different GM brain regions (Dineen RA et al 2009).

Relationship between impaired WM tract and GM atrophy has been investigated with a combination of TBSS and VBM analysis of high-resolution T1 weighted scans. In

fact, in early PPMS an anatomical and quantitative correlation between NAWM tract damage and volume reduction in GM regions has been found (Bodini B et al 2009) whereas in SPMS thise correlations were detected only between regional deep GM and connected WM tracts (Steenwijk MD et al 2015).

2.4.2 Proton MRI Spectroscopy

Spectroscopy (Magnetic resonance Spettroscopy-MRS) allows a non-invasive detection of some biochemical changes of lesions compared to the NAWM. In acute lesions, even at an early stage, MRS shows increase of choline (Cho), lactate (Lac), lipids (LIP) and diminution of creatine (Cr) (with an increase in the Cho / Cr ratio), myo-inositol (Mi), of glutamate / glutamine (Glx). The increase in Cho and LIPs comes from the increase of membrane phospholipid levels (phospholipids and glycerol / phosphocholine) and of myelin released during myelinolysis. The increase in Lac, a marker of anaerobic glycolysis, derives instead from the ischemic phenomena secondary to inflammation. In large demyelinating acute lesions it can also be observed a decrease in Cr, which is an element of energy metabolism and reflects probably axonal loss. The short-time echo spectra show transient increases in MI, which is slightly reduced in acute plaques and increases in chronic lesions, as it reflects the increased glial content.

Finally, the pathogenetically decreased Glx (astrocytic marker) is reported in various inflammatory conditions of the CNS. Cronic brain plaques in MS have different characteristics: in a period of some day / week after the acute phase there is

a progressive normalization of the Lacs and of the Cho; months are needed for LIP and Mi to return to normal. Chronic plaques show a decrease in the peak of N-acetyl aspartate (NAA), as a possible marker of axonal and neuronal dysfunction / loss, much more evident in black holes. Reduced NAA feedback without a corresponding normal value of the Cho peak is a useful element in the differential diagnosis with glial neoplasms (which in fact also present an increase in the Cho peak (Barker PB et al 2010).

As for the lesion evolution over time, the signal intensity of NAA may be low or show partial recovery, starting early after the acute phase and lasting for many months (De Stefano N et al 1995), thus correlating with the clinical evolution (Ciccarelli O et al 2010) and with a mechanism of repair. Indeed, decreased NAA levels in the cerebral cortex may be absent in early stage of MS but marked in PPMS whereas a reduction of NAA in deep GM is mostly found since the early stages of disease (Wylezinska M et al 2003; Geurts JJ et al 2006).

2.4.3 Magnetization Transfer Imaging

Magnetization transfer imaging (MTI) is based on the interactions between free-water protons and protons bound to macromolecules, providing an index of general tissue integrity called magnetization transfer ratio (MTR) (Filippi M et al 2007, Schmierer K et al 2004).

Variable degrees of MTR decrease have been found in acute and chronic WM lesions, and obviously the most marked abnormalities are present in black holes.

Some authors revealed that average MTR of NABT and cerebral cortex are respectively the factor most significantly correlated with cognitive dysfunctions in RRMS (Filippi Met al, 2000) and benign MS (Amato MP et al 2008).

A more severe MTR decrease has been obsverved in SPMS over the course of new lesions during 3 years and in RRMS after 1 year, thus predicting the disability worsening (Rocca MA et al 1999; Agosta F et al 2006).

Therefore other studies demonstrated reduced MTR in NAWM at the level of corpus callosum, front-occipital tracts, external capsule and optic radiations, of CIS and MS. These regional MTR abnormalities were also associated with physical disability and worse cognitive performance (Ranjeva JP et al 2005).

2.4.4 Functional MRI (FMRI) and brain plasticity

FMRI measures blood-oxygen-level dependent (BOLD) signal in regions of GM involved in the performance of a task or during a rest condition (Giorgio A et al 2016).

Task-FMRI, exploring the visual, cognitive and sensorimotor systems, demonstrated functional cortical changes in a wide spectrum of MS phenotypex, compared to normal controls, with hyperactivation of regions normally recruited for performing a specific task and/or the recruitment of additional areas (Filippi M et al 2011).

By measuring changes in the concentration of what can be considered as a physiological contrast (e.e., deoxyhaemoglobin), FMRI is contributing to defining the role of brain reorganization in limiting the cognitive impact of disease-related tissue

in MS (Audoin B et al 2005, Mainero C et al 2004, Rocca MA et al 2009).

Functional MRI abnormalities occur early in the MS course. In a 1-year study, CIS patients who developed clinically defined MS had a higher cortical activation when compared with those who did not, suggesting and early cortical reorganization following tissue injury (Rocca et al 2005). CI in MS is reflected by changes of structural but also functional brain connectivity. Indeed, not all MS patients present cognitive dysfunctions despite having GM and WM pathology, and these discrepancies can depend on compensatory functional brain reorganization.

The role of functional connectivity (compensatory vs maladaptive) remains, to date, still unclear.

The functional adaptation or reorganization (increased recruitment of cortical regions) helps to limit the functional disease burden associated to structural damage, in part explaining the favorable clinical outcome in these patients (Giorgio et al 2010, Giorgio et al 2016). Nevertheless, increased cortical recruitment does not proceed indefinitely, and a lack and /or exhaustion of adaptive mechanisms has been considered as a possible factor responsible for clinical worsening in the advanced stage of MS (Rocca et al 2005).

3 Aims of this thesis

Multiple sclerosis (MS) is a prototype of diffuse chronic inflammatory demyelinating disease of the CNS, representing the most common non-traumatic cause of disability in young adults. Demyelination and neurodegeneration in the MS brain is associated

with a marked astroglia reaction, forming a dense glial scar in long-standing established lesions. For some time, the view on MS pathology centered on focal demyelinated plaques in the WM. Given its sensitivity in revealing focal WM abnormalities, MRI has become an indispensable tool for the diagnostic MS workup. It is also extensively used in monitoring of abnormalities over time and elucidating the mechanisms of disease progression and clinical disability (Giorgio et al 2016).

Moreover, in the last few years, conventional MRI has been significantly improved by quantitative and advanced MRI techniques, which have shown greater sensitivity and specificity to the heterogeneous pathological substrates of the disease, not only in focal T2-visible WM lesions, but also in normal-appearing gray matter (GM) and white matter (NAWM). The latter can be assessed quantitatively with various MRI methods. In particular, diffusion tensor imaging (DTI), by assessing tissue damage through the quantification of water motion direction (Chabriat et al., 1999; Rovaris et al., 2009),

DTI) is one of the preferred MRI methods for WM investigation in vivo, detecting macro- and microscopic tissue abnormalities. To date, several DTI-derived indices (eg, fractional anisotropy [FA], mean diffusivity [MD], axial [AXD], and radial diffusivity [RD]) have been proposed as reflecting different aspects of WM microstructure and damage (Iadecola et al 2013). Histogram analysis has been largely used to analyze data coming from MRI for the investigation of various neurological disorders (Holtmannspotter et al 2005; Lema et al 20017; Giulietti et al 2018).

The aim of this thesis is to report the results of 3 studies, carried out from November 2016 to June 2019, based on the application of some conventional and non-conventional quantitative MRI techniques such as quantification of brain lesion volumes (SIENAX and JIM softwares), DTI in a different group of relapsing-remitting MS. The first and the second studies are focused on the application of a new MRI marker named peak width of skeletonized mean diffusivity (PSMD) and its role in detecting the diffuse WM microstructural damage in demyelinating disorders, particularly in MSs, taking into account also the correlations between brain structures changes and clinical measures (physical disability and cognitive impairment). In the third and last study we reported the pleminiary results about the relevance of cognitive reserve (CR) on the association between cognitive impairment (CI) and WM microstructural damage in mild disability MS.

4. Peak width of skeletonized mean diffusivity (PSMD) as marker of widespread tissue damage in multiple sclerosis.

4.1 Introduction

Cerebral white matter (WM) diseases include a wide spectrum of disorders with damage mostly to the myelinated fibers of the central nervous system–and an MRI pattern ranging from small multifocal to confluent or extensive WM lesions (Sarbu et al., 2016). Multiple sclerosis (MS) is a diffuse chronic inflammatory demyelinating disease of the adults, primarily due to damage of myelin sheaths and oligodendrocytes as result of perivascular inflammation and lymphocytemediated immune reaction targeting myelin basic protein and proteolipids (Lassmann, 2018). On the other hand, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencepahlopathy (CADASIL) is an increasingly recognized inherited non-atherosclerotic and amyloid-negative type of severe small-vessel disease (SVD) with autosomal dominant transmission, related to a mutation of the NOTCH3 gene on chromosome 19 (Di Donato et al., 2017). Despite the different pathogenic mechanisms, both MS and CADASIL lead to physical and cognitive disability, which are not always reflected by the brain damage visible on MRI. In both MS and CADASIL, macroscopic lesions may have similar appearance on brain MRI, particularly at early disease stages, although usually their extent is smaller in the former. It is well known that the neuropathologic process is not limited to the WM lesion areas as identified by conventional MRI and the damage is rather widespread,

also involving the normal-appearing WM (NAWM) (Giorgio et al., 2016; Di Donato et al., 2017). The latter can be assessed quantitatively with various MRI methods. In particular DTI, by assessing tissue damage through the quantification of water motion direction (Chabriat et al., 1999; Rovaris et al., 2009), may be a valuable option for a more comprehensive understanding of brain pathology in vivo. In this context, a new fully automated, robust, fast and easy-to-use DTI-derived imaging marker, named as peak width of skeletonized mean diffusivity (PSMD), has been recently proposed as a markeroutperforming traditional DTI measures (Baykara et al., 2016). By combining "skeletonization" and histogram analyses of DTI-derived mean diffusivity (MD) images, PSMD has shown to provide robust and clinically relevant information on the global brain tissue damage in both acquired and inherited SVD, with values that were increasing withhigher WM lesion load (Baykara et al., 2016). Against this background, we aimed here to explore the PSMD status and its relevance in a demyelinating disorder such as MS. To do so, we performed group analyses of PSMD values across the brain of relapsingremitting (RR) MS patients compared to CADASIL patients and normal controls (NC).

4.2 Materials and methods

4.2.1. Population

We consecutively included 100 subjects divided into three groups (see Table 1): i) RRMS patients (n=47, age: 43 ± 9.4 years, 76% females) diagnosed according with the revised McDonald criteria (Polman et al., 2011), ii) CADASIL patients (n=25,

age: 46.9 ± 10.5 years, 36% females), all with a genetically confirmed diagnosis (Di Donato et al., 2017) and iii) NC (n=28, age: 45.2 ± 12.3-years, 46% females).

