Universitat Autònoma de Barcelona

Study of diagnostic and prognostic clinical, biological, and magnetic resonance imaging markers at the time of a clinically isolated syndrome

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Departamento de Medicina

Facultad de Medicina

Tesis Doctoral

Programa de doctorado en Medicina (RD: 1393/2007)

Study of diagnostic and prognostic clinical, biological, and magnetic resonance imaging markers at the time of a clinically isolated syndrome

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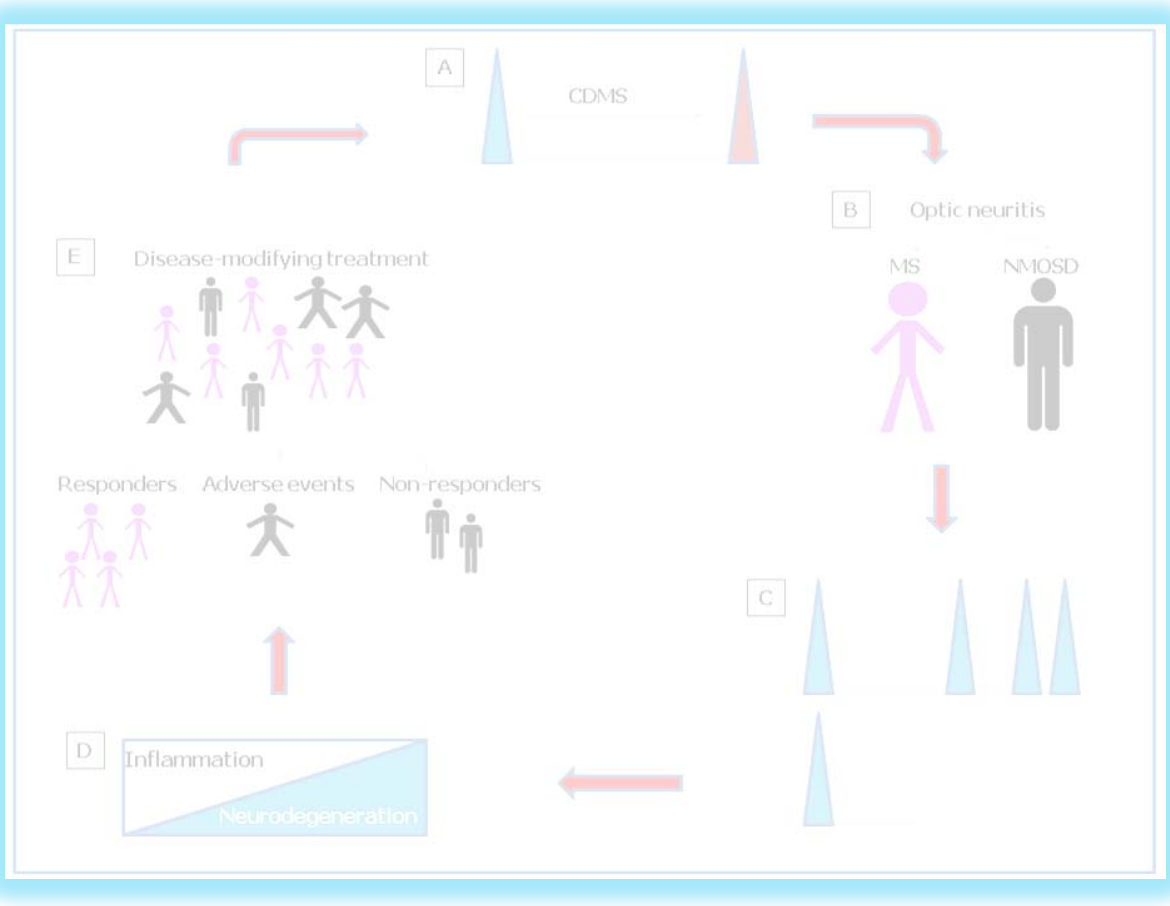
Barcelona, noviembre de 2015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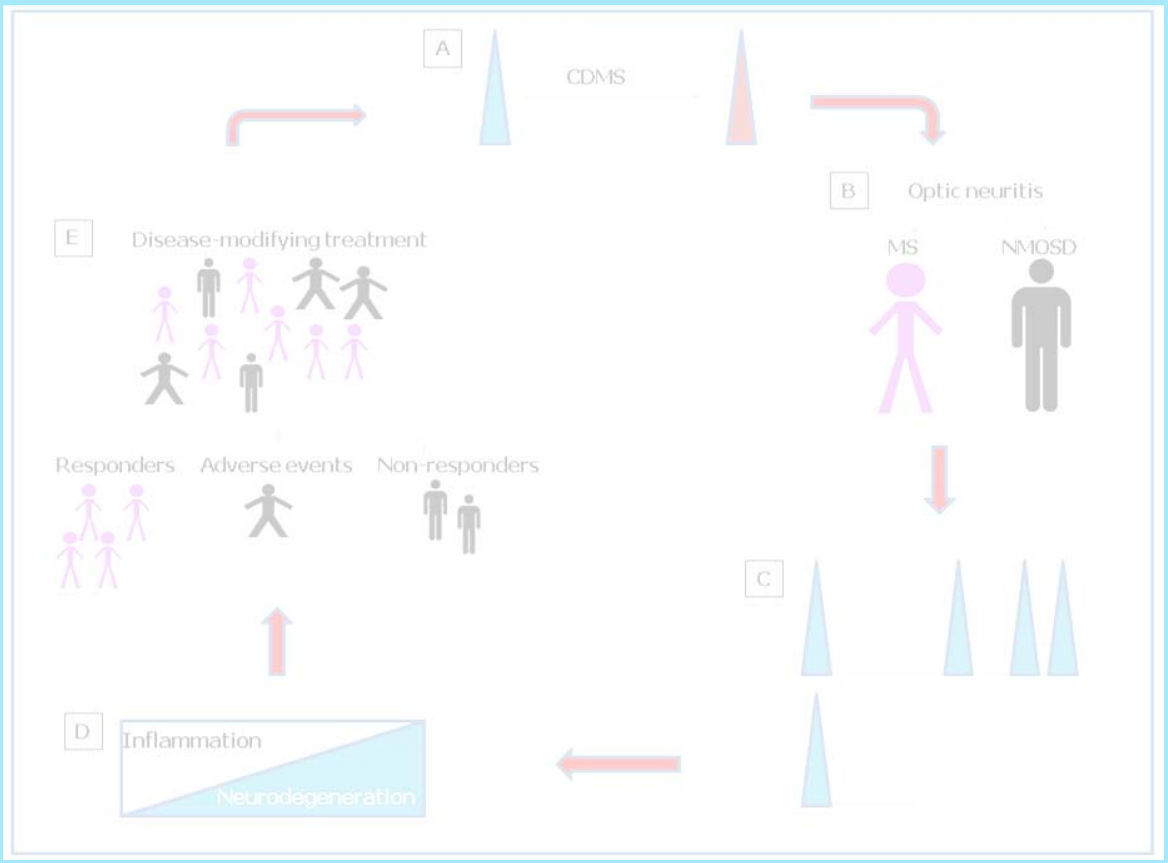


i. Acknowledgements

i. Acknowledgements

First of all, I would like to thank Xavier Montalban and Mar Tintore for considering me worthy of belonging on their team ever since we met in Mexico several years ago (time flies!). I hope the tequila and the micheladas did not play a (big) part in their decision. Similarly, I want to thank Carmen Espejo for her guidance on laboratory procedures and for her support during complicated times. To the three of them, many thanks for sharing their knowledge and experience. I also want to thank Prof. Vicenç Fonollosa for accepting to be my tutor, which shows his confidence on the team at the MS centre.

I thank all the Cemcat team for the great working atmosphere. More specifically, I'd like to thank Eva, Berta, Susana, Sergio, Elena, Juanfra, and Manel for their enormous support on updating and improving the CIS database: without them, the present work would not have been possible. I also want to thank Herena, Ester, Carme, Mireia, and Zoraida for showing me how to handle biological samples, and Ester again for "saving me" when a problem arose during one of the ELISA tests and for sharing the task of sample classification ("buenos", "malos") with me. I'd like to thank Xavier Vidal for all the statistical support, especially at the beginning, as well as Joaquín, Anka, and Nico for sharing extra-academic thoughts, dreams, and experiences, and Alex Rovira, Cristina Auger, and Deborah Pareto for their support regarding MRI studies.

I want to thank people who are no longer at the MS centre but with whom I shared work, some knowledge, and leisure: Carmen Tur, Filipe Palavra, Ana Wing, and Laura Negrotto. Of course, many thanks to the Fundació Esclerosi Múltiple and to all the patients: without them, none of the research carried out at the Multiple Sclerosis Centre would have been possible.

I want to thank my family. Special mention to my parents, who have been able to cope with having a restless daughter and for giving me all their support in any decision I have taken. Thank-you, mum, for the then insufferable discipline, for encouraging my reading and writing habits, for your insistence on me

learning English from a young age (that helped learn other languages with relative ease), and for encouraging me to be physically active through ballet and Spanish dance. Thanks, dad, for your patience and sense of humour, as well as for introducing me to music, film, photography, National Geographic, and México Desconocido. Thanks to my brother and sister for reminding me not to take myself too seriously, and also for being close to my parents (and pets) whenever I cannot be with them in person.

Another special mention to Colin for his support when going through difficult phases (in his own, pestering-like style) and all those dinners he cooked whenever I had to work from home, for compromising during our trips, and for planning a future together. With you and our friends in Barcelona I've "re-learned" to appreciate nature and be more adventurous. And now that I mentioned our friends in Barcelona, many thanks to the "Team Pepino": Vero, Sarah, Esther, Maria, and Silvia. Six more years and we'll arrive to Santiago de Compostela! And Vero and Berni, thanks a lot for sharing a good number of travel experiences. Good luck with your start-up!

I shouldn't forget my neurology training years, and therefore I'd like to thank many people from the Instituto Nacional de Neurología y Neurocirugía in Mexico City, starting with Dr. Teresa Corona. Thank-you to Pepe Flores, a good friend and MS colleague, and friends Juan Calleja and Mayela Rodríguez, as well as Prof. Enrique Otero (rip) and his "Dead Neurologists Society".

I also owe a lot to people I met during my Internal Medicine training: fellow residents César and Alex, and also Lily, Abel, and Ever, Drs. Martín Hernández, Manuel de la Maza, Luis Morales, Tere Sánchez, Ignacio Rangel, and Sergio Garza, among many others.