MS patients had no evidence of vascular conditions (arterial hypertension, coagulopathies, heart diseases [e.g., patent foramen ovale]). Physical disability was measured with Expanded Disability Status Scale (EDSS, median=2.1 [range: 1–4.5]) (Kurtzke, 1983) and cognition was assessed with the Rao Brief Repeatable Battery (BRB) (Rao, 1990), which categorized patients into cognitively preserved (CP, 36%) and cognitively impaired (CI, 64%), using the normative data for the Italian population (Amato et al., 2006). Briefly, BRB incorporates tests of verbal memory acquisition and delayed recall (Selective Reminding Test [SRT]), visual memory acquisition and delayed recall (10/36 Spatial Recall Test [SPART]), attention, concentration and speed of information processing (Paced Auditory Serial Addition Test [PASAT]; Symbol Digit Modalities Test [SDMT]) and verbal fluency on semantic stimulus (Word List Generation [WLG]). Failure of a test was defined as a score at least two standard deviations below the mean normative values of the Italian population (Amato et al., 2006). Significant cognitive impairment was defined as failure on at least two tests of the BRB (Rao, 1990; Amato et al., 2006).

Diagnosis of CADASIL was based on clinical, genetic, neuropathological and MRI data (Di Donato et al., 2017). In particular, genetic analysis in all CADASIL patients showed a cysteine-altering mutation in exons 2–24 of the NOTCH3 gene (Di Donato et al., 2017). Patients had minimal physical disability, assessed with modified Rankin Scale (mRS, 0.32 ± 0.85 [range 0–3]) (Wilson et al., 2002).

NC subjects were recruited among laboratory and hospital workers and included if they had normal neurological examination and no history of neurological disorder. Informed written consent was obtained from all subjects of the study, which was approved from the local Ethics Committee (Azienda Ospedaliera Universitaria Senese).

Table 1: demographic, clinical and MRI data of the study groups

GROUP	VARIABLES VALUES	
MULTIPLE SCLEROSIS (N = 47)	AGE (YEARS)	43 ± 9.6
	SEX: FEMALE (PERCENT)	76.6
	EDSS	2.1 [1–4.5]
	COGNITIVE IMPAIRMENT (PERCENT)	34%
	LV (CM3)	8.6 ± 8.2
	PSMD (X 10(4 MM2/SEC)	4.2 ± 1.2
CADASIL (N = 25)	AGE (YEARS)	46.9 ± 10.5
	SEX: FEMALE (PERCENT)	36
	RANKIN SCALE	0.32 ± 0.85
	MMSE	28.8 ± 6.4
	LV (CM3)	24.4 ± 17.4
	PSMD (X 10(4 MM2 /SEC)	4.5 ± 1.3
NORMAL CONTROLS (N = 28)	AGE (YEARS)	45.2 ± 12.3
	SEX: FEMALE (PERCENT)	46
	PSMD (X 10(4 MM2/SEC)	2.8 ± 0.3

EDSS= Expanded Disability Status Scale, **LV**= lesion volume,

PSMD = peak width of skeletonized mean diffusivity

4.2.2. MRI data acquisition

All study subjects were examined using an identical MR protocol. MRI examinations were acquired on a 1.5 T clinical scanner (Philips Medical Systems, Best, The Netherlands). The MRI protocol included the following sequences, acquired in the axial plane parallel to the bicommissural line: dual-echo, turbo spin-echo sequence (TR/TE1/ TE2=2075/30/90 ms, voxel size=1×1×3 mm) yielding proton density (PD) and T2-weighted (T2-W) images; fluid attenuated inversion recovery (FLAIR) (TR=9000 ms, TE=150 ms, inversion recovery delay=2725 ms, voxel size=1×1×3 mm); DTI data consisted of echo-planar imaging (TR=8500 ms; TE=100 ms; voxel size=2.5 mm3), with diffusion weighting distributed along 32 directions and b-value=1000 sec/mm2).

4.2.3. MRI data analysis

T2-lesion volume (LV) was computed by a single observer using a semiautomated user-supervised segmentation technique based on local thresholding (Jim 7.0, Xinapse System, http://www.xinapse.com/Manual/, Leicester, UK). Lesion borders were determined on PD images but information from T2-W and FLAIR images was also considered to increase confidence in lesion detection. LV was computed by multiplying lesion area by slice thickness.

Analysis of DTI data was performed with tools of FSL (FMRIB Software Library, www.fmrib.ox.ac.uk/fsl/, Oxford, UK). First, raw DTI data were corrected for eddy

currents and head motion with the eddy_ correct tool, then were brain-extracted using BET (Brain Extraction Tool) and finally entered into the program DTIFit which, by fitting a diffusion tensor model at each voxel, created DTI images, including mean diffusivity (MD) and fractional anisotropy (FA).

PSMD was computed across the brain in a fully automated fashion (i.e., without user intervention) via a simple shell script (freely available at www.psmd-marker.com) containing all the processing steps, from DTI data through "skeletonization" of WM tracts with TBSS (Tract- Based Spatial Statistics) (Smith et al., 2006) and diffusion histogram analysis, as previously described (Baykara et al., 2016). Briefly, FA images, nonlinearly aligned with FNIRT (FMRIB Nonlinear Image Registration Tool) into a common space (standard space FMRIB 1mm FA template) were first projected onto the WM skeleton, which was derived from the standard-space template. Then, MD images were projected onto the same skeleton, using the FA-derived projection parameters. Finally, PSMD was calculated as the difference between the 95^{th} and 5^{th} percentiles of the voxel-based MD values within the WM skeleton.

4.2.4. Statistical analysis

Group heterogeneity for age and sex were assessed, respectively, with one-way analysis of variance (ANOVA) and Chi-square. Group differences in PSMD across the brain were measured with ANOVA corrected for age and sex and then, in order to assess the contribution of WM lesions, also for LV (log-transformed to obtain normal distribution), including Bonferroni-corrected pairwise comparisons. A T-test was used to compare the log-transformed LV between MS and CADASIL.

In each patient group, correlation of PSMD with LV was tested with Spearman correlation coefficient. Significance was set at $p<0.05$ for all statistical analyses, which were performed with SPSS (www.ibm.com).

4.3. Results

No group heterogeneity was found for age and sex (Table 1). As expected, MS had lower LV than CADASIL patients (8.6 ± 8.2 vs 24.4 ± 17.4 cm^3, $p<0.001$). After correction for LV, PSMD values in MS were higher than in CADASIL patients (adjusted mean values: 4.5 vs 3.9×10−4 mm^2/s,

$p=0.03$) (Fig. 1) and in both patient groups were higher than in NC (2.8 ± 0.3×10−4 mm2/s, $p<0.001$). In both patient groups, PSMD values correlated with LV ($r=0.8$, $p<0.001$ in MS; $r=0.6$, $p=0.002$ in CADASIL). In MS, PSMD correlated with disease duration ($r=0.4$, $p=0.011$) and with all BRB tests (r values between 0.4 and 0.5, $p<0.01$, with the closest correlation for SDMT ($r=0.6$, $p<0.001$), but not with EDSS ($r=0.2$, $p=0.2$). Data are summarised in Table 2.

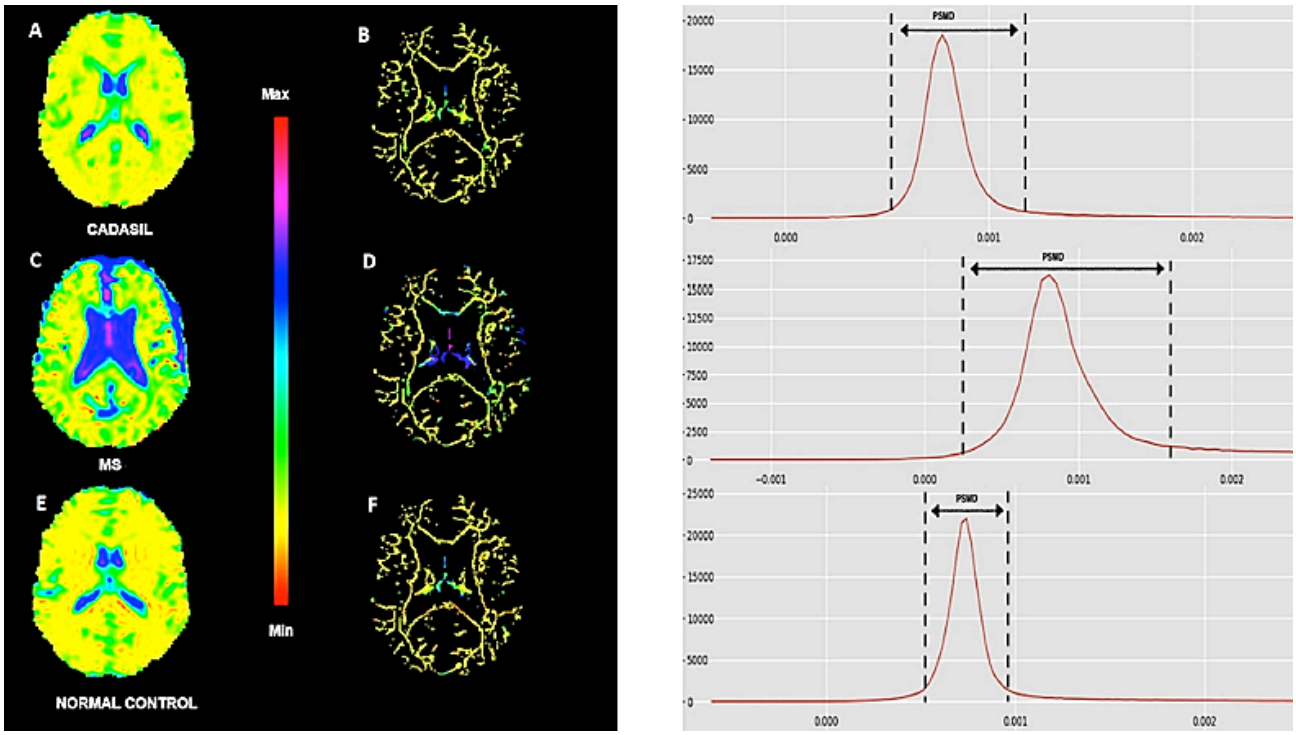


Fig 1: On the left, illustrative examples of MD maps projected onto the standard WM skeleton from a CADASIL patient (A-B), MS patient (C-D) and normal control (E-F). On the right, the corresponding histogram analysis of the MD is shown, PSMD is calculated as the differences between the 95th and 5th percentile. See text for abbreviations.