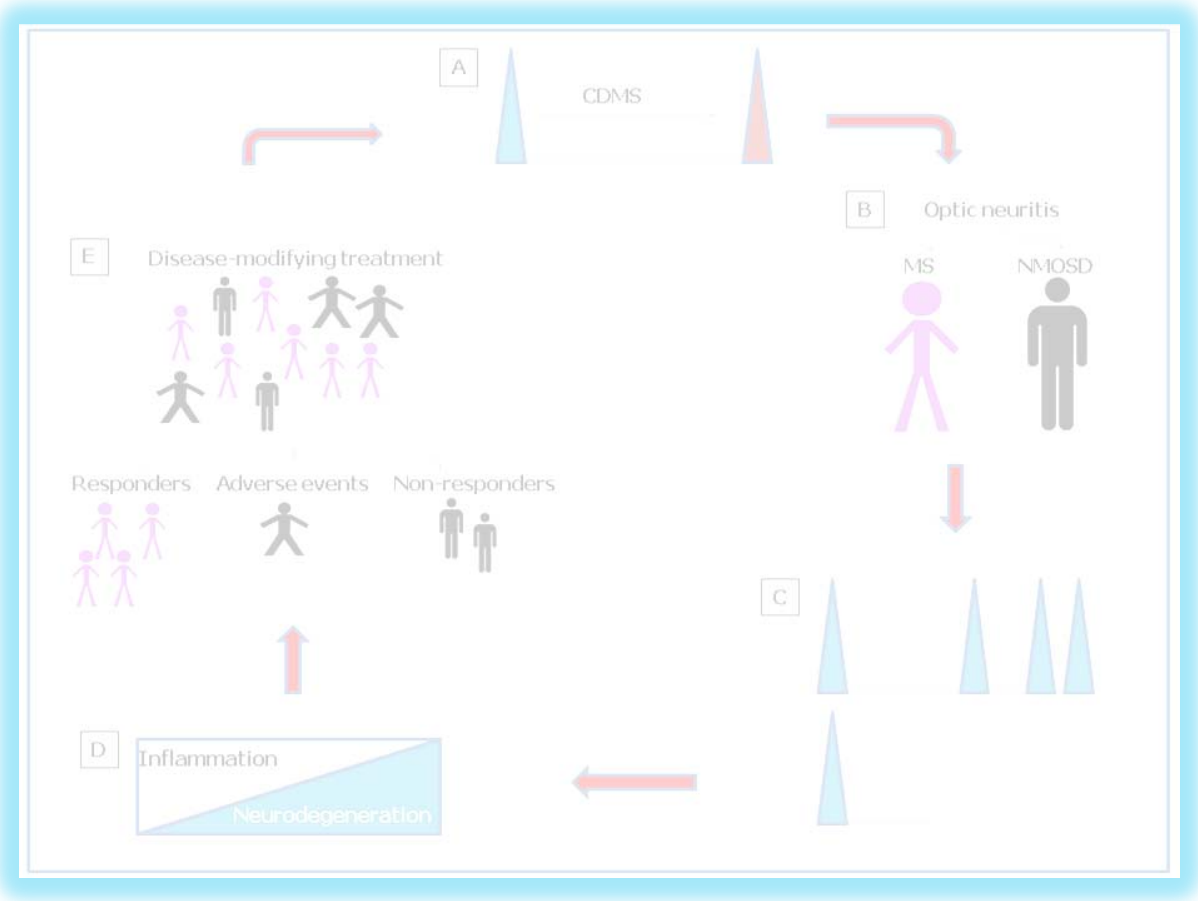
Many thanks to friends I met the first time I lived abroad, for showing me different lifestyles and that it's possible to adapt to life in a different country: Torbjörn, Tomas, Guigone, Elena, Stefano, and Karsten. We still have to organise a second post-Heidelberg reunion! Also thanks to Holger, the first

German classmate to talk to me (I hope everything goes well with your daughter), and to Mirjam, Chris, Dado, and Marcel.

I spent a lot of time with the "Hakunas and associates" in medical school, during class and night shifts but also during parties and weekend getaways. Thanks for being there, Bárbara, Tere, Teté, Eric, Arturo A., Arturo C., Charlie, Lalo, "Moe", and Julio.

Long-time friends from secondary school are still there and continue being supportive (thank-you, Whatsapp and Facebook): Silvia, Ivy, Rouss, Vivi, Nyx, Paco "Bolo", and Vero: it´s nice to see you every time I go back to Mexico. Finally, also thanks to friends who have been there for a longer time (about 35 years and counting!!!) in good and bad times: Claudia, Elsa, Eme, Euge, Mary, Kary, Lucy, Xóchitl, and Paty.

All these people have helped shape who I am and have supported me, in one way or another, through my years in Barcelona.



ii. Presentation

ii. Presentation

The work herein presented has been carried out at the laboratory and clinical premises of the Multiple Sclerosis Centre of Catalonia (Cemcat), Vall d'Hebron Institut de Recerca (VHIR), Vall d'Hebron University Hospital. This PhD thesis has been co-directed by Dr. Carmen Espejo, Dr. Mar Tintoré, and Prof. Xavier Montalban, the Cemcat director.

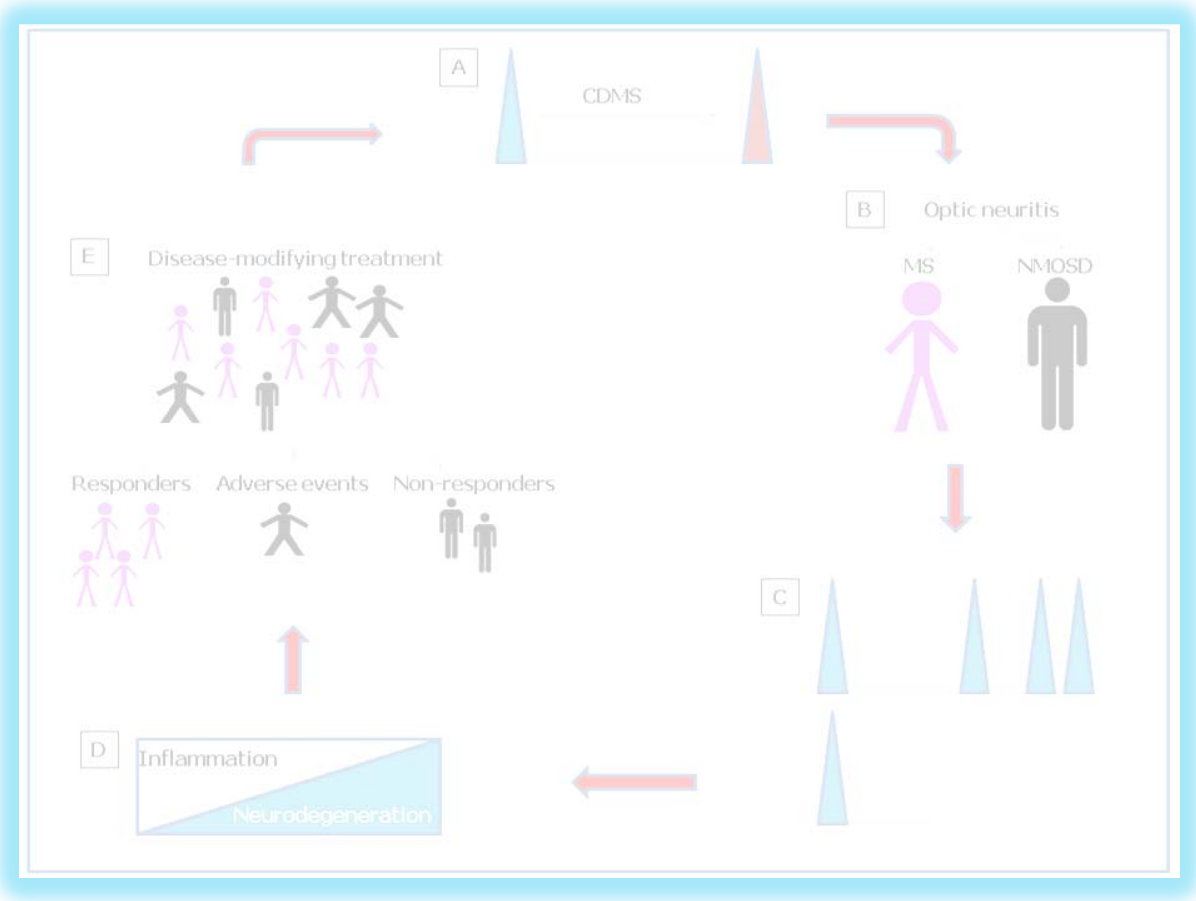
The Cemcat is committed to clinical activity, research, and teaching in the field of multiple sclerosis (MS). The centre is formed by an experienced multidisciplinary team comprised by clinicians (neurologists, neuropsychologists, physiotherapists, speech and occupational therapists, and nurses), basic researchers (neurologists, biologists, biotechnicians, a veterinarian, and a biochemist), technical support personnel (laboratory and informatics technicians), as well as research and teaching managers (an economist, accountants, and administrators). This arrangement provides patients with an integrated health care system, further increasing the quality of clinical care by means of regular clinical sessions and high quality clinical and basic research with translational potential, consequently turning the Cemcat into an international reference in MS.

Research at this centre is especially focused in broadening the knowledge of pathogenic mechanisms and new therapeutic approaches in MS. The present research project is concerned with finding clinical, biological or radiological diagnostic and prognostic markers at the time of a clinically isolated syndrome (CIS). Given that presenting a CIS indicates the possibility of developing MS, it is considered crucial to identify which patients will present a second attack and to determine the degree of disability accumulation over the medium to long-term.

The results of this project have been published in three peer-reviewed articles, one more manuscript has been submitted for consideration, and a fifth one is currently being drafted. Besides, a revision on prognostic markers has also been published [Arrambide G, Sastre-Garriga J. Predictive markers of disease

evolution after a CIS in everyday practice. J Neurol Sci. 2014;343(1-2):8-14], as well as one comment on neuromyelitis optica spectrum disorders [Arrambide G, Rovira A, Tur C, Montalban X. NMO spectrum disorders: how wide is the spectrum? Mult Scler. 2014;20(10):1417-9] and another on neurofilaments [Arrambide G, Espejo C, Tintore M. The only certain measure of the effectiveness of multiple sclerosis therapy is cerebrospinal neurofilament level-NO. Mult Scler. 2015;21(10):1240-2].

This project was supported by FIS PI08/0788 and FIS PI12/01313 from the Fondos de Investigación Sanitaria (FIS) of the Ministry of Economy and Competitiveness of Spain, and by McDonald, MAGNIMS, and ENS fellowships awarded to Georgina Arrambide, whose ongoing work involving the CIS database is currently supported financially by Novartis.



iii. Abbreviations

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ΔAUC	Area under the curve change
ADEM	Acute disseminated encephalomyelitis
aHR	Adjusted hazard ratio
AUC	Area under the curve
BBB	Blood-brain barrier
BENEFIT	Betaferon/Betaseron in Newly Emerging Multiple Sclerosis for Initial Treatment
BPF	Brain parenchymal fraction
BPFΔ	Brain parenchymal fraction percentage change
Breg	Regulatory B cells
BSA	Bovine serum albumin
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CARE-MS I	Comparison of Alemtuzumab and Rebif Efficacy in Multiple Sclerosis study 1
CARE-MS II	Comparison of Alemtuzumab and Rebif Efficacy in Multiple Sclerosis study 2
CD4	Cluster of differentiation 4
CDMS	Clinically definite multiple sclerosis
CHAMPS	Controlled High-Risk Subjects Avonex Multiple Sclerosis Prevention Study

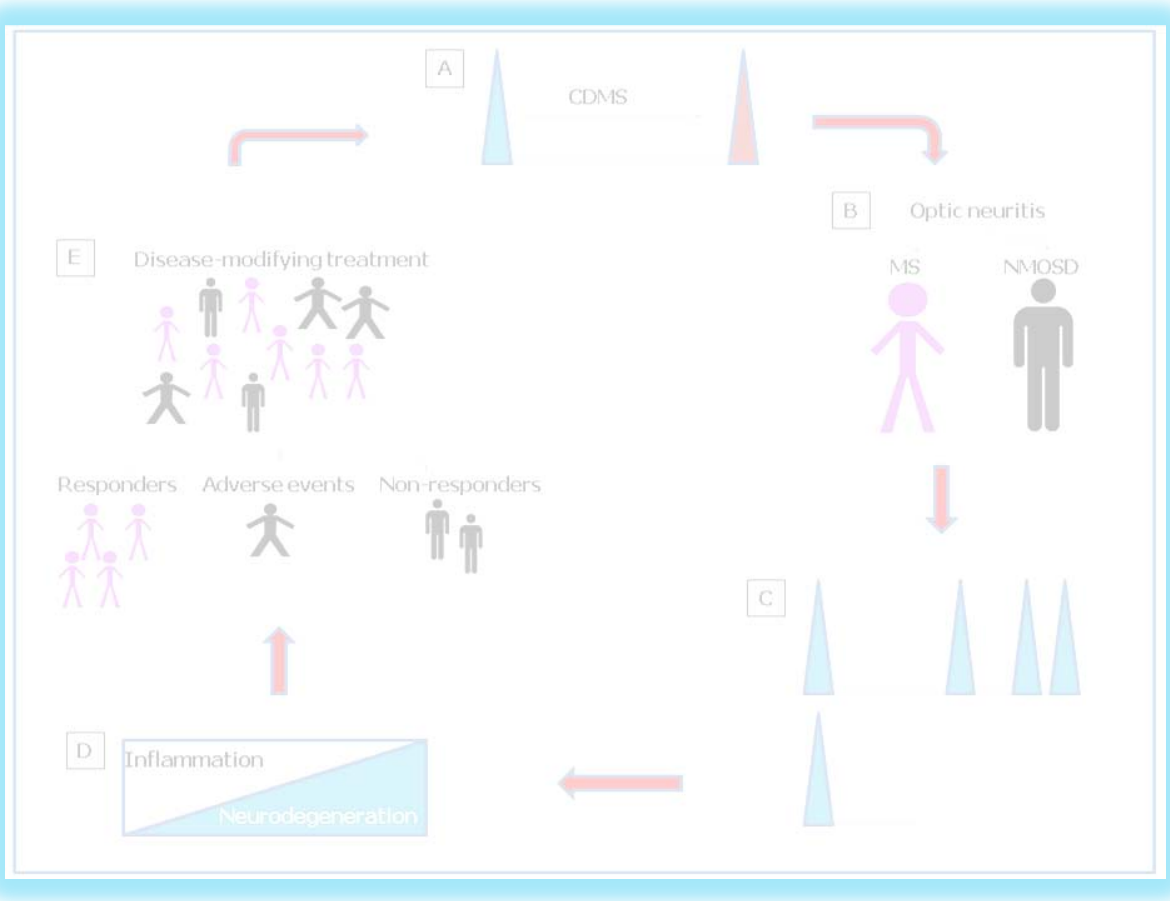
CHAMPIONS	Controlled High Risk Avonex Multiple Sclerosis Prevention Study In Ongoing Neurological Surveillance
CI	Confidence interval
CIS	Clinically isolated syndrome
CNS	Central nervous system
CONFIRM	Comparator and an Oral Fumarate in RRMS
CSF	Cerebrospinal fluid
CV	Coefficient of variation
DEFINE	Determination of the Efficacy and Safety of Oral Fumarate in RRMS
DIS	Dissemination in space
DIT	Dissemination in time
DMT	Disease-modifying treatment
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EBNA-2	Epstein Barr virus nuclear antigen 2
EBV	Epstein Barr virus
ECL	Enhanced chemiluminescence
EDSS	Expanded disability status scale
EDTA	Ethylenediaminetetraacetic disodium salt
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ETOMS	Early Treatment of Multiple Sclerosis trial

EU	ELISA units
FLAIR	Fluid-attenuated inversion recovery
FoxP3	Forkhead box P3
FITC	Fluorescein isothiocyanate
FSL	Oxford Centre for Functional MRI of the Brain (FMRIB) Software Library
Gd	Gadolinium
GFAP	Glial fibrillary acidic protein
Glc	Glucose
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HR	Hazard ratio
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IQR	Interquartile range
IL	Interleukin
IL2RA	Interleukin 2 receptor alpha
IL7RA	Interleukin 7 receptor alpha
IFN	Interferon
iNOS	Inducible nitric oxide synthase
IRF8	Interferon regulatory factor 8

LETM	Longitudinally extensive transverse myelitis
LINGO-1	Neurite outgrowth inhibitor receptor-interacting protein-1
MAGNIMS	MAGNetic resonance Imaging in Multiple Sclerosis group
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSSS	Multiple sclerosis severity score
NADPH	Nicotinamide adenine dinucleotide phosphate
NfH	Neurofilament heavy subunit
NfL	Neurofilament light subunit
NK	Natural killer cells
NMO	Neuromyelitis optica
NMOSD	Neuromyelitis optica spectrum disorders
Nogo	Neurite outgrowth inhibitor
NPV	Negative predictive value
OCB	Oligoclonal bands
OD	Optical density
PBS	Phosphate buffered saline
PLP	Proteolipid protein

PPV	Positive predictive value
PreCISe	Study to Evaluate the Effect of Early Glatiramer Acetate Treatment in Delaying the Conversion to CDMS in Subjects Presenting With a Clinically Isolated Syndrome
PBVC	Percentage brain volume change
REFLEX	REbif FLEXible dosing in early multiple sclerosis
RIS	Radiologically isolated syndrome
ROC	Receiver operating characteristic
RRMS	Relapsing remitting multiple sclerosis
SD	Standard deviation
Sema3A	Semaphorin 3A
Sema 3F	Semaphorin 3F
SIENA	Structural Image Evaluation, using Normalization, of Atrophy
SMI 35	Neurofilament heavy subunit, hypophosphorylated
SPMS	Secondary progressive multiple sclerosis
SPSS	Statistical Package for the Social Sciences
STIR	Short tau inversion recovery
SWI	Susceptibility weighted imaging
TBS	Tris buffered saline
TGF-β	Transforming growth factor β
Th	T helper cell
TNF-α	Tumour necrosis factor α

TOPIC	Oral teriflunomide for patients with a first clinical episode suggestive of multiple sclerosis
Treg	Regulatory T cell
VCAM-1	Vascular cell adhesion molecule 1
VDRE	Vitamin D response element
VLA-4	Very late activation antigen 4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I. ABSTRACT

I. Abstract

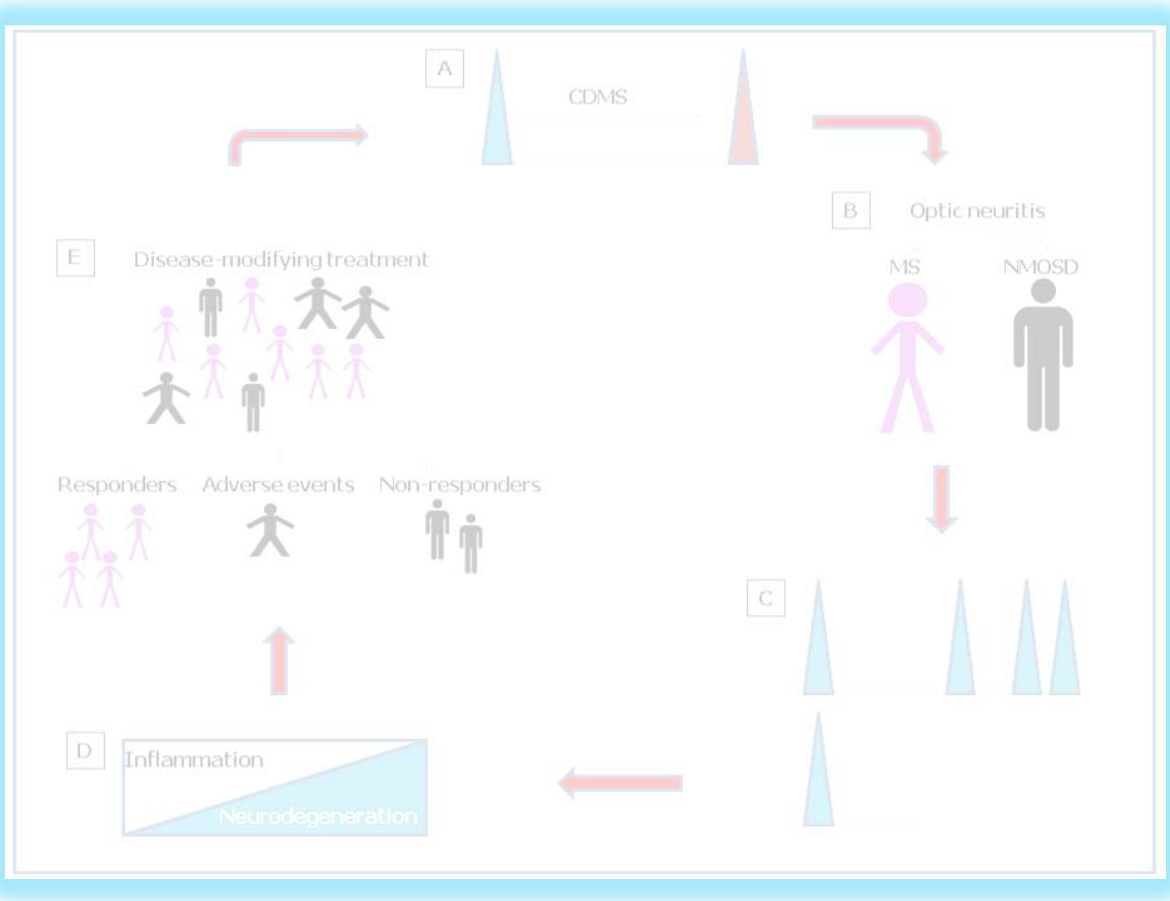
The present work is concerned with finding clinical, biological or radiological diagnostic and prognostic markers at the time of a clinically isolated syndrome (CIS). Given that presenting a CIS indicates the possibility of developing multiple sclerosis (MS), it is considered crucial to identify which patients will present a second attack and to determine the degree of disability accumulation over the medium to long-term. To date, brain magnetic resonance imaging (MRI) remains the most reliable diagnostic and prognostic marker, and oligoclonal bands also play a role in predicting conversion to MS. However, the disease heterogeneity hinders a more individualized prognosis. Therefore, the search for markers that capture the different aspects of this disease is still considered necessary, particularly if they demonstrate to be useful in the daily clinical practice. And so we aimed to determine the diagnostic and prognostic value of a number of clinical, biological, and radiological markers available at the time of the CIS. This was possible thanks to the large, ongoing inception CIS cohort with systematic and prospective data collection operated by the Cemcat.

With this objective in mind, several research proposals were considered.

First, recognizing neuromyelitis optica spectrum disorders (NMOSD) as one of the main differential diagnoses of MS after a first attack, we decided to assess the value of systematically determining the NMO-IgG status at the time of a CIS, observing that such approach is not necessary since the antibody determination was negative in most patients. Therefore, other clinical and radiological characteristics should also be taken into account during the differential diagnosis and this test could be considered in indeterminate cases.

Next, a collaborative work was established with Aurélie Ruet and Bruno Brochet, from the Groupe Hospitalier Pellegrin, Centre Hospitalo-Universitaire (CHU) de Bordeaux, INSERM-CHU centre d'Investigation Clinique, Université de Bordeaux, France, to further assess the added value of presenting ≥2 predictive factors for MS, previously identified by them, in patients not fulfilling the 2010

criteria for dissemination in space, and observed that although lower, patients with combinations of these predictive factors are still at risk of developing MS and should be monitored closely. Likewise, the usefulness of a baseline spinal cord MRI at the time of a CIS is still somewhat controversial. Therefore, we analysed its added diagnostic and prognostic value, observing that although the diagnostic value is modest, the presence of spinal cord lesions does pose an increased and independent risk for both evolution to MS and disability accumulation. One more international collaboration was established with the Israeli company Glycominds, Inc., when researchers Jennifer Yarden, Nir Dotan, and Avinoam Dukler contacted us to validate the predictive value of gMS-Classifier2, an algorithm incorporating serum IgM anti-glycan antibodies, designed with the aim of identifying CIS patients at risk of a second demyelinating attack. Surprisingly, gMS-Classifier2 turned out to be an independent risk factor for clinically definite MS, although MRI findings still posed a higher risk. Finally, we evaluated a number of biological markers in cerebrospinal fluid during a screening and a validation phase that, again, involved a couple of collaborative works, first with Jens Kuhle, Ludwig Kappos (Department of Neurology, University Hospital Basel, in Switzerland), and Giulio DiSanto (Neurocentre of Southern Switzerland, Ospedale Civico, Lugano, Switzerland) to determine neurofilament heavy subunit levels, and later with Luisa María Villar, José Carlos Álvarez-Cermeño, and Carmen Picón, from the Departments of Neurology and Immunology, Multiple Sclerosis Unit, Hospital Ramón y Cajal, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS) in Madrid, Spain, to evaluate neurofilament light subunit levels during the validation phase. Similarly to the gMS-Classifier2 findings, baseline neurofilament light subunit levels were independent risk factors for MS with MRI findings again posing the highest risk but, interestingly, they showed very strong correlations with brain volume changes at five years of follow-up.