PSMD correlations in MS	Rho	p-value
T2LV	0.8	< 0.001
EDSS	0.2	0.2
Disease duration	0.4	0.011
SRT LTS	0.4	< 0.001
SRT CLTR	0.5	< 0.001
SRT recall	0.5	< 0.001
SPART	0.4	0.003
SPART recall	0.4	0.003
SDMT	0.6	< 0.001
PASAT 3	0.3	0.013
WLG	0.5	0.001
PSMD correlations in CADASIL		
T2LV	0.6	0.002
RANKIN SCALE	0.4	0.06

Table 2: Spearman correlations of PSMD with LV and clinical variables in the two patients groups. **T2LV=** T2 lesion volume, **EDSS =** Expanded Disability Status Scale, **SRT**= Selective Reminding Test, **SPART=** Spatial Recall Test, **PASAT**= Paced Auditory Serial Addition Test, **SDMT=** Symbol Digit Modalities Test, **WLG**= Word List Generation.

4.4. Discussion

In the present study, PSMD was evaluated in MS patients and its values were compared to those of NC and an inherited form of severe SVD such as CADASIL. In the only previous study published thus far (Baykara et al., 2016), PSMD showed significant increases, compared to NC, in acquired and genetic SVD (including CADASIL) and consistently outperformed conventional MRI markers (i.e, volumes of WM hyperintensities, lacune and brain), providing higher clinical relevance towards tissue damage (i.e, closer association with processing speed deficits). Indeed, in that study increased PSMD was found exclusively in SVD but not in neurodegenerative disorders (mild cognitive impairment and Alzheimer disease). Our results add to the previous study in showing that PSMD is significantly increased, compared to NC, also in a demyelinating disorder such as MS and therefore suggesting that this new, fully automated imaging marker of tissue damage is not exclusively linked to vascular pathology (Baykara et al., 2016). Our study showed increased PSMD values in MS with respect to CADASIL patients, despite the presence of higher WM LV in the latter group. This seems particularly interesting as the previous study clearly showed in SVD an increase of PSMD with higher WM LV (Baykara et al., 2016). Since PSMD is a measure of WM tissue damage that goes beyond visible WM lesions, our results seem to indicate the presence of a more pronounced WM microstructural damage in MS than in CADASIL patients.

Several studies have shown close association of abnormal DTI measures (i.e., increased MD/apparent diffusion coefficient [ADC] and/or decreased FA) with

myelin content and axonal count in the NAWM of MS brains, probably due to Wallerian degeneration, perivascular infiltration and microglia activation, with a clinical relevance for both physical disability and cognitive impairment (Ciccarelli et al., 2003; Inglese and Bester, 2010). Moreover, the increase of ADC in NAWM can predate the development of new lesions, highlighting the role of progressive widespread brain tissue abnormalities beyond the resolution of conventional MRI (Inglese and Bester, 2010). Similarly to MS, several studies have reported in CADASIL clinically relevant alterations of DTI measures in NAWM, probably due to chronic ischemia or Wallerian degeneration from small infarcts (Chabriat et al., 1999; O'Sullivan et al., 2005; Mascalchi et al., 2017). However, the presence in the NAWM of MS brain, with respect to non-immune-mediated conditions, of a more pronounced inflammation due to higher VCAM-1 (vascular cell adhesion molecule-1) expression, higher density of perivascular lymphocytes, focal perivascular inflammation and ongoing acute axonal damage (Vercellino et al., 2017) lends support to the hypothesis that the higher PSMD in MS with respect to CADASIL can be due to a more pronounced microstructural damage in the NAWM. In this pilot study, we explored the clinical relevance of PSMD values in MS. We found that while PSMD values did not correlate with EDSS, they showed a moderate-to-close correlation with all cognitive tests of the BRB and in particular with SDMT. This confirms in MS patients the great relevance for cognition of this new MRI biomarker, as found in the previous work for SVDs (Baykara et al., 2016). In MS, the pathogenic substrate of cognitive impairment seems to be closely related to GM damage (Amato

et al., 2007; Calabrese et al., 2009). However, macro- and microscopic WM damage plays a role in the cognitive impairment of MS patients, leading to altered anatomical connectivity among widely distributed brain regions with a "disconnection" that could be responsible, at least in part, of the cognitive deficits (Dineen et al., 2009). Results reported here lend further support to this hypothesis. PSMD has potentials for an extensive use in clinical MS studies.

Although more extensive studies are needed to confirm whether it can have a role in monitoring diffuse tissue damage in patients with MS, it has shown to provide robust and user-unbiased results. It exploits the advantages of the combined "skeletonization" of WM tracts using TBSS (Smith et al., 2006)) with diffusion histogram analysis (Baykara et al., 2016). Skeletonization process is able to reduce contamination of DTI data from cerebrospinal fluid and other non-brain structures by focusing on the central part of the WM tracts and, as such, PSMD may enhance the sensitivity towards the total brain tissue damage. Histogram analysis is able to detect subtle brain tissue abnormalities and is particularly suitable in presence of diffuse brain disorders such as MS. Indeed, it provides a summary measure from the whole WM in the form of a curve, which then can be easily characterized by a metric as simple as PSMD.

In conclusion, significantly high PSMD values do not seem to be limited to SVD (Baykara et al., 2016) but, in our population, turned out to be significantly more altered in MS than in an inherited form of severe SVD such as CADASIL with higher cerebral lesion load. These data suggest that PSMD is a useful marker in MS

potentially supporting conventional MRI in providing additional information on microstructural tissue damage. The assessment of PSMD is fast and fully automated and has previously demonstrated to be robust and accurate in multicenter datasets (Baykara et al., 2016). Therefore, this new promising MRI marker has proven to be potentially of great value in quantifying widespread microscopic tissue damage in a wide spectrum of WM conditions and can be applied retrospectively to existing DTI data sets. In spite of this, larger and above all longitudinal studies are warranted in order to confirm PSMD usefulness in terms of sensitivity to changes over time and response to treatment.

5. Peak width of skeletonized mean diffusivity (PSMD) and Cognitive Functions in RRMS.

5.1 Introduction

Cognitive impairment (CI) is estimated to affect 40-70% of MS patients (Chiaravalloti et al 2008). It tends to worsen over time but it might be relevant even at earliest stages (Amato MP et al. 2006) and it is primarily characterized by reduced information processing speed, visual learning, working and long-term memory and executive functioning.

These cognitive dysfunctions are often associated with depression and represent a considerable disease burden because of the negative impact on various functions, including maintaining employment, social and daily living activities (Benedict et al 2006).

The pathologic substrate of CI seems to be closely related with GM damage (cortical atrophy and cortical lesions) while this relationship is less certain with macroscopic WM damage (Amato et al 2007). Furthermore, MS lesions are associated with altered anatomical connectivity among widely distributed brain regions, and for this reason MS is deemed as “disconnection” syndrome (Dineen et al 2009).

Despite the exact mechanism of cognitive impairment in MS is not yet know, imaging studies have investigated the relationship between cognition and brain pathology, focusing their attention on the effects of lesions and atrophy, measured through conventional MRI.

More recently, advanced imaging techniques, in particular diffusion tensor imaging (DTI), have provided further insights into the understanding of CI in people with MS, highlighting the importance of the subtle damage in the normal-appearing brain tissue (NABT) which goes undetected by conventional imaging.

Recently, interest is growing for a novel fully automated, robust and fast DTI-derived imaging marker named as peak width of skeletonized mean diffusivity (PSMD), derived from a combination of "skeletonization" and histogram analysis of DTI mean diffusivity (MD), that provide a clinically crucial information on whole brain tissue damage in some WM disorders.

In cerebral small vessels diseases (SVD) higher PSMD values, resulted correlated with increased WM lesion load and reduced information processing speed (IPS) performance better than other structural brain MRI indices (Baykara et al 2016).

Based on these premises, we aimed here to investigate whether in a population of RRMS, PSMD is able to predict cognitive dysfunction, more than other imaging measures, taking into account the correlations between each of these MRI biomarkers and individual cognitive tests. In detail we tested whether PSMD had an incremental contribution in predicting cognitive variation in situation where structural brain variables were simultaneously included into the analysis. We also performed TBSS analysis, in order to explore which WM regions subtend cognitive performance in MS, after controlling for age, sex, T2LV, disease duration and disability.

5.2 Materials and Methods

We studied a total of 75 partecipants, of whom MS patients (n=60, age: 43.1±9.9 years, sex: 76.7 % female) with clinically definite MS and RR course according with the revised McDonald criteria (Polman et al 2011) who were age-matched with normal controls (NC, n= 15, age: 42 ±10 years, sex: females 46.7 %). Patients were recruited among those who were consecutively referring to the MS Clinics of the Universities of Siena and Florence during the study period. All the patients were relapse-free and were not taking steroids for at least one month prior to the assessment. No subject was taking psychoactive drugs or substances that might interfere with neuropsychological performance.

All patients underwent a neurological evaluation, which included assessment of the Expanded Disability Status Scale (EDSS) (Kurtzke JF et al 1983), neuropsychological evaluation and conventional MRI scans within one-week interval.

5.2.1 *Neuropsychological assessment*

In both centers, cognitive functioning was tested using the previously validated Italian translation of Rao Brief Repeatable Battery (BRB) (Rao et al 1990), using the normative data for the Italian population (Amato et al 2006), as previously reported (Vinciguerra et al 2019).

5.2.2 *MRI data acquisition and analysis*

Lesion volume (LV) was computed by a single observer using a semiautomated segmentation technique based on local thresholding (Jim 7.0, Xinapse System, Leicester, UK), as previously described.

DTI data were corrected for MRI eddy currents and head motion, then images were brain-extracted using Brain Extraction Tool (BET) and entered into the program DTIFIT which fits a diffusion tensor model at each voxel and creates DTI images, including mean diffusivity (MD) and fractional anisotropy (FA), Axial diffusivity AD), Radial diffusivity (RD).

Brain volumes (total brain, GM, cortical and deep GM, WM) were measured on high-resolution (1mm3) T1W images by using the new SIENAX 2.0 (Battaglini et al 2019). PSMD was computed across the brain in a fully automated fashion (i.e., without user intervention) via a simple shell script (freely available at www.psmd-marker.com) containing all the processing steps, from DTI data through "skeletonization" of WM tracts with TBSS (Tract- Based Spatial Statistics) (Smith et al., 2006) and diffusion histogram analysis, as previously described (Baykara et al., 2016, Vinciguerra et al 2019).