II. INTRODUCTION

II. Introduction to clinically isolated syndromes (CIS)

A clinically isolated syndrome (CIS) is a term used to define an acute or subacute episode suggestive of central nervous system (CNS) inflammatory demyelination (McDonald WI, 2001). A CIS, in turn, suggests the possibility of developing multiple sclerosis (MS) over time in a percentage of patients ranging from 20.0% to 80.0%, depending on the presence of certain baseline features (Miller D, 2005). Current evidence suggests that disease modifying treatment (DMT) should be started at this stage since it is likely to have an important impact on the evolution of the disease (Tintore M, 2008a). Furthermore, several clinical trials in CIS have demonstrated that DMT delays conversion not only to clinically definite MS (CDMS) (Jacobs LD, 2000; Comi G, 2001; Kappos L, 2006; Comi G, 2009), but also to McDonald MS (Kappos L, 2006). On the other hand, arguments against early treatment include exposing patients who will not evolve to MS to medication adverse events (Bunyan RF, 2012) or modest clinical benefits on the long run (Pittock SJ, 2007). Thus, accurately identifying which patients will present a second demyelinating episode and, above all, determining the degree of disability they could develop over the medium- to long term is considered crucial for a more individualized treatment.

1. Nosology

Possibly, the first available description of an MS case dates back to 1421 (Medaer R, 1979); however, it was until 1868 that an association between symptoms and CNS damage was established by Jean Martin Charcot, who defined MS or *sclérose en plaques disséminées* as a disease (Clanet M, 2008). But it was until 1965, with the publication of the Schumacher criteria for diagnosis of MS (Schumacher GA, 1965), when current terminologies originated, such as the definition of relapse as well as the concepts of dissemination in space (DIS) and time (DIT) in a purely clinical context. In 1983, paraclinical studies such as IgG oligoclonal band (OCB) determination and multimodal evoked potentials were incorporated into a new set of criteria to

support the diagnosis of MS, in which presence of a second clinical attack in a different topography from the first established the diagnosis of clinically definite MS (CDMS). For the first time, mention is made of one clinical attack supported by clinical or paraclinical evidence of separate lesions, or of presence of OCB or increased IgG levels in the cerebrospinal fluid (CSF) to make the diagnosis of laboratory-supported definite MS or of probable MS. Additionally, terminology such as "clinical attack" was refined (Poser CM, 1983). After demonstrating the high sensitivity of magnetic resonance imaging (MRI) for detection of demyelinating plaques in the CNS, the McDonald criteria were created (McDonald WI, 2001). Although clinical symptoms were still considered important, the role of MRI became very relevant since, for the first time, the term CIS was incorporated and this allowed the diagnosis of MS in patients with a first attack if presence of demyelinating lesions fulfilling DIS and DIT on MRI was determined. Herein, the definition of attack contemplated the presence of neurological symptoms or signs "seen on MS" that lasted longer than 24 hours in the absence of concomitant fever or infection. Paroxysmal symptoms could also be considered an attack if appearing repetitively for more than 24 hours and, to define two attacks separated in time at least 30 days should have elapsed between them (McDonald WI, 2001). Finally, in 1996, a standardization of terminology to describe the different patterns and clinical courses of MS into four categories was proposed, although CIS was included as part of the MS phenotype spectrum until its revision in 2013 (Lublin FD, 1996; Lublin FD, 2014) (**Figure II-1**).

2. Epidemiology and risk factors

The cause of a CIS and, therefore, of MS, is unknown. However, it is believed to occur as a result of a combination of environmental factors triggering the disease in genetically susceptible individuals (Compston A, 2008).

2.1. Age and gender. Being the first demyelinating attack that could lead to the development of MS, a CIS occurs more typically in young adults aged 20-40 years in 70.0% of cases with a mean age of 30 years (Miller DH, 2008), but suggestive symptoms can present at older and younger ages. Females are more

commonly affected than men, with the female-male gap increasing over the last 50 years from 1.4 to 2.3 or more, probably related to lifestyle changes and environmental factors (Alonso A, 2008; Alonso and Hernán MA, 2008; Koch-Henriksen N, 2010; Dunn SE, 2013; Kotzamani D, 2012).

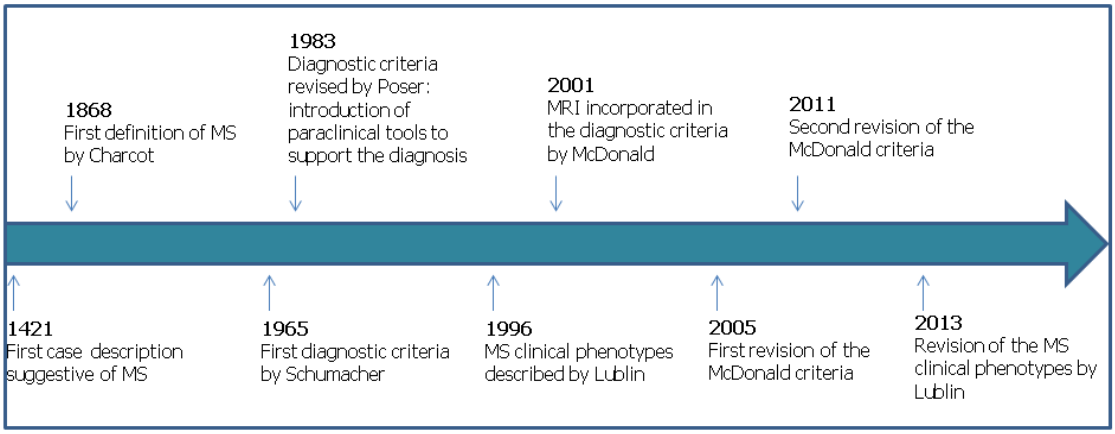


Figure II-1. Simplified timeline showing the evolution of MS description and diagnosis.
Abbreviations: MS: multiple sclerosis; MRI: magnetic resonance imaging.

2.2. Geography. Both incidence and prevalence vary: areas with a high prevalence (60 per 100000 inhabitants or more) include Europe, Southern Canada, Northern United States, New Zealand, and Southeast Australia (Ebers GC, 2008; Simpson S Jr, 2011). This could be partly due to racial differences: people of northern European origin appear to be the most susceptible, whereas people of Asian, American Indian or African ancestry have the lowest risk (Aguirre-Cruz L, 2011; Giampaolo D, 2013; Langer-Gould A, 2013). Nevertheless, an increase in prevalence has been observed in Latin America (Aguirre-Cruz L, 2011) and a higher incidence (10.2) of MS was determined in African Americans in a recent publication, with black women having the highest risk of evolving to MS (Langer-Gould A, 2013). Besides, persons who migrate from a high to a low risk area after puberty retain their former risk, whilst those who migrate at earlier ages appear to acquire the risk of the new area to which they migrated (Marrie RA, 2004), suggesting that exposure to certain environmental factors at a young age may be key to developing the disease.

There has been an attenuation of the north-south gradient after the 1980s, probably due to an increased incidence in lower latitudes (Hernán MA, 1999; Alonso A and Hernán MA, 2008). Conversely, although prevalence has increased in Australia, New Zealand, Western Europe, and North America, incidence has not in the latter two regions (Koch-Henriksen, 2010), meaning that the observed latitudinal gradient of prevalence could be explained by factors such as survival time or diagnostic accuracy.

2.3. Genetic risk factors. Risk of developing MS is associated with class II alleles of the major histocompatibility complex (MHC) and HLA-DRB1 in particular. Presence of the vitamin D response element (VDRE) on the HLA-DRB1*15:01 promoter region suggests a functional interaction between the locus determining genetic susceptibility and vitamin D intake because VDRE enhances gene expression when stimulated by vitamin D (Ramagopalan SV, 2009). Furthermore, the risk of MS can vary according to HLA-DRB1 allele type, observing a protective effect with HLA-DRB1*04, *07, and *09 alleles that express a non-responsive VDRE motif, whereas an elevated risk is observed with the DRB1*15, *16, and *08 alleles. However, most of the HLA-DRB1 alleles expressed a functional VDRE sequence, including alleles that had no apparent effect on MS risk (Nolan D, 2012). MS risk is also associated with other non-MHC susceptibility genes, like IL2RA, IL7RA, and IRF8 (Hafler DA, 2007; De Jager PL, 2009; Sawcer S, 2011). Nevertheless, genetic factors may have only a modest effect, as shown by the concordance rate for MS of 20.0-35.0% among monozygotic twins. For first-degree family members of people with MS, including dizygotic twins, the risk of developing the disease is sevenfold higher than in the general population; however, familial risk is only 3.0-5.0% (Sadovnick AD, 1993; Nielsen NM, 2005).

2.4. Environmental risk factors

2.4.1. Vitamin D. Closely related to latitude is the exposure to sunlight and the resultant metabolism of vitamin D in the risk of MS (Ascherio A, 2007). Several studies have shown that taking supplements of vitamin D reduces the risk of developing MS or that serum levels of 25-hydroxy vitamin D are inversely associated with the risk of MS (Munger KL, 2004, Ascherio A, 2014).

2.4.2. Infections. Neurotropic viruses have been studied as potential triggers of MS. To date, no specific evidence linking viruses directly to development of CIS and MS exists, but there is increasing attention on the Epstein Barr Virus (EBV) as a cause or trigger of MS, observing a relative risk increase of 2.3 times [95% confidence interval (CI) 1.7-3.0] after infectious mononucleosis and elevations of anti-EBV antibody titres before MS onset, especially antibodies against the EBV nuclear antigen 2 (EBNA-2) (Thacker EL, 2006; Ascherio A, 2001; Levin LI, 2005). In a more recent study, when compared to controls, CIS patients had increased immune responses to the EBV-encoded nuclear antigen-1 (EBNA1), but not to other EBV-derived proteins (Lünemann JD, 2010). Nevertheless, proving a link between the EBV and MS is complex given that serological evidence of EBV exposure can be found in up to 90.0% of the adult general population and only about 1 in 500 will develop MS, meaning other factors must be critical to MS development (Kim SK, 2005; Ascherio A, 2010). Other hypothesis proposes that infections during early life could attenuate the immune response to self-antigens and confer some protection against autoimmunity later in life (Bach JF, 2002; Ponsonby AL, 2005).