5.2.3 *Statistical Analysis*

Group heterogeneity for age and sex were assessed, respectively, with one-way analysis of variance (ANOVA) and Chi-square. Differences in PSMD and others MRI measures between MS patients and NC were tested with ANOVA corrected for

age, sex and T2 LV (log-trasformed to allow normal distribution) and a T-test was applied to compare PSMD and brain volumes measures between the two groups. In the MS group, associations between brain imaging parameters and cognitive test scores were conducted using a linear regression with p values adjusted for the false discovery rate (FDR) (Benjamini et al 1995). Voxel-wise statistical analysis employed is based on a non-parametric approach utilizing permutation test with a standard general linear model design matrix. The permutation testing was performed using the program Randomise, part of FSL. In order to detect the imaging biomarker with the highest relative importance in the independent associations with attention and memory tests, we included all measures into a multiple linear regression model, using a stepwise hierarchical regression including age, sex, EDSS, disease duration and all MRI parameters. Significance was set a $p<0.05$ for all the analyses, using SPSS (ww.ibm.com).

5.3 Results

Demographic, clinical and brain imaging descriptive results are shown in table 1.

As expected in MS group, all MRI measures significantly differed from those of the NC group ($p<0.001$). Correlations between MRI measures and each BRB test are shown in table 2. In detail, the strongest correlations were found between PSMD and SMDT ($R2= 0.54$, $p<0.001$) and between thalamic volumes and SRT-LTS, CLTR, D (R2 values respectively 0.32, 0.23 and 0.13 , $p<0.001$, $p= 0.004$) (fig 3 and fig 5) . TBSS analysis in the MS group showed significant correlation between lower SDMT

score and lower FA in WM clusters in the right anterior corona radiata, superior right and left longitudinal fasciculus (temporal part), right anterior thalamic radiation and significant correlation between lower SMDT and higher MD in a cluster in the body of corpus callosum.

Moreover, higher PSMD resulted significantly correlated with higher T2LV and T1LV ($R= 0.52$, $p<0.001$), lower WM and GM volumes ($r=-0.50$, $p<0.001$ for both), lower FA ($r=-0,6$, $p<0.001$), higher MD and RD ($r= 0.40$ $p<0.001$). see table 2.

PSMD contributed most to the multiple regression model than the other MRI markers to determine IPS variability in SDMT performances as well as thalamus volumes for memory scores in SRT tests.

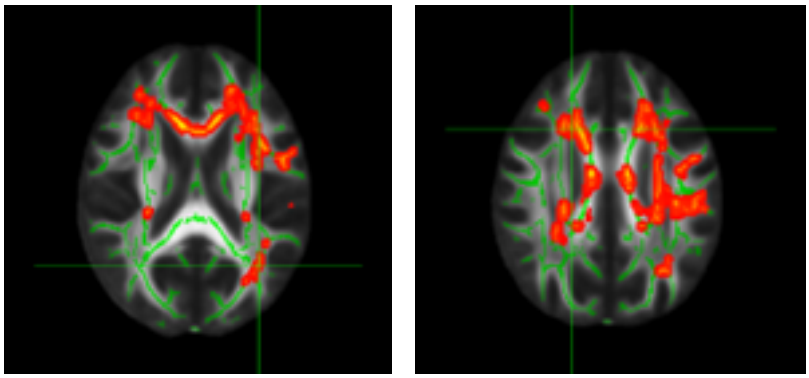
Further two separeted hierarchical stepwise regressions were performed, resulting in a firts final model that include only PSMD, using as indipendent variable SMDT, and a second one in which thalamus resulted retained when all SRT components (LTS, CLTR and SD) were tested as indipendent variables.

VARIABLE	MS (N= 60)	NC (N=15)	P VALUE
AGE (mean, standard deviation) years	43.1 ± 9,9	42 ± 10	ns
EDSS (median, range)	1.5 (1-3)	n.a.	
FEMALE N %	46 (76.7 %)	7 (46.7 %)	
DISEASE DURATION	9.53 ± 7,43	n.a.	
COGNITIVE IMPAIRMENT (number percentage)	22 (36.7 %)	n.a.	
T2LV cc	5 ± 4.6	n.a.	
T1LV cc	2,6 ±2,7	n.a.	
Vscaling	1.44 ±0.11	1.40 ±0,10	ns
NBV cc	1465 ± 40.8	1491.2 ± 27	0.002
NGMV cc	1465 ± 40.8	835,3 ± 25.4	0.04
p-NGMV cc	599.4 ±33.5	615.6 ±17,3	ns
NWMV cc	652,3 ± 23	655,9 ± 18,7	ns
CSF cc	547.7 ± 38.5	524.8 ± 26.5 cc	0.035
v-CSF cc	46.6 ±21.5	30,2 ±9	0.001
ACCUMBENS cc	1.14 ± 0.3	1.41 ± 0.2	0.001
AMYGDALA cc	3 ± 0,54	3,3 ± 0,4	ns
CAUDATE cc	8.9 ± 1.2	9.6 ± 1.4	ns
HIPPOCAMPUS cc	10 ± 1.3	11.1± 0.6	0.005
PALLIDUS cc	4.6 ± 0.56	5 ± 0.4	0.004
PUTAMEN cc	12,3 ± 1.7	13.5 ± 1.4	0.02
THALAMUS cc	19.3 ±2.1	21.7 ± 1	<0.001
FRACTIONAL ANISOTROPY	0.4840407±0.0312	0.210234±0.0153	<0.001
MEAN DIFFUSIVITY	0.00080637±0.0000048784	0,00000410283±0,00000212928	<0.001
PSMD 10-4 mm2/s	4,2 ± 1,3 10-4	2,9 ± 0,6	<0.001

Table 1: Demographic, clinical and MRI data of study population.
T2LV= T2-lesion volume; **T1LV**= T1-lesion volume; **NBV** = normalized brain volume; **NGMV** = normalized gray matter volume; **NWMV**= normalized white matter volume; **p-NGM** = partial normalized gray matter; **CSF** = cerebrospinal fluid; **v-CSF** = ventricular cerebrospinal fluid; **PSMD** = peak width of skelethonized mean diffusivity.

MRI measures	srtLTS	srtCLTR	SPART	SDMT	PASAT3	srtD	spartD	WLG
T2LV	p=0.001	p=0.002	ns	p=0.001	ns	ns	ns	ns
T1LV	p=0.003	p=0.005	ns	p=0.004	ns	ns	ns	ns
NBV	p=0.03	p=0.03	ns	p=0.003	ns	ns	ns	Ns
NGM	ns	ns	p=0.03	ns	ns	p=0.03	ns	Ns
p-NGM	ns	ns	ns	ns	ns	ns	ns	Ns
NWM	ns	ns	ns	ns	ns	ns	ns	Ns
CSF	ns	ns	ns	ns	ns	ns	ns	Ns
v-CSF	p=0.02	p=0.014	ns	ns	ns	ns	ns	Ns
ACCUMBENS	ns	ns	ns	ns	ns	ns	ns	Ns
AMYGDALA	ns	ns	ns	ns	ns	ns	ns	Ns
CAUDATE	ns	ns	ns	ns	ns	ns ns	ns	Ns
HIPPOCAMPUS	p=0.01	p=0.02	ns	ns	ns	ns	ns	ns
PALLIDUS	0.039	ns	ns	ns	ns	ns	ns	ns
PUTAMEN	p<0.001	p<0.001	ns	ns	ns	p=0.010	ns	p=0.012
THALAMUS	**p<0.001**	**p<0.001**	ns	p=0.019	ns	**p<0.001**	ns	ns
FRACTIONAL ANISOTROPY	ns	ns	ns			ns	ns	ns
MEAN DIFFUSIVITY	ns	ns	ns	ns		ns	ns	ns
PSMD	p=0.02	p=0.016	p=0.02	**p <0.001**	ns	ns	ns	p=0.04

Table 2: correlations between MRI measures and Rao-Battery Tests.
P values are adjusted according to Benjamini et al method. **T2LV** = T2-lesion volume; **T1LV** = T1-lesion volume; **NBV** = normalized brain volume; **NGMV** = normalized gray matter volume; **NWMV** = normalized white matter volume; **CSF** = cerebrospinal fluid; **v-CSF** = ventricular cerebrospinal fluid; PSMD= peak width of skelethonized mean diffusivity. Selective Reminding Test **(SRT),** the 10/36 Spatial Recall Test **(SPART),** the Symbol Digit Modalities Test **(SMDT),** the Paced Auditory Serial Addition Test **(PASAT),** delayed recall of the SRT, delayed recall of the **(SPART)** and Word List Generation **(WLG)**



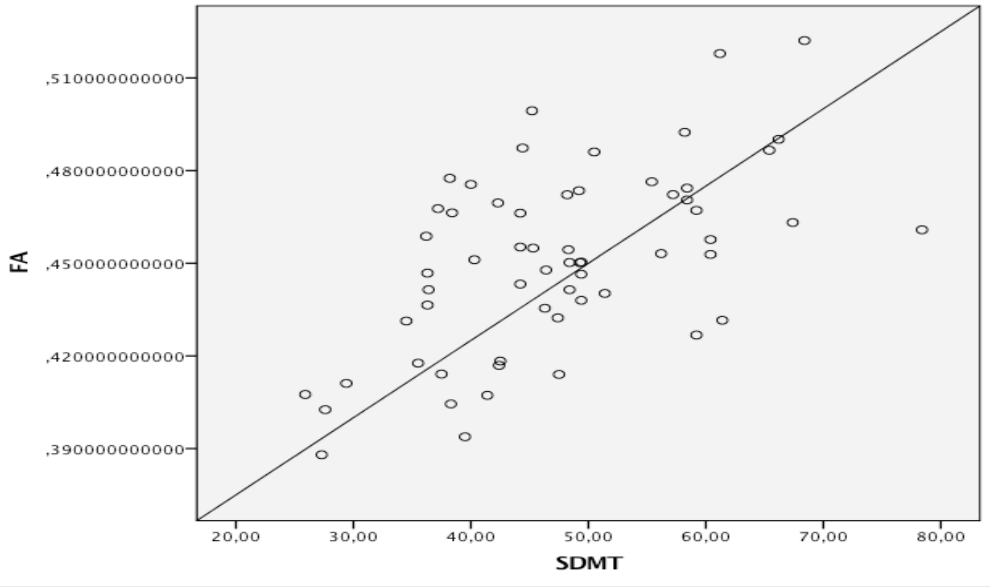
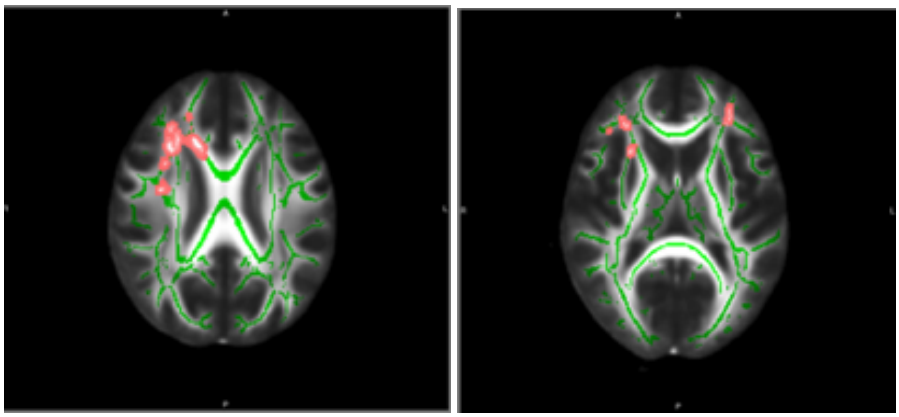


Figure 1: Correlations between Fractional Anisotropy **(FA)** in the White Matter clusters regions and symbol digit modalities test **(SDMT).**



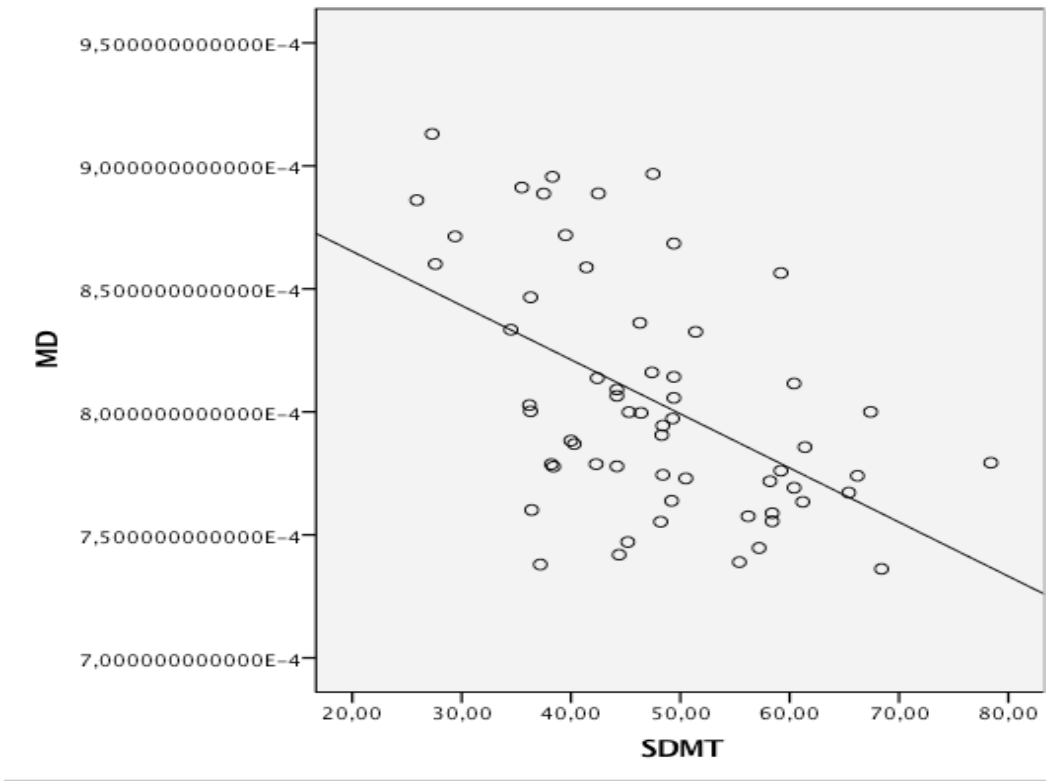


Figure 2: Correlations between mean diffusivity (MD) in the White Matter clusters regions and symbol digit modalities test (SDMT).

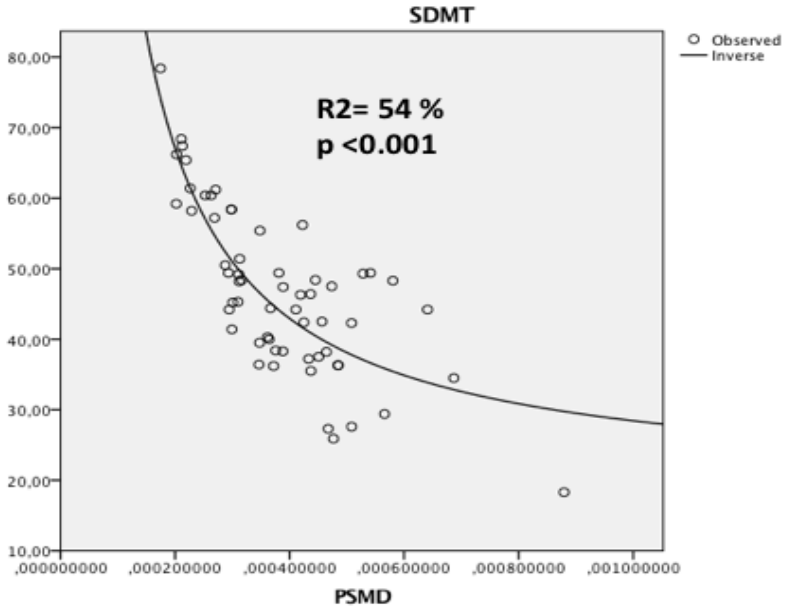


Fig 3 Simple linear regression between peak width of skeletonized mean diffusivity (PSMD) and information processing speed scores detected with symbol digit modalities test (SDMT) in the MS group.

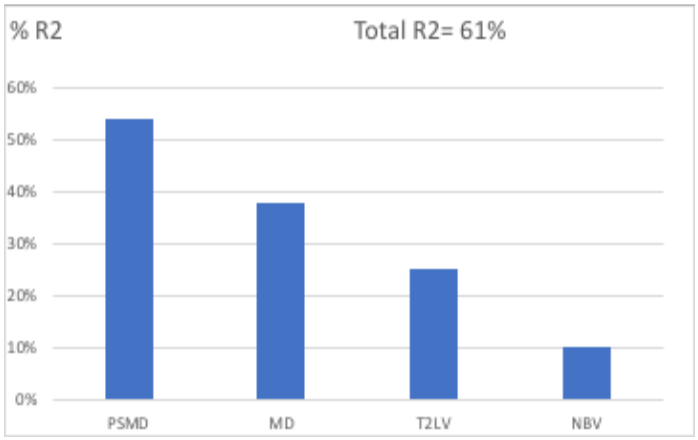
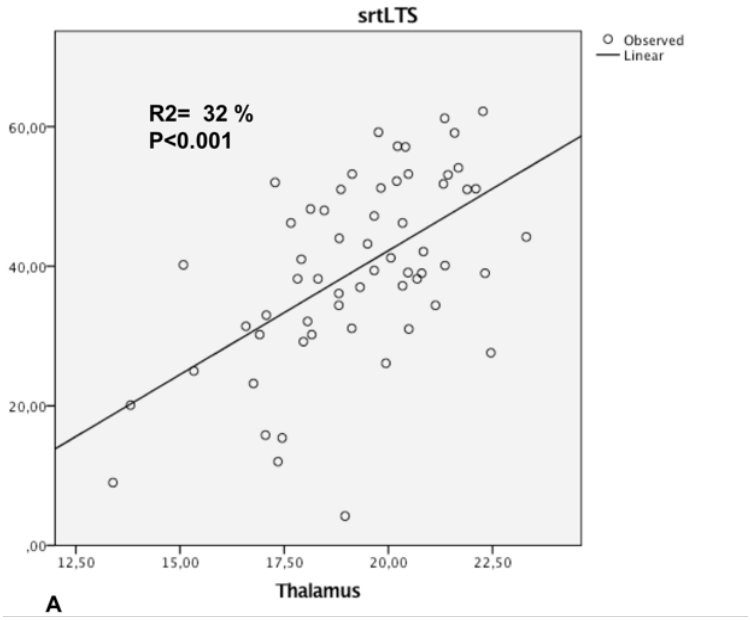
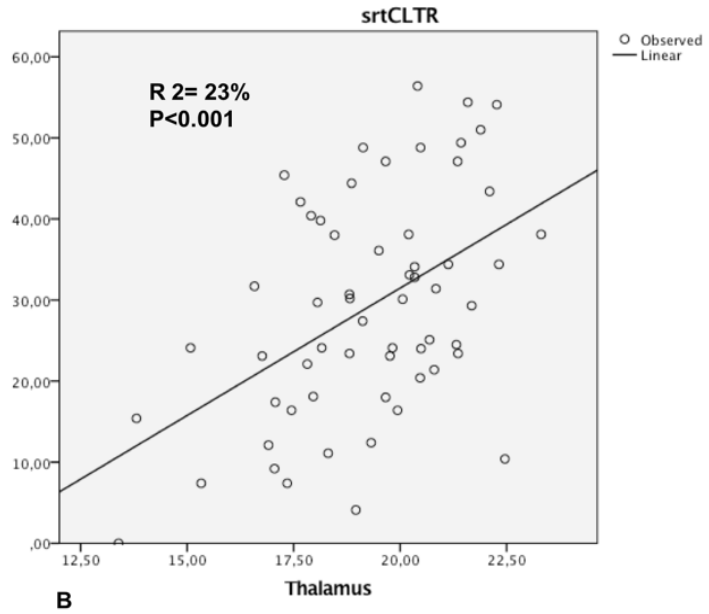


Figure 4 Bar chart depicts the contribution of each regressor (PSMD, Mean diffusivity [MD], T2 lesion Volume [T2LV] normalized brain volume [NBV]) to the multiple regression models in predicting information processing speed deficit. Note that in all cases PSMD contributes most to the models

PSMD	Information processing speed		
	beta	SE	P-value
	-0,736	3,193	<0.001

Table 3: Multivariate models showed that the best predictor of information processing speed deficit from all brain variables, entered simultaneously in the hierarchical regression, is the Peak width of skelethonized mean diffusivity (PSMD). SE: standard error.