2.4.3. Other environmental risk factors. An association between smoking and a higher risk of CIS and MS has been shown in a number of studies (Riise T, 2003; Franklin GM, 2003; Ascherio A, 2007). Month of birth has been implicated as a risk factor for MS, with higher probabilities of developing MS in persons born in April and May (Dobson R, 2013a), but it is possible that this birth effect is a false positive that results from confounding factors such as seasonal variation in birth rates, due to excess births in March, April, and May (Fiddes B, 2013). Recent studies suggest that the daily diet and gut microbiota can act as an MS trigger (Montalban X and Tintore M, 2014). Two studies in particular, carried out in *in vitro* and animal models, showed that increased salt concentrations induced IL-17-producing Th17 cells and led to severe symptom worsening in mice with experimental autoimmune encephalomyelitis (Wu C, 2013; Kleinewietfeld M, 2013). Finally, a higher body mass index during childhood and adolescence has been associated with an increased risk of developing MS, especially in females (Munger KL, 2013; Gianfrancesco MA,

2014). Other studies suggest that interactions between obesity and HLA-DRB1*15 as well as with past infectious mononucleosis could trigger the autoimmune response (Hedström AK, 2014; Hedström AK, 2015). **Figure II-2** summarizes the main genetic and environmental risk factors.

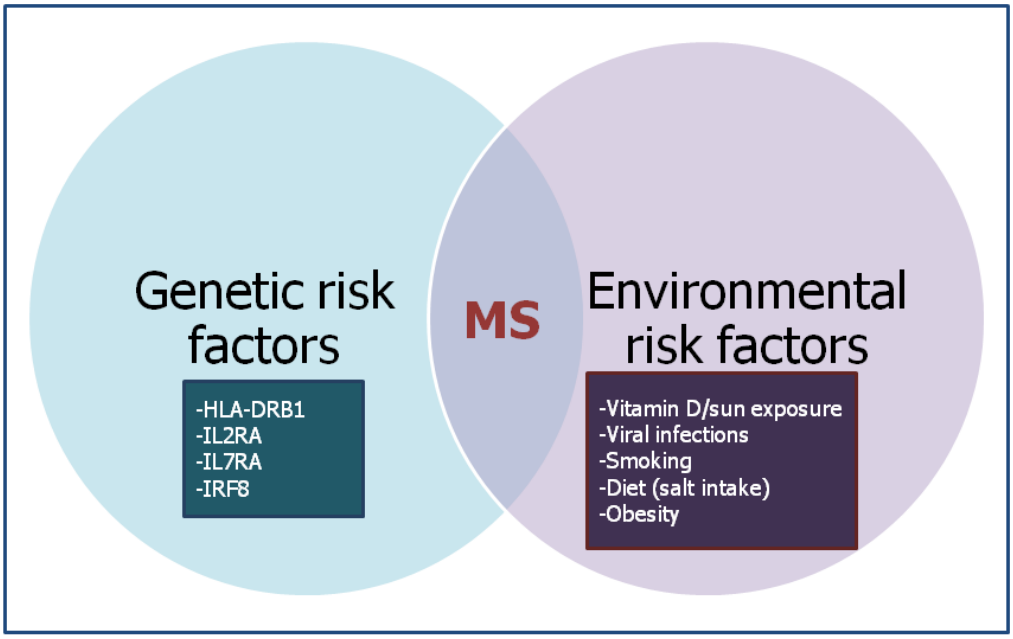


Figure II-2. Main genetic and environmental risk factors for multiple sclerosis (MS).

3. Pathogenesis

3.1. Immunopathogenesis. The most accepted theory is that MS begins as an inflammatory autoimmune disease mediated by autoreactive lymphocytes crossing the blood-brain barrier (BBB) and that later in the disease course it is dominated by microglial activation and neurodegeneration (Weiner HL, 2004; Compston A, 2008). This theory stems from the similarities observed between MS and experimental autoimmune encephalomyelitis (EAE), the animal model of the disease, which is induced by immunizing the animals with myelin-derived proteins such as proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) or myelin basic protein (MBP) (Comabella M and Khoury SJ, 2012).

3.1.1. Peripheral activation. Myelin-specific autoreactive T cells can be found in peripheral blood and in the CSF of both MS patients and healthy

controls, although in the former case, myelin-reactive T cells are more frequently activated and have a memory phenotype compared to the resting, naïve phenotype observed in controls (Lovett-Racke AE, 1998; Frohman EM, 2006a). It is not yet clear how these T cells become activated in MS, but several processes have been suggested as triggers in genetically susceptible individuals exposed to the right environmental factors, such as molecular mimicry (cross-reaction with self-myelin epitopes of T cells generated against non-self epitopes) (Wucherpfennig KW, 1995; Münz C, 2009), peripheral constitutive myelin antigens in cervical lymph nodes according to EAE models (Furtado GC, 2008; Zhang H, 2008), or a deficient immunoregulatory control in which regulatory T lymphocytes fail to suppress effector cells, allowing them to begin an immune response within the brain (Viglietta V, 2004).

3.1.2. Migration of inflammatory cells into the CNS. Transmigration across the BBB is mediated by adhesion molecules, chemokines, and matrix metalloproteinases (MMP). The first steps in this sequential process involve interactions between adhesion molecules expressed on endothelial and immune cells. One relevant adhesion molecule implicated in cell extravasation is the α4β1 integrin (VLA-4, very late activation antigen 4) expressed on the activated lymphocyte surface, which binds to the vascular cell adhesion molecule 1 (VCAM-1) of the brain endothelium (Steinmann L, 2014). Its role in MS etiopathogenesis has been underscored by the significant reduction of disease activity in patients treated with natalizumab, a humanized monoclonal antibody that blocks the α4 subunit of VLA-4 (Steinmann L, 2014). MMPs are proteolytic enzymes that not only disrupt the BBB by degrading the extracellular matrix and basement membrane, but also appear to have a role in cytokine activation, demyelination, and axonal damage (Leppert D, 2001). Worth mentioning is MMP-9, with elevated levels in CSF and blood of MS patients compared to controls, and associated with clinical and radiological activity (Waubant E, 1999). Chemokines are cytokines that regulate recruitment and migration of immune cells from peripheral blood to the CNS in the case of MS. They are displayed at the endothelial lumen and bind to receptors expressed on circulating leukocytes, thus determining which subsets will extravasate into the

CNS (Holman DW, 2011). Altered chemokine or chemokine receptor levels have been reported in blood, CSF, and brain lesions from MS patients, emphasizing their role in the immunopathogenesis of this disease (Holman DW, 2011). Once within the CNS, auto-reactive CD4 lymphocytes are reactivated by myelin antigens presented, in the context of HLA class II molecules, by antigen presenting cells like macrophages and microglia. This reactivation triggers the release of pro-inflammatory cytokines that disrupt further the BBB and stimulate chemotaxis, resulting in a larger wave of inflammatory cell recruitment into the CNS (Comabella M and Khoury SJ, 2012).

After peripheral activation, IL-12 induces the differentiation of naïve CD4+ T cells into Th1 cells, producing pro-inflammatory cytokines such as interferon (IFN) γ that activates macrophages (Loleit V, 2014). On the other hand, differentiation into Th2 cells induces an anti-inflammatory response through cytokines such as IL-4 (McFarland HF and Martin R, 2007). A dysregulation in the balance between Th1 and Th2 cytokines has classically been implicated at the core of MS immunopathogenesis, but over the years, other cell subsets have been discovered that also play a role in autoimmune diseases. Among them, Th17 cells undergo expansion after exposure to IL-23 produced by macrophages and dendritic cells. This cell lineage secretes IL-17, a pro-inflammatory cytokine (Korn T, 2009; Loleit V, 2014), as well as IL-16 and TNF-α (Langrish CL, 2005; Iwakura Y, 2006). The number of Th17 cells in CSF has been demonstrated to be higher during MS relapses and in comparison to patients with non-inflammatory neurological diseases (Brucklacher-Waldert V, 2009). Nevertheless, it appears that it is the Th17 to Th1 ratio that is associated with T cell infiltration of the brain parenchyma (Stromnes IM, 2008).

Pathological studies in MS patients have demonstrated that IL-17, IFNγ, and IL-22 disrupt the BBB, allowing penetration of mostly Th17 cells into the brain (Kebir H, 2007; Tzartos JS, 2008). Since Th17 cells have a higher expression of activation markers and adhesion molecules than Th1 cells, it is now considered that Th17 cells play a central role in MS etiopathogenesis (Brucklacher-Waldert V, 2009). IL-17 further promotes differentiation and B cell and macrophage activation (Frohman EM, 2006a; Hofstetter H, 2009) and allows penetration of

mostly Th17 cells into the brain (Kebir H, 2007; Tzartos JS, 2008). The antigen specificity of Th17 cells, however, is unresolved (Compston A, 2008).

So whilst Th17 and Th1 cells promote inflammation, naturally occurring regulatory T cells (CD4+CD25+ Treg) and Th2 cells have a role in the resolution of the inflammatory process by inhibiting T cell proliferation through down-regulation of MHC-II expression and reducing the release of inflammatory cytokines (Raphael I, 2015). Although the number of Treg cells in both blood and CSF seems to be similar between MS patients and healthy controls, there might be defects in the capacity of Tregs to suppress the peripheral activation of myelin-specific T cells in MS patients (Viglietta V, 2004). Furthermore, FoxP3+ Treg cells have not been detected in MS brain tissue (Tzartos JS, 2008). This could indicate either a lack of Treg-mediated suppression in the CNS or defects in the migration or survival of Tregs within the CNS.

Classically, MS was defined as a CD4+ cell-mediated disease. There is evidence that other cell lines also contribute to the disease immunopathogenesis. Regarding CD8+ cells, they are prominently found in MS lesions and, in some studies, CD8+ cells outnumber CD4+ cells (Babbe H, 2000). They may promote CNS vascular permeability and the number of infiltrating CD8+ cells in MS lesions correlate with axonal damage (Johnson AJ, 2007; Huseby ES, 2001). In this sense, *in vitro* studies show they may transect neurites in an MHC-I/peptide-restricted fashion, suggesting CD8+ cells may participate in axonal damage by directly attacking neurons (Medana I, 2001). Additionally, adoptive transfer of activated myelin-specific CD8+ cells can induce EAE, suggesting a role as effector cells in MS pathogenesis (Huseby ES, 2001).

As for B cells, they can act as antigen presenting cells and, once they mature into plasmatic cells, they can produce auto-antibodies that bind to their target cells and activate the complement cascade or induce macrophage antibody-mediated phagocytosis. B cells can also produce demyelination through secretion of antibodies against oligodendrocytes that can be mediated or not by complement (McFarland HF and Martin R, 2007). The role of humoral immunity in MS immunopathogenesis is further supported by the persistent intrathecal production of oligoclonal immunoglobulins, a finding that, although not specific

for MS, has been part of the diagnostic criteria in one way or another (McDonald WI, 2001; Polman CH, 2005; Polman CH, 2011). Another finding supporting the role of the humoral immune response is the presence of follicle-like aggregates with germinal centres in the meninges of patients with secondary progressive MS (SPMS), meaning that B-cell responses like proliferation, antigen-driven maturation selection, and differentiation into plasma cells can be maintained locally inside the CNS and may contribute to the pathogenic process (Magliozzi R, 2007). Finally, B cell ablative therapy with rituximab, an anti-CD20 monoclonal antibody that depletes naïve and memory B cells, reduces clinical relapses and inflammatory activity on brain MRI, probably by decreasing antigen presenting capacity and cytokine production by B cells (Hauser SL, 2008).

Once T and B cells, plasma cells, and macrophages accumulate in the CNS, proinflammatory cytokines increase the immune response by activating microglia. Contact is then established between the activated microglia and components of the oligodendrocyte-myelin unit, which is opsonised with ligands for both microglial Fc and complement receptors, delivering a lethal signal through the surface-bound TNF-α resulting in demyelination and oligodendrocyte loss (Zajicek JP, 1992; Hemmer B, 2002). Additionally, it has also been suggested that astrocytes could play a more active role in facilitating a glutamate-mediated axonal degeneration and oligodendrocyte damage (Williams A, 2007a; Cambron M, 2012) (**Figure II-3**).

3.1.3. Remission of inflammation. Although Treg cells are dysregulated in MS, their number increases during remission phases (Steinman L, 2014). Treg cells produce anti-inflammatory cytokines like IL-10, IL-4, and TGF-β (Comabella M and Khoury SJ, 2012; Raphael I, 2015). Whilst IL-4 is associated with a decreased Th1 response, the role of TGF-β appears to be dual since it does have an important inhibitory activity over T cells, but in presence of IL-6 or IL-21 favours differentiation into Th17 cells (Steinman L, 2014). As for IL-10, besides suppression of effector T cells in EAE through downregulation of MHC-II and co-estimulatory molecules on antigen presenting cells, it favours

differentiation of naïve CD4+ cells into induced Treg cells (iTreg), also known as type 1 Treg cells (Tr1) (Raphael I, 2015).

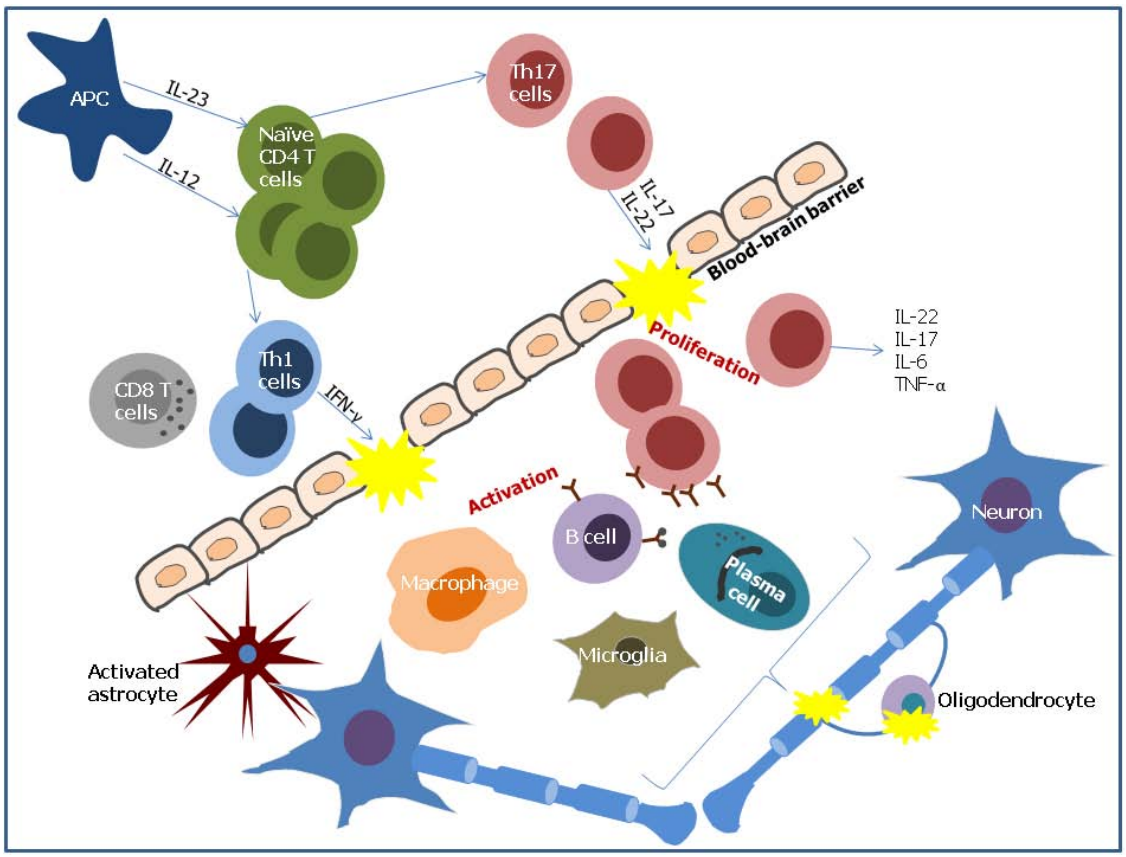


Figure II-3. Schematic representation of MS immunopathogenesis.
Abbreviations: APC: antigen presenting cell.

More recently, regulatory B (Breg) cells have been described. They also express FoxP3 and produce IL-10 and TGF-β (Correale J and Equiza TR, 2014). Although usually reduced, the number of Breg cells can increase during relapses and their main anti-inflammatory effects are mediated by IL-35 (de Andrés C, 2014; Krumbholz M and Meinl E, 2014). Natural killer (NK) cells also play an immunomodulatory role acting as regulatory cells (CD16+, CD56+) by inducing apoptosis through Fas-FasL (CD95) signalling, observing increased numbers in MS (van Kaer L, 2015; Rodríguez-Martín E, 2015). Correlations have been shown between reduced inflammatory activity in MS patients treated with

humanized monoclonal antibody anti-CD25 daclizumab, and $CD56^{bright}$ regulatory NK cell expansion (Elkins J, 2015).

Once inflammation subsides, oligodendrocyte progenitor cells migrate towards the sites of demyelination, expand, and develop into myelinating oligodendrocytes to myelinate axons and repair local damage (Scolding NJ and Franklin RJ, 1997; Hemmer B, 2002).

3.2. Pathological findings. The most characteristic change in MS brains is the formation of demyelinated plaques. Traditionally seen as an inflammatory disease affecting the white matter of the CNS, it is now known that demyelination also occurs in the cortex and that diffuse injury affects the normal appearing white matter (Lassmann H, 2007). Disease mechanisms appear to change during the natural course of MS and can show interindividual differences (Lassmann H, 2007; Lassmann H, 2013a). Specifically in CIS and early relapsing remitting MS (RRMS), inflammation plays a major role in the formation of white matter plaques (Frohman EM, 2006a; Steinman L, 2014). The most pronounced inflammation is seen in classical active lesions, the predominant type of white matter lesion in CIS and RRMS (Lassmann H, 2013a; Lucchinetti CF, 2011). It is characterized by lymphocytic inflammation and massive infiltration by macrophages containing myelin debris (Frischer JM, 2009). MHC class I-restricted CD8+ T cells predominate but CD4+ T cells are also found (Frohman EM, 2006a; Gay FW, 1997; Hayashi T, 1988; Friese MA, 2009). Active demyelination at the edge of the lesions is associated with a variable degree of acute axonal injury and destruction that correlates with T cell and microglial activation (Kuhlmann T, 2002) (**Figure II-4**). Inactive plaques are sharply demarcated areas of demyelination without inflammatory activity at the edges, but these lesions are not common in early stages of the disease (Lassmann H, 2013a). Remyelinated lesions can be seen at all stages (Patrikios P, 2006) (**Figure II-4**). They can be extensive in some patients whilst remyelination fails in others. Lesion location and the availability of viable axons within plaques might be important factors. Besides the well-known white matter lesions, cortical lesions can be seen in all stages of MS as well, but they are sparse in CIS and early RRMS (Kutzelnnig A, 2005). They can be found as

purely intracortical, as combined white matter/grey matter lesions, and as subpial demyelination (Geurts JJ, 2008).

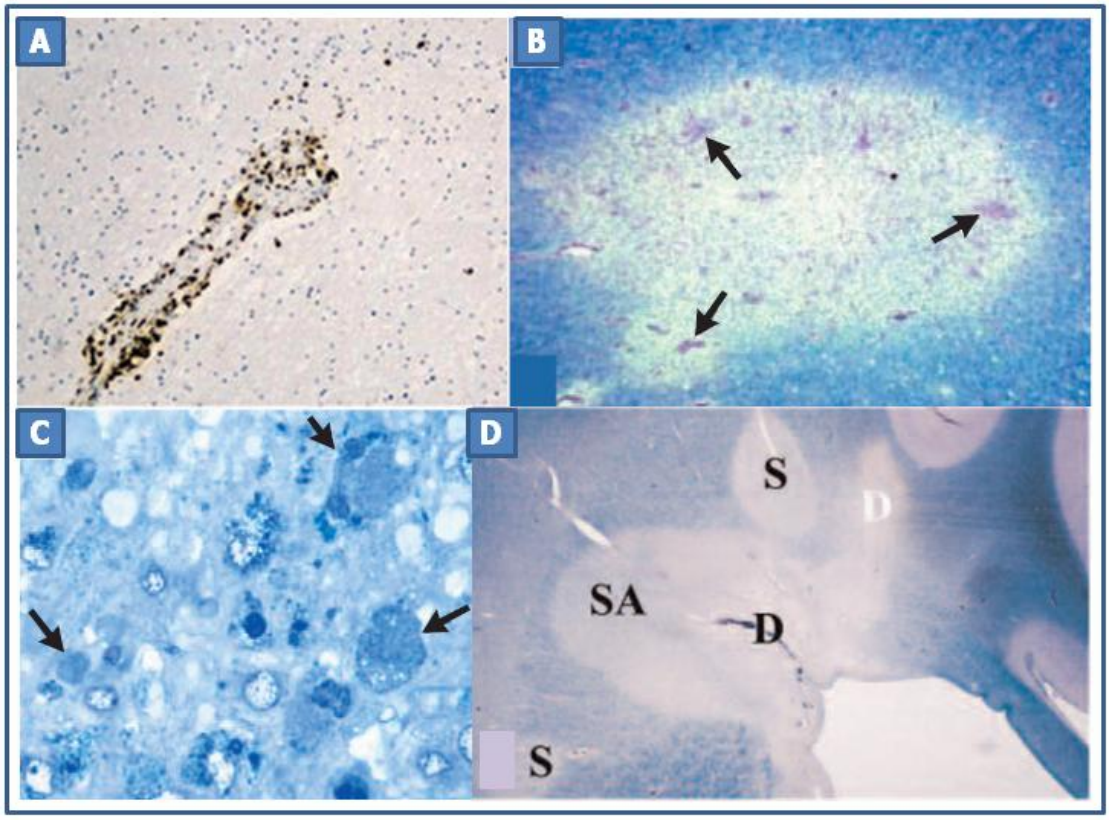


Figure II-4. Classical pathological findings in the multiple sclerosis lesion. A. Inflammation: immunohistochemistry stain showing perivascular CD8 positive lymphocytes (Adapted from Lassmann H et al, 2007). B. Demyelination: luxol fast blue stain demonstrating a demyelinated plaque; arrows show perivascular infiltrates. C. Axonal damage: toluidine blue stain of a demyelinated plaque; the arrows show the transacted axons forming spheroids (B and C adapted from Frohman EM et al, 2006a). D. Remyelination: luxol fast blue stain; SA indicates a shadow plaque within a demyelinated area; S indicates shadow plaques; D represents completely demyelinated periventricular lesions (Adapted from Patrikios P et al, 2006).