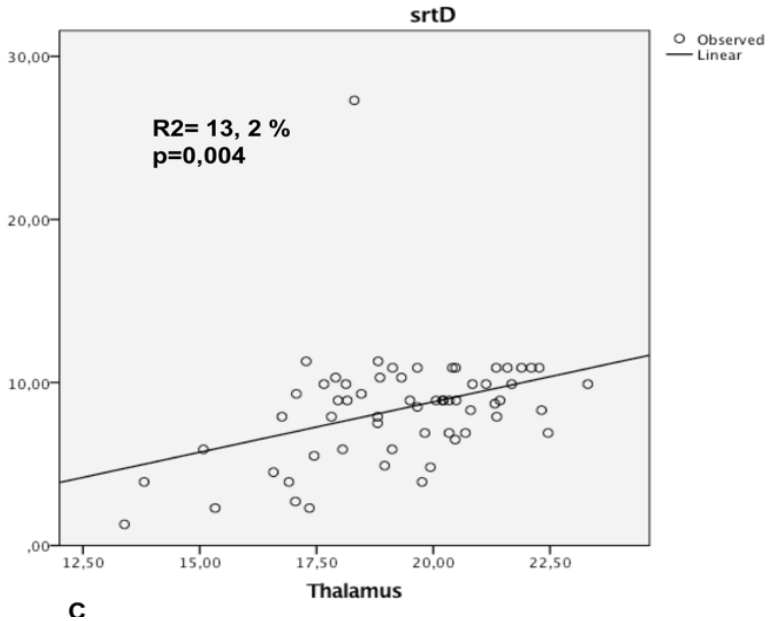


Fig 5 (A, B, C): Simple linear regression between peak width of skeletonized mean diffusivity (PSMD) and memory tests scores detected with selective reminding test long-term storage (SRT-LTS), consistent long term retrieval (CLTR) and recall (D) in the MS group (panel A, B, C, respectively).

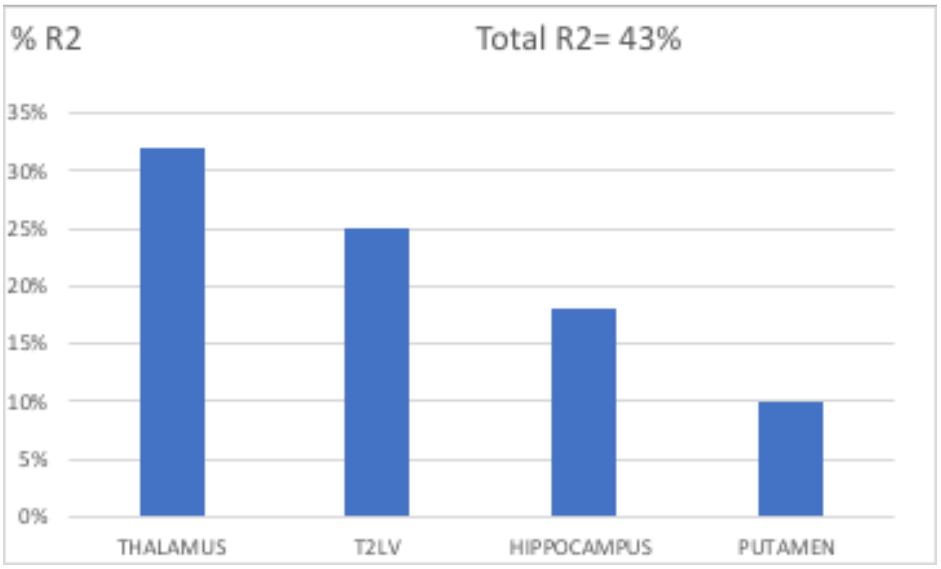


Fig 6 Bar chart depicts the contribution of each regressor (thalamic volume, T2-lesion volume [T2LV], hippocampal and putamen volumes) to the multiple regression models in predicting memory deficit. Note that in all cases thalamic volumes contributes most to the models

Thalamic volume	Verbal memory		
	beta	SE	P
SRT-LTS	0.563	0.684	<0.001
SRT-CLTR	0.480	0.755	0.001
SRT-D	0.363	0.208	0.004

Table 4: Multivariate models showed that the best predictor of memory deficit from all brain variables, entered simultaneously in the hierarchical regression, is the thalamic volume

5.4 Discussion and conclusions

In this study, we extended the previously reported association between PSMD and SDMT (Vinciguerra et al 2019), including a higher number of brain MRI measures in relation to a wider spectrum of cognitive domains in a group of RR-MS. We tested the possibility that PSMD showed significantly stronger associations with IPS than with other domains and whether they were significantly stronger or weaker associations compared to other brain MRI variables.

In our sample, as expected, most of MRI measures significantly correlated with BRB tests. In particular, the most relevant association was observed on the one hand between PSMD and SDMT ($r= -0.70$, $p <0.001$), on the other hand between thalamic volume and SRT tests ($r= 0.60$, $p <0.001$).

Higher PSMD values correlated significantly with poorer IPS while reduced thalamic volume resulted associated with lower scores in memory tests (SRT-LTS, CLTR, D). Moreover, TBSS analysis showed that lower performance in SDMT was associated with a reduction of FA and increase of MD respectively in different WM regions.

These data were strengthened by multiregression analysis that confirmed the strongest incremental contribution and indipendent association of PSMD and thalamic volume in predicting, respectively, dysfunction in IPS and memory functions in a group of RR-MS.

In one study, the increases in PSMD were linked to vascular but not to neurodegenerative disease such as Alzheimer diseases, showing a closer association

with deficit in IPS and capturing cerebral SVD better than the other imaging markers (Baykara et al 2016).

A recent work conducted on elderly brains, showed that in the latter IPS performances are worse than those of healthy young brains, where even PSMD values result lower (Deary IJ et al 2019). In this study, normal appearing gray and white matter volumes, and brain atrophy had similar associations to PSMD with IPS measures. In detail, PSMD was not strongly associated with IPS compared to other cognitive domains; indeed, it correlated to similar levels with the visuospatial ability and the general cognitive ability, less strongly with verbal memory and crystallized ability, contributing to the visuospatial and general congitive performances variability much more than other MRI markers.

In MS brain instead, PSMD resulted significantly increased, when compared to CADASIL and NC, suggesting that this novel MRI biomarker is not exclusively related to vascular tissue pathology (Vinciguerra et al, 2019). .

These data therefore reinforce the role of PSMD in detecting WM microstructural pathology, even in an inflammatory disease such as MS, where damage is probably more widespread and pronounced than in vascular forms.

Our work confirms the clinical relevance of PSMD in detecting IPS deficit better than other MRI markers also in a demyelinating disease as MS. However, this is not equally valid for the variability in memory performances, where instead the thalamic volume turns out to be the most involved measure. IPS deficits represent the most prevalent, primary and early cognitive dysfunction in MS, referring to the speed with

which one can process information in the brain. (Costa et al, 2016 e Meijer et al 2018).

Higher order association areas in prefrontal cortex and temporal and parietal lobes, as well as their subcortical connections, are considered to have an important role in this cognitive function.

MS patients take more time to complete tasks in cognitive testing, because they require greater neural recruitment. Given the multifocal white matter pathology that occurs in multiple sclerosis, it is conceivable that cognitive impairment in multiple sclerosis is, at least in part, caused by disconnection and that NAWM abnormality makes an important contribution to cognition (Dineen et al 2009), without excluding other mechanisms such as grey matter pathology (Amato et al., 2004; Morgen et al., 2006).

Compared to normal controls, MS patients presented with differences in shape and volumes of thalamus, associated with a decreased cognitive performance, especially information processing speed (Bergsland et al 2016).

Other authors claimed that neuropsychological measures had a strong correlation with MRI brain atrophy and that thalamic area resulted the most sensitive imaging marker for memory and psychomotor speech, predicting clinically cognitive decline in RR patients (Papathanasiou et al 2015; Pravatà et al 2017). Another study indicated the role of anterior thalamic nuclei in episodic memory performance of MS people (Kletnik et al. 2019).

Another recent study showed that left thalamic volume predict performance in verbal

memory, supporting the growing evidence of the involvement of thalamus in cognitive impairment in MS and its association with verbal memory deficits (Tremblay et al 2018).

Also basal ganglia, thalamus and neocortex are thought to have a key role for efficient information processing, nevertheless, the specific relative contribution of these structures for MS-related IPS impairment is to date poorly understood. Some authors confirm the significant role of thalamus and putamen atrophy in MS-related IPS slowing, pointing out the independent and significant role of these deep gray matters structures in cognitive impairment (Batista S et al 2012). Selective and parallel decreases of neocortical volumes with cognitive deteriorations with a good correlation between cortical volumes and cognitive measurements were reported in a longitudinal study performed in a population of early RRMS in terms of a strong correlation between brain volumes and verbal fluency performance. (Amato et al 2007; Bisecco et al 2015).

Our study once again confirms the role of MRI markers in explaining the variability of cognitive impairment in MS patients. We tested PSMD, as it promising in explaining the deficit in IPS, also in other white matter pathologies. Our results confirm the greater relevance of this marker in predicting worse SDMT performance, compared to other MRI markers, even in multiple sclerosis. The same is not true for verbal memory performances, which in our population, were better predicted by the thalamic volumes.

We believe that in RR-MS also microstructural brain pathology can play an important

role in affecting cognitive performance, expecially before brain pathology burden becomes macroscopically more evident. This could explain how MS is an inflammatory but at the same time neurodegenerative disease and how IPS deficiency is also largely related to the widespread microstructural damage rather than only to atrophy, especially in a mild and moderate stages of disease.

The present study confirms the PSMD clinical utility in detecting IPS dysfunctions performance in RRMS. Being free available and being able to be calculated retrospectively on DTI data, PSMD seems to have a very high potential for extensive use in clinical practice.

Furthermore, the histogram analysis, equipped with a simple metric, provides a summary measure of the entire brain WM damage, which is particularly useful in diffuse brain disorders such as MS. However, our result may be due to the small sample size and the limits of the lack of a follow-up period, therefore they would be worthy of further longitudinal studies, extended to a wide range of clinical and non-clinical populations, in order to clarify and confirm the role of this promising MRI marker in MS as well as in many others neurological diseases.

6. Cognitive reserve mediates the association between cognition and white matter microstructural damage in mild disability MS.