Pancortical lesions have been described rarely (Peterson JW, 2001). Lesions are characterized by CD3+ T cell infiltrates and macrophage-associated demyelination can be observed. Although it is mostly a feature of later stages, meningeal inflammation has been topographically associated with cortical demyelination in early MS (Lucchinetti CF, 2011). In the case of pure intracortical lesions, T and B cells are barely found, whereas important microglia activation has been demonstrated (Bo L, 2003). At early stages of the disease, there is little damage of the normal appearing white matter, which

predominates in progressive forms of the disease (Kutzelnigg A, 2005). Different immune mechanisms can be involved in the formation of active lesions leading to different patterns of demyelination (Lucchinetti C, 2000). Type I lesions are dominated by T cells and macrophages, whereas in type II, accumulation of immunoglobulins and complement has been observed. Type III lesions are caused by production of oxygen and nitric oxide radicals, probably inducing oligodendrocyte mitochondrial dysfunction which leads to histotoxic hypoxia. Type IV lesions have been described in very few cases and are associated with mild inflammation and oligodendrocyte degeneration (Miller DH, 2002; Frohman EM, 2006a; Lucchinetti C, 2000). This schema has been questioned by other authors, who suggest the findings seen in acute lesions are initiated by extensive oligodendrocyte cell death and changes in the myelin sheath. Such lesions contain few or no lymphocytes or myelin phagocytes. They also have observed that the different subtypes can be found in one single patient, suggesting that such heterogeneity might be a reflection of the evolution of one single pathophysiological process that may be modified by individual genetic factors (Barnett MH, 2004; Barnett MH, 2006; Barnett MH, 2009). Recent findings, however, challenge these views and reinforce the concept of patient-dependent immunopathological heterogeneity (Metz I, 2014). Nevertheless, more recent information could indicate a need to modify the four previously described types of demyelination, since evidence points to a mechanism of tissue injury that is related to oxidative damage mediated by activated macrophages and microglia, which leads to mitochondrial injury, a prominent finding in active MS lesions (Mahad D, 2008; Lassmann H, 2011) (**Figure II-5**). Mitochondrial injury, in turn, could explain pathological features of MS lesions, like demyelination, oligodendrocyte apoptosis, and axonal degeneration (Lassmann H, 2013b; Witte ME, 2014). Radical production in active MS lesions is reflected by the expression of inducible nitric oxide synthase (iNOS), NADPH oxidase complexes, and myeloperoxidase in microglia in the zones of initial tissue injury (Liu JSH, 2001; Fischer MT, 2012; Gray E, 2008). Presence of oxidized DNA and lipids in the involved cells also supports the role of oxidative injury (Haider L, 2011), whereas iron deposition in active

demyelinating MS lesions appears to amplify oxidative injury in the presence of oxygen radicals (Haider L, 2014).

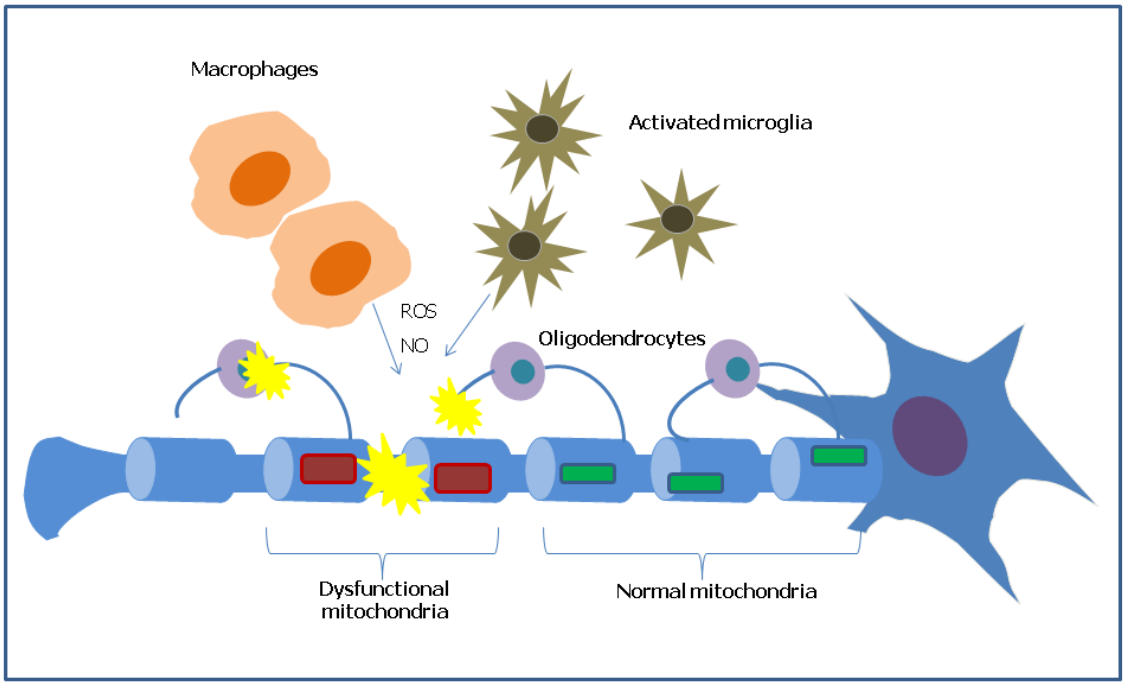


Figure II-5. Mitochondrial injury in multiple sclerosis.
Abbreviations: ROS: reactive oxygen species; NO: nitric oxide.

As for remyelination, it is incomplete and heterogeneous: it is more active during early stages of the disease but it can also occur once progression begins, due to a combination of factors that either inhibit or facilitate the process, such as neurite outgrowth inhibitor (Nogo) receptor-interacting protein-1 (LINGO-1), Nogo-A or oligodendrocyte transcription factor Olig2 (Rudick RA, 2008; Lee JY and Petratos S, 2013; Wegener A, 2015). So whilst remyelinated lesions can be seen at all stages (Patrikios P, 2006), they can be extensive in some patients and it may fail in others. Lesion location and the availability of viable axons within plaques might be important factors. Besides, remyelination may be less effective in chronic phases: even though active oligodendrocytes are still observed in lesions, they do not seem to succeed in remyelinating axons (Hemmer B, 2002).

4. Clinical findings

A CIS usually presents as optic neuritis, syndromes of the brainstem, cerebellum or spinal cord, or with long tract signs and symptoms (Confavreux C, 2000; Miller DH, 2012). Although a CIS is, by definition, monofocal, approximately 10.0-15.0% of patients present with multifocal abnormalities (Brownlee WJ and Miller DH, 2014). There are no pathognomonic clinical findings, but some of them are highly characteristic of the disease, like the following (Miller DH, 2005; Miller DH, 2012): optic neuritis usually presents as acute or subacute unilateral eye pain accentuated by ocular movements, followed by a variable degree of visual loss affecting mostly central vision. Brainstem and cerebellar syndromes usually involve diplopia, most classically manifested as internuclear ophthalmoplegia. If bilateral, it can be associated with vertical nystagmus on upward gaze. Impairment of cerebellar pathways can lead to gait imbalance, difficulty in performing coordinated actions, and slurred or scanning speech. Physical examination can reveal dysmetria, decomposition of complex movements, hypotonia or intention tremor. Ocular findings include nystagmus and ocular dysmetria. Vertigo is usually associated with symptoms reflecting dysfunction of adjacent cranial nerves, such as hyper- or hypoacusis, facial numbness or diplopia. Sensory disturbances are a common initial symptom. Usual complaints include numbness, tingling, pins-and-needles, tightness, coldness or swelling of the limbs or trunk. Radicular pain can be present, as well as an intense itching sensation. Motor symptoms present as weakness of the lower extremities more often than of the upper extremities. Physical findings may include spasticity, brisk or exaggerated deep tendon reflexes or extensor plantar responses. Lhermitte phenomenon, a transient sensory symptom described as an electric shock radiating down the spine or into the limbs with flexion of the neck (Kanchandani R, 1982), can also be described by patients.

5. MRI findings

To this day, MRI is the test of choice to support the clinical diagnosis of MS (Filippi M, 2011).

5.1. Conventional brain MRI. The characteristic lesion is the plaque. Plaques are typically found in the periventricular region, corpus callosum, centrum semiovale, brainstem, and cerebellum. Other regions that can be affected to a lesser extent include the deep white matter structures and basal ganglia. Lesions are arranged at right angles to the corpus callosum (Horowitz AL, 1988; Ge Y, 2006) (**Figure II-6, panel A, yellow arrows**). They usually have an ovoid appearance and appear hyperintense on proton density and T2-weighted images. If visible on T1-weighed images, they appear hypointense. Acute lesions tend to be larger with somewhat ill-defined margins and become smaller with sharper margins upon resolution, probably reflecting decrease of oedema and inflammation (Horowitz AL, 1988; Ge Y, 2006). Gadolinium (Gd), a paramagnetic contrast agent that crosses the disrupted BBB, has been used to assess plaque activity by increasing signal intensity on T1-weighted images (Tortorella C, 1999), but Gd enhancement can diminish or disappear after corticosteroid treatment. Gd-enhancing lesions often correspond to areas of high signal on T2-weighted images and low signal intensity on unenhanced T1-weighted images, probably due to oedema. Thus, accumulation of Gd in plaques is associated with new or newly active lesions (Cotton F, 2003). The majority of these enhancing lesions are clinically asymptomatic, but this suggests continuing disease activity. Gd enhancement usually disappears after 30-40 days, but it may rarely persist for up to eight weeks. Prolonged persistence of enhancement is a red flag against the diagnosis (Smith ME, 1993). Several Gd enhancement patterns can be observed. Concentric ring-enhancing lesions with central contrast pallor, which can occur in an open ring, are more specific for MS than for infections or neoplastic diseases (Miller DH, 1988; Morgen K, 2001; He J, 2001; Rovira A, 1999) (**Figure II-6, panel B**).

On T1-weighted images, most lesions are isointense, but some can appear hypointense or as black holes, particularly in the supratentorial region (Paolillo A, 2000; Simon JH, 2000).

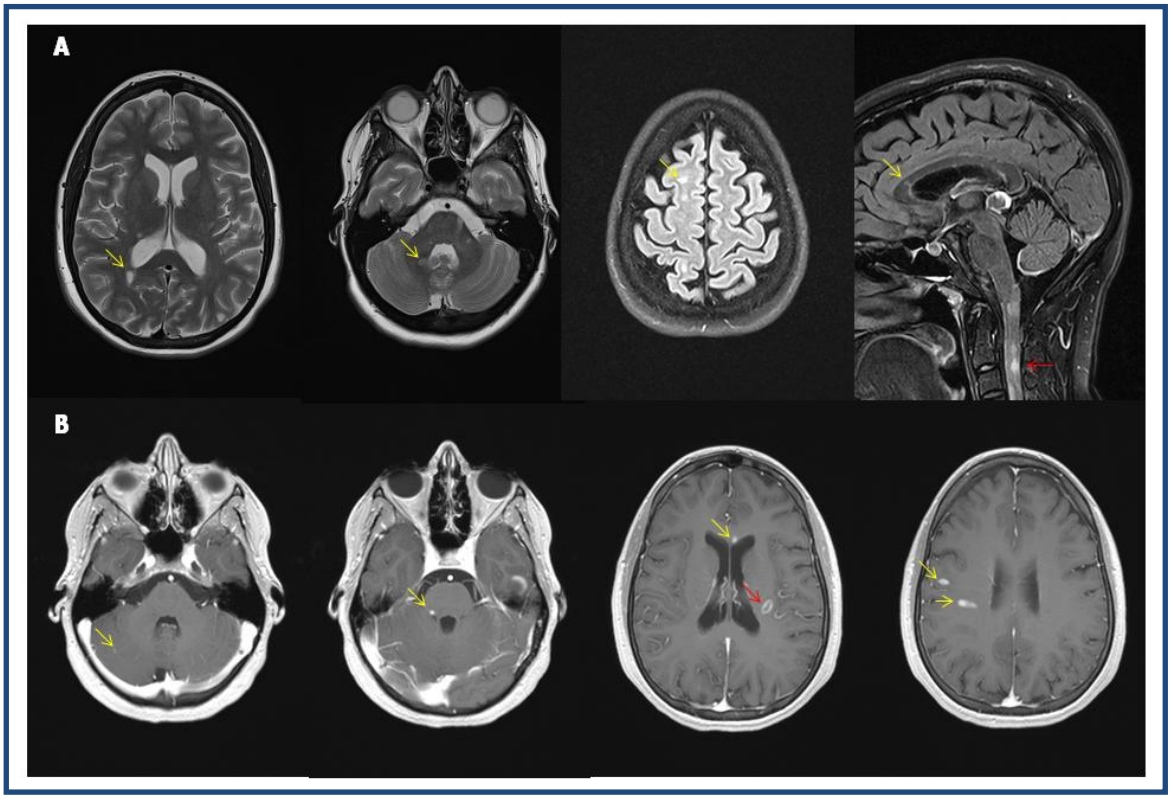


Figure II-6. Characteristics of MS lesions on MRI. Panel A: typical topographies in the brain (yellow arrows) and spinal cord (red arrow). Panel B: common gadolinium enhancement patterns include nodular (yellow arrows) and ring (red arrow) patterns.