6.1 Introduction.

Cognitive impairment (CI) is very common in people with Multiple Sclerosis (MS). Nevertheless, not all patients present cognitive dysfunctions, despite having brain tissue damage. As in Alzheimer's disease and several other neurological disorders (traumatic, neoplastic, infective and vascular brain injury), also in MS brain atrophy does not fully explain variance in cognitive performance (Sandroff et al 2016). This concept was recognized by two different constructs of reserve (brain and cognitive), a kind of protection against clinical manifestations of neurological damage. Recently, several works suggest that individuals with larger brain reserve (BR) presumably have more neurons to lose before CI becomes evident. Noteworthy, BR is considered a fixed construct of brain capacity (i.e brain size/synapse count/extent of dendritic arborization) and is largely genetically determined. However, this model does not take an individual's premorbid or compensatory behavior into account (Stern et al 2002). Instead, theory of cognitive reserve (CR) posits that individuals who process cognitive demands more efficiently (perhaps due to higher educational /occupational attainment and/or participation in cognitively stimulating activities, intellectual enrichment) can withstand greater neural damage before CI becomes clinically relevant. This cognitive efficiency might involve using alternate cognitive strategies (i.e., recruitment of differential brain networks, compensation), to maximize

cognitive performance in response to neural damage (Sandroff et al 2016; Sumowski et al 2013.). We believe that the reserve can be built and maintained over time based on ongoing participation in cognitively stimulating activities and its maintenance (cerebral, cognitive) could play a protective role against disease progression, particularly at early stage of disease, allowing to cope better with damage and before brain tissue loss occurs. Previous studies mostly investigated the relationship of CR with brain atrophy and functional connectivity. We aimed to explore the relevance of CR on the association between CI and microstructural withe matter (WM) damage in MS.

6.2 Materials and Methods

We analyzed 56 of the 60 patients, already enrolled for the previous study reported in this thesis.

The local Ethics Committee approved the study and we obtained a written informed consent from all participants.

All patients underwent the same MRI examination, clinical and neuropsychological testing to assess cognitive status and cognitive reserve.

Disease duration was reported for each patient and expressed in years. Clinical disability was measured using the Expanded Disability Status Scale (EDSS) (Kurtzke et al 1983). Cognitive functioning was tested as previously reported (Amato et al 2006).

6.2.1 Cognitive reserve assessment

CRI was evaluated including educational level, premorbid intelligence quotient (IQ), and leisure activities (Stern et al 2009). Education level will be expressed as years of education. Premorbid IQ was estimated through the Italian version of the National Adult Reading Test (Colombo L et al 2002). Leisure activities were assessed using a previously published questionnaire that gathered information about participation not only in the premorbid stage, but also during the disease. The questionnaire included 7 items (producing arts, reading books, magazines or newspapers, writing, participation in hobbies, playing a music instrument or structured games) with frequency of participation during the year, month, week, day (Bukowski et al 2010; Varghese et al 2003, Scarmes et al 2003, Amato et al 2013, Verghese et al 2003, Scarmeas N et al 2003, Amato et al 2006). Thus, the 3 CR variables were combined as follows: scores on each variable were normalized trough a z-score transformation and the mean value of the 3 z-scores for each patient provided the global CRI (Amato et al 2010).

6.2.2 ***MRI data acquisition and analysis***

MRI data were acquired with an 8-channel head coil on a 3 Tesla MR scanner (Philips Medical Systems, Best, The Netherlands) located at Meyer University Hospital, Florence. A sagittal survey image was used to identify anterior and posterior commissures. Sequences were acquired in the axial plane parallel to the bicommissural line. A dual-echo, turbo spin- echo sequence (repetition time [TR]/echo time [TE]1/TE2 = 4000/10/100 ms, voxel size = 1×1×3 mm) yielded

proton density (PD) and T2-weighted (T2-W) images. DTI data were an echo-planar imaging sequence (TR = 7036 ms; TE = 196 ms; voxel size = 2.5 mm3) with 32 diffusion directions and b-value = 900 s/mm2.

Lesion Volume was computed by a single observer using a semiautomated segmentation technique based on local thresholding (Jim 7.0, Xinapse System, Leicester, UK).

After T1-hipointense lesion refilling (Chard et al 2010), normalized brain volume (NBV), GM volumes (GMV), WM volumes (WMV) were assessed on 3Td1-weighted images using the SIENAX 2.0 software (Battaglini et al 2019).

DTI data were pre-processed through the FSL program Eddy, which corrects for eddy current- induced distortions, subject movement and signal dropout (Andersson et al 2016). Then, we used FDT (FMRIB Diffusion Toolbox) (Behrens ET AL 2003), which allowed to obtain images of fractional anisotropy (FA), axial, radial and mean diffusivity (AD, RD, MD) by fitting a diffusion tensor model at each voxel.

BR was quantified based on intracranial volume (ICV) through the measurement of the SIENAX 2.0-derived scaling factor. ICV is considered an accurate measure of head size and a proxy of maximal lifetime brain growth (Sandroff et al 2016, Sumowski et al 2013). Larger values of scaling factor represent larger ICVs. Given that men have larger ICV than women we adjusted ICV measurements for sex.

6.2.3 *Statistical Analysis*

Group heterogeneity (between cases and NC) for age and sex were assessed, respectively, with one-way analysis of variance (ANOVA) and Chi-square. A T-test was used to compare brain atrophy volumes (NBV, NGM, NWM , ICV) between MS and NC.

Partial correlations, adjusted for age, sex, disease duration and EDSS, were calculated between CRI and cognitive tests as well as MRI measures (T2LV, NBV, GMV, WMV, FA, MD, RD, AD) using Pearson's coefficient. T2LV was transformed on log-scale before calculation. Then, hierarchical linear regression models were used to evaluate the role of CRI in explaining fractions of cognitive test score variance. Models included in a within-block stepwise method, age, sex, disease duration, EDSS, MRI (block 1); CRI values (block 2). Each MRI variables were considered separately .

Finally a mediation analysis was used to explore the mechanism through which CRI, as mediator, influence the association between SDMT performance and WM microstructural brain damage.

This analysis was carried out by comparing the regression coefficient of the MD or FA with the same coefficient when the CRI was introduced and in particular was calculated as the difference between the regression coefficients of MD or FA without and with CRI divided by the coefficient without CRI into the model. In this way we calculated the percentage reduction of the association between MD (FA) and SDMT when CRI was took in account.

Significance level was set at $p<0.05$ for all statistical analyses, which were performed with SPSS, version 22.0.

Anatomical connectivity among WM tracts was assessed at group level, using a voxelwise Tracted-Based Spatial Statistics (TBSS) after using, at single subject level (cases and controls). All voxelwise statistical analyses on DTI data were performed in the general linear model (GLM) framework, using *randomise,* a nonparametric permutation test also part of FSL, including age, sex, duration disease, EDSS and ICV as covariates. Then, multiple regression models for regional WM assessment, were used to assess their correlations with CRI and cognitive functions. The level of significance was set at $p<0.05$.

6.3 Results.

We enrolled a group of 56 RRMS (76% females;, age: 43.1±10 years, MS duration: 6±4.5 years, median EDSS: 1 [range 1-2.5], Cognitively Impaired in 38%) and 15 normal controls (age 40.6±10.1, females 50 %).See table 1.

Cognitive tests showed that in the group of RR-MS patients worse symbol digit modalities test (SDMT) performance correlated with lower CRI ($r=0.38$, $p=0.005$), higher T2-lesion volume ($r=-0.26$, $p=0.061$), lower fractional anisotropy (FA) in clusters of R corona radiata, R anterior thalamic radiation , WM of planum temporale ($r=0.43$, $p<0.001$), higher mean diffusivity (MD) in clusters of callosal body and WM

of inferior frontal gyrus (r=-0.46, p<0.001) whereas no significant correlations were found for axial and radial diffusivity (**table 2**) .We didn't find significant correlations between CRI and others cognitive tests. As expected, all DTI parameters (FA, AD,RD,MD) were significantly altered in the MS group compared to the NC, (p <0.001).

In multivariate analyses (**table 3**), adjusted for age, sex, EDSS and disease duration as the partial correlations, logT2 (p=0.65) not resulted more significantly associated with SDMT and wasn't considered into the model.

After the introduction of CRI into the multivariable model the association of both MD and FA with SDMT was consistently reduced.

The mediation analysis, in fact, showed that CRI mediated 30.4% of the MD effect and 41.4% of the FA effect on SDMT.

6.4 Discussion

The present cross-sectional study explored the relationships of CR with cognitive functions and MRI measures in a group of RRMS patients. In particular, we aimed to assess for the first time the relevance of CR in the association between cognition and WM microstructure in mild disability MS.

Widespread inflammatory changes in the normal-appearing white matter (NAWM), distant from focal demyelinating lesions, are a distinctive feature of MS (Kutzelnigg et al., 2005; Filippi et al, 2005; Zeis et al., 2008; Frischer et al., 2009)

DTI provides quantitative informations about the structural integrity of the WM

(Grafton et al 1991, Pierpaoli et al 1996). This technique is based on the principle of diffusion of water molecules and provides two parameters: FA that reflects the directionality of diffusion and MD that measures the degree of bulk diffusivity. FA decreases and MD increases are typical indications of impaired structural integrity (Sen et al 2005). However, the relative importance of each of these MRI parameters in explaining the variance of CI in patients with MS, is still not very clear. To date, it is not yet well known which conventional MRI parameters better explain most of the variance in cognitive function and to what extent the microstructural integrity of NAWM contributes to cognitive deterioration in MS.

Certainly, TBSS analysis reveals functionally relevant WM tract injury underlying CI in MS (Dineen et al 2009), reflecting Wallerian degeneration secondary to spatially remote lesions in connected WM tracts (Lin et al 2007), and supporting the definition of MS as a "disconnection" syndrome.

Some studies confirmed that CR is able to moderate the negative effect of brain atrophy on cognition and that a larger BR protect against CI.

Amato et al, argued that higher CR may be a relevant mediator between cognition and brain atrophy, but its protective role decreased with increasing levels of brain tissue loss (Amato et al 2010).

In a recent work (Rocca et al 2018) authors found that CR moderates the effect of thalamic atrophy on cognitive performance, in particular for memory deficit rather than cognitive efficiency (usually measured with SDMT), without any effect on longitudinal cognitive changes. These results were in line with previous one that

demonstrated a significant thalamic atrophy in cognitively impaired MS patients, suggesting a role of CR in attenuating the effect or regional atrophy on cognition (Amato et al 2013; Sumowski et al 2009; Sumowski et al 2013). Another recent paper (Santangelo et al 2018) showed that reduced WM volumes contribute to poor performance on SDMT, supporting previous data about a role of WM atrophy on impaired IPS (Preziosa et al 2016; Sacco et al 2015; Sanfilipo et al 2006).

Conversely, Sumoswki et al (Sumowski et al 2009) found that the impact of brain atrophy on IPS was moderated by estimated premorbid intelligence and patients with more severe atrophy and grater CR, showed better cognitive performance. The same was not true for patients with minimal atrophy.

Benedict et al (2010) confirmed that grater CR as indexed by higher premorbid intelligence or more years of education, protects against the progressin of CI, moderating the decline in information processing speed, according to different degree of brain atrophy.

Our results add to the previous studies in showing that CR may also play a role in attenuate the impact of microscopic brain damage on CI in MS people, expecially in a stage of with mild disabibily and moderate macroscopic brain pathology.

This study contains some limits that should be mentioned: i) we did not include in the analysis the possible confounding effects of some behavioral symptoms (fatigue or depression) wich can negatively impact on cognitive performance, ii) small sample size and iii) lack of a follow-up period.

The main results of our study is that the mediation analysis enhanced the CRI ability

in moderating the association between IPS performance and microstructural WM integrity, reflected by MD and FA changes.

In most studies using the BRB battery, the test with the most frequent impaired outcome is the SDMT. Performance in this test, which is mainly related to IPS, has been shown to be impaired in 43-54% in RRMS since frome the early stage of disease (Deloire et al, 2005, Nocentini et al 2006; Benedict et al 2006), demonstrating to be a better test for the detection of CI in MS, while the other cognitive domain alterations, including memory, might be involved at a later time (Feinstein et al 1992, Achiron et al 2003).

In this scenario, NAWM abnormalities make an important contribution to cognitive dysfunction in MS, as previously claimed (Zizadinov et al 2001, Rovaris et al 2002 di Dineen). Therefore we speculate that in the earliest stage of disease, CR (premorbid leisure and current intellectual encrichment) may exercise a positive effect on global cognitive efficiency, protecting against the disease progression.

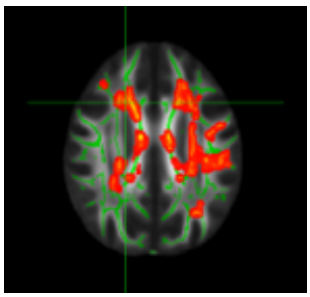
Therefore, our data could open new therapeutic scenarios, supporting the opportunity of a suitable time window for cognitive rehabilitation strategies, useful since the first phases of illness, where intellectual enrichment and leisure activities could play a crucial role.

In conclusion**,** measures of higher CR and good IPS performance, resulted associated with WM microstructural integrity. Higher CR may alleviate the impact of WM microstructural damage on CI even at relatively mild MS stage.

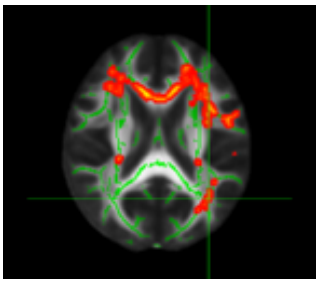
Table 1: Clinical, MRI and demographic data of study population.
EDSS: expanded disability status scale; LV (lesion volume).

GROUP	VARIABLES VALUES	
RELAPSING REMITTING MULTIPLE SCLEROSIS (N=56)	AGE (YEARS)	43.1±9.9
	SEX: FEMALE (PERCENT)	76.7
	EDSS	1. [1-2.5]
	COGNITIVE IMPAIRMENT (PERCENT)	38.3%
	LV (CM^3)	4.5 ±4.6
	COGNITIVE RESERVE INDEX (CRI)	0.2±0.7
	DURATION DISEASE (YEARS)	6±4.5
NORMAL CONTROLS (N=15)	AGE (YEARS)	41±10.1
	SEX: FEMALE (PERCENT)	46.7

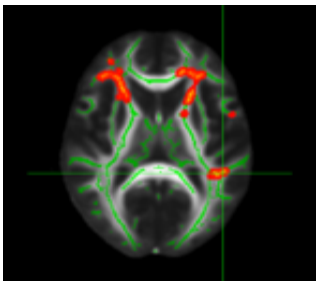
Figure 1: Statistical mapping of brain regional WM clusters significantly correlated with SMDT and corresponding scatterplots: in red-yellow FA clusters (callosal body, inferior longitudinal fascicle panels A, B, C respectively) , in blu-ligh MD clusters (callosasl body, inferior frontal gyrus, panels D, E respectively)



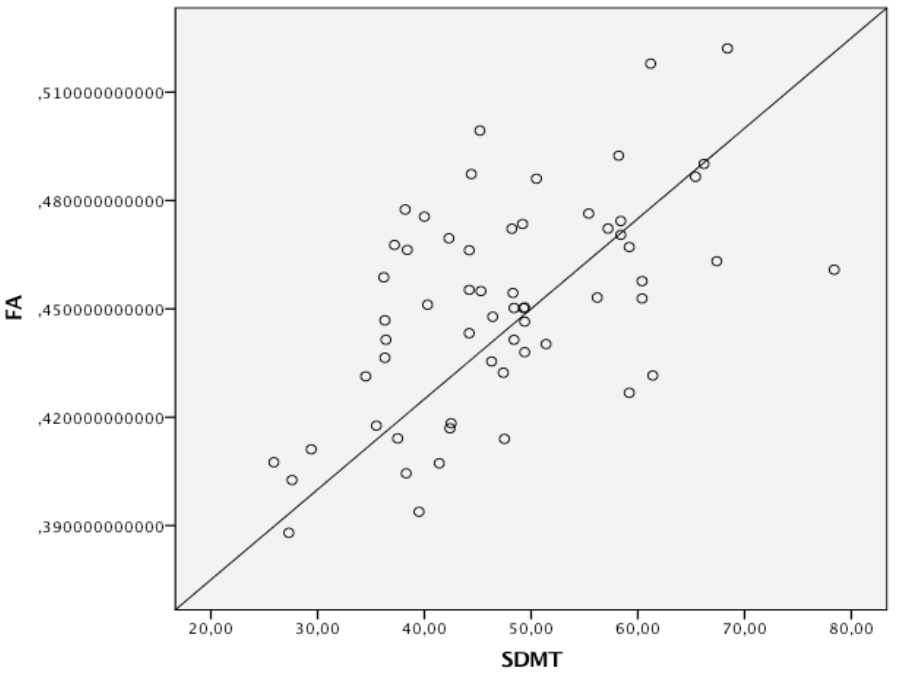
A

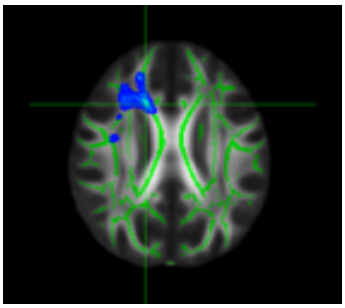


B

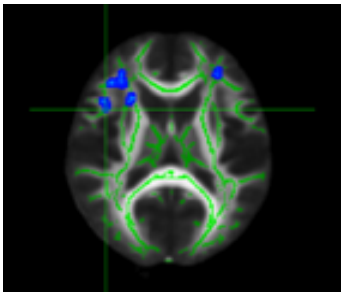


C

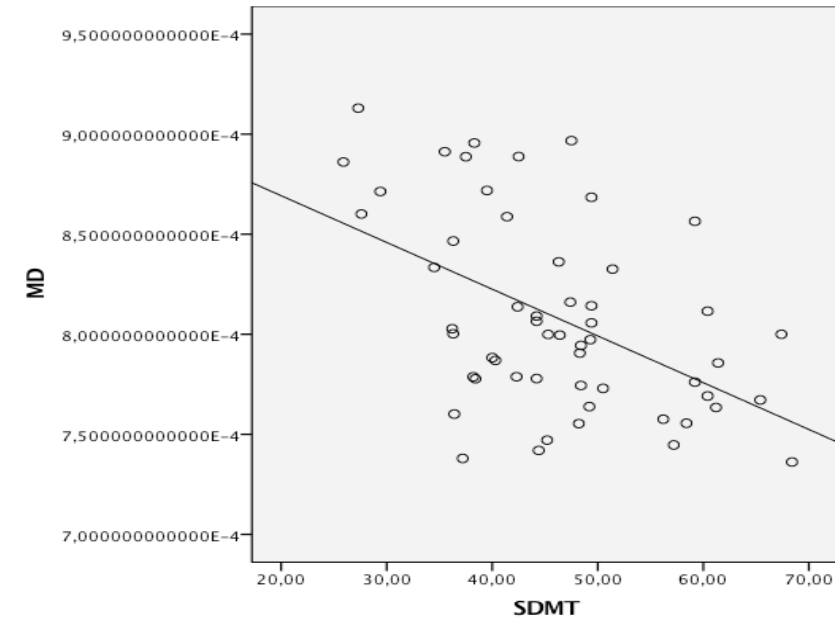




D



E


9,5000000000000E-4
9,0000000000000E-4
8,5000000000000E-4
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7,5000000000000E-4
7,0000000000000E-4
MD
20,00
30,00
40,00
50,00
60,00
70,00
SDMT

Table 2: Partial correlations between SDMT, cognitive reserve index (CRI) and microsctural WM regional clusters in the MS group. SDMT: symbol digit modalities test. MNI: Montreal Neurological Institute.FA: fractional anisotropy. MD: mean diffusivity.

Cognitive test	Variables	MNI coordinates X, Y, Z	B	p
SDMT	CRI	-	0.43	<0.001
	FA in callosal body	21,16,29	0.43	<0.001
	FA in inferior longitudinal fascicle	-34,-60,20	0.40	0.001
	FA in the WM of planum temporale	-44,-40,10	0.38	0.004
	MD in callosum body	17,7,27	-0.50	p<0.001
	MD in the WM of inferior frontal gyrus	42,10,14	0.37	0.003

Table 3: Partial correlations adjusted for age, gender, EDSS, disease duration. **T2LV**= T2 lesion volume; NBV = normalized brain volume; **GMV** = normalized gray matter; **WMV** = normalized white matter volume; **FA** = fractional anisotropy; **MD** = mean diffusivity; **RD** = radial diffusivity; **CRI** = cognitive reserve index ; **SDMT** = symbol digit modalities test.

Cognitive test	Variables	r	p-value
SDMT	T2LV	-0.26	0.061
	NBV	0.049	0.73
	GMV	0.20	0.16
	WMV	-0.18	0.21
	FA	0.35	0.011
	MD	-0.40	0.003
	RD	-0.24	0.083
	CRI	0.38	0.005

Table 4: *R-square change after introduction of CRI into the model; **: represent the relative change of MD or FA coefficient after the correction for CRI. All models are adjusted for Age, gender, EDSS, disease duration.

Cognitive test	Predictors	Coefficient B (95% CI)	p-value	Mediation of CRI (%)**
SDMT	**First block**			
	MD (*10000)	-11.5 (-18.9 to -4.1)	0.003	-
	Second block			
	MD (*10000)	-8.0 (-16.5 to 0.5)	0.065	30.4%
	CRI	4.0 (-1 to 9.0)	0.11	
	First block			
	FA (*100)	1.52 (0.37 to 2.66)	0.011	-
	Second block			
	FA (*100)	0.89 (-0.43 to 2.21)	0.18	41.4%
	CRI	4.6 (-0.54 to 9.68)	0.079	

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