Non-enhancing black holes can be present in approximately 40.0% of CIS patients (Mitjana R, 2014). They are nonspecific at a given time point, since nearly 50.0% will revert to normal in a few months, most likely due to remyelination and resolution of oedema (Bitsch A, 2001). Although evidence is limited, persistent black holes are thought to be markers of severe demyelination and axonal loss (van Walderveen MA, 1998), whereas another study suggests they are associated with remyelination (Barkhof F, 2003).

5.2. Conventional spinal cord MRI. In contrast to the increasing frequency of hyperintense signals seen in older individuals on brain MRI, the frequency of abnormal signs on spinal cord MRI in normal individuals is about 3.0%. Spinal cord findings typical of MS include little or no cord swelling, unequivocal hyperintensity on T2-weighted images visible in two planes (axial and sagittal), size of at least 3mm but less than two vertebral segments in length, and usually

involve only a part of the cord in cross-section (Brex PA, 1999; Bot JC, 2004) (**Figure II-6, panel A, red arrow**).

5.3. Non-conventional MRI. Conventional MRI has limitations concerning a comprehensive assessment of the underlying pathology, lacking the capability to recognize grey matter lesions or changes in the normal-appearing brain tissue, features that can be better assessed with non-conventional MRI techniques (Poloni G, 2011; Cecarelli A, 2012). Diffusion-weighted and diffusion tensor MRI imaging and magnetic resonance spectroscopy may provide information about axonal loss and diffuse abnormalities in normal appearing white and grey matter (Guo AC, 2002; Oh J, 2004; Rovaris M, 2005). Although studies in CIS patients are scarce, abnormalities at this stage of the disease have been reported (Tavazzi E, 2007; Cappellani R, 2014; Sbardella E, 2013; Sbardella E, 2011). Quantitative methods like magnetization transfer ratio can be used to assess myelin content and axonal count (Schmierer K, 2004). Variable degrees of magnetization transfer ratio-detectable damage on white and grey matter can be present at the time of the CIS (Gallo A, 2007; Rocca MA, 2008; Jure L, 2010; Crespy L, 2011). Susceptibility weighted imaging (SWI) is a relatively novel MRI technique based on the differences in the magnetic properties existing in biological tissues (Haacke EM, 2004). Using SWI, areas of intralesional susceptibility signal, likely corresponding to iron deposits, have been observed in most chronic but also in acute MS plaques (Haacke EM, 2009). Some studies have shown that these intralesional susceptibility signals can aid in differentiating CIS from other neurological diseases with focal white matter lesions (Hagemeier J, 2012; Hagemeier J, 2014) (**Figure II-7**).

SWI also allows differentiating CIS and MS patients from controls by identification, in the former, of central venules within demyelinating periventricular lesions as well as by decreased visibility of cerebral medullary veins in the cerebral hemispheres (Rovira A, 2012; Beggs CB, 2012; Kau T, 2013).

Grey matter involvement can be observed in MS (Calabrese M, 2010; Geurts JJ, 2012). The visualization of focal cortical lesions has been partly improved with the combination of two- and three-dimensional fluid-attenuated inversion

recovery imaging, double inversion recovery, and phase-sensitive inversion recovery T1-weighted sequences (Geurts JJ, 2005; Calabrese M, 2007a; Nelson F, 2007), leading some authors to propose the inclusion of cortical lesions in the diagnostic criteria for MS (Filippi M, 2010); nevertheless, although they can be observed in CIS, a high interobserver variability exists (Calabrese M, 2012; Geurts JJ, 2011).

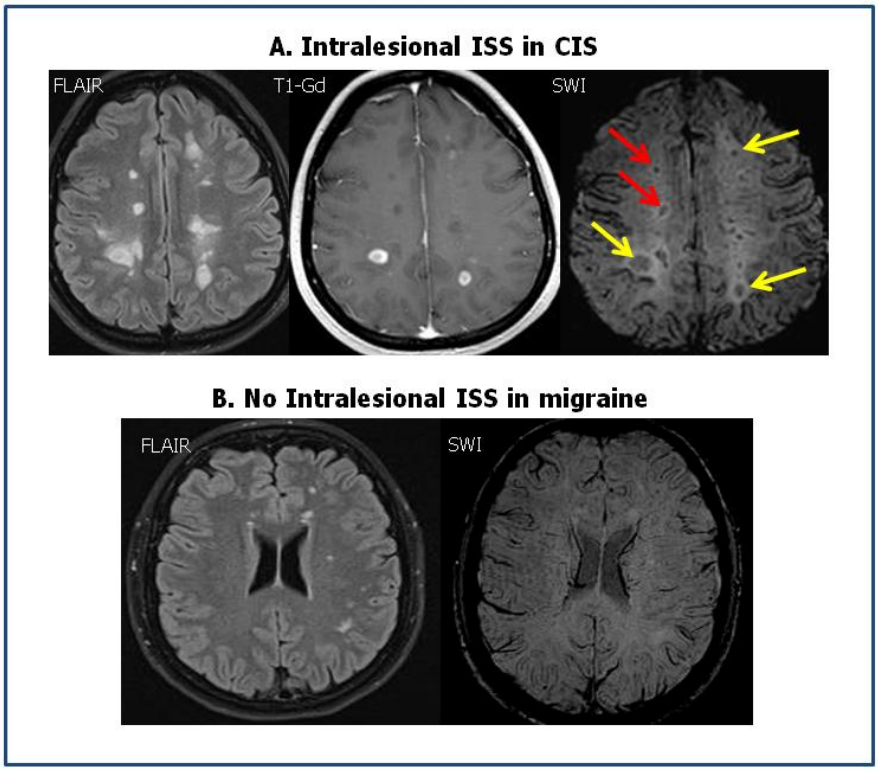


Figure II-7. SWI sequence may aid in differentiating MS lesions (A) from white matter lesions observed in other conditions like migraine (B). The arrows show the ISS in a CIS case.
Abbreviations: FLAIR: fluid attenuated inversion recovery; Gd: gadolinium; SWI: susceptibility weighted images; ISS: intralesional susceptibility signal.

Diffuse grey matter damage can be measured through image analysis software to segment total grey matter or specific structures (Geurts JJ, 2012; Chard DT, 2002), by estimating the cortical thickness using voxel-based morphometry (Ashburner J, 2000) or by means of a cortical deformation modelling software that estimates changes in mean global or regional cortical thickness (Fischl B, 2000; Nakamura K, 2011; Fischl B, 2012). There is evidence of grey matter

volume loss early in CIS in some, but not all, studies (Dalton CM, 2004; Calabrese M, 2007b; Ceccarelli A, 2008). Although non-conventional MRI adds valuable information in CIS and MS, it is important to bear in mind that its use in a more generalized clinical setting is still limited.

5.4. Radiologically isolated syndrome. With the advent of MRI, a particular issue has arisen due to incidental brain MRI findings highly suggestive of MS in the absence of characteristic symptoms and signs, grouped in the term radiologically isolated syndrome (RIS) (Okuda DT, 2009), for which even diagnostic criteria have been proposed. These patients are at risk of developing a CIS and MS, but data are limited to assess the risk. Follow-up studies in small cohorts have demonstrated appearance of new T2 lesions, Gd enhancement or enlargement of pre-existing lesions in almost 60.0% of cases. A CIS or MS occurred in 33.0-34.0%, with time to CIS ranging from 0.8 to 10 years and a median time of 5.0-5.4 years (Okuda DT, 2009; Lebrun C, 2009; Okuda DT, 2014). Other studies in RIS have suggested that presence of spinal cord lesions, male gender or a younger age are risk factors for developing a CIS or MS (Okuda DT, 2011; Okuda DT, 2014). Of note, cortical lesions have been identified in some patients with RIS (Giorgio A, 2011).

6. CSF

6.1. General findings. CSF appearance and pressure are usually normal. Total leukocyte count is normal in approximately 60.0% of patients, exceeds 15 cells/µl in less than 5.0% of cases, and rarely exceeds 50 cells/µl (a finding that should be considered a red flag) (Pohl D, 2004). Lymphocytes are the predominant cell type. CSF protein levels are usually normal.

6.2. IgG OCB. Elevation of CSF immunoglobulin levels relative to other proteins suggests intrathecal synthesis. Such increase is predominantly IgG, although IgM and IgA synthesis can be increased as well. Positive CSF is based on the finding of either OCB different from any such bands in serum or by an increased IgG index. IgG level is usually expressed by use of the IgG index (normal value <0.66-0.90, depending on the laboratory) or by use of formulae

for intrathecal fluid synthesis of IgG. Such abnormality can be found in up to 90.0% of patients with CDMS (McLean BN, 1990; Giesser BS, 2011; Dobson R, 2013b). OCB represent limited classes of antibodies that are depicted as discrete bands on agarose gel. Around 8.0% of CSF samples of patients without MS also contain OCB, and most are the result of chronic CNS infections, viral infections, and neuropathies. Of the five OCB patterns, types 2 (OCB in CSF but not serum) and 3 (OCB in CSF plus additional identical OCB in CSF and serum) indicate intrathecal IgG synthesis (Freedman MS, 2005). Expert recommendations on evaluation of CSF in patients suspected of having MS indicate that the most informative analysis is qualitative assessment of CSF for OCB, best performed using isoelectric focusing on agarose gel followed by immunodetection by blotting or fixation, as this method achieves the best sensitivity and specificity (Freedman MS, 2005).

7. Multimodal evoked potentials

Multimodal evoked potentials are the electrical events generated in the CNS by peripheral stimulation. They are used to detect abnormal CNS function that may be clinically undetectable. Detection of such subclinical lesion in a site remote from the region of clinical dysfunction supports the diagnosis of MS, and may also help define the anatomical site of the lesion in tracts not easily visualized by imaging (optic nerves, dorsal columns). The most commonly used are somatosensory, visual, and brainstem auditory evoked potentials. Patients with CDMS have abnormal visual evoked potentials in 85.0% of cases, but ocular or retinal disorders should be excluded. Somatosensory evoked potentials are abnormal in 77.0% of cases, including approximately 50.0% of those who do not have sensory symptoms or signs. Brainstem auditory evoked potentials abnormalities are present in 67.0% of cases. Thus, visual evoked potentials are probably useful, whereas somatosensory evoked potentials are possibly useful, and there is insufficient evidence to recommend brainstem auditory evoked potentials as a test for diagnostic purposes (Gronseth GS, 2000).

8. Diagnosis

In the context of a first demyelinating event suggestive of MS, diagnostic criteria can be applied. As mentioned before, the McDonald criteria incorporated MRI as a robust tool to demonstrate DIS and DIT of CNS lesions. Clinical findings are also considered to determine fulfilment of DIS and DIT (McDonald WI, 2001).

8.1. DIS. The original McDonald criteria as well as their revision in 2005 established the concept of DIS on MRI by using the Barkhof-Tintoré criteria (**Table II-1**). At least three of the four criteria must be present to fulfil DIS (**Table II-2**) (Barkhof F, 1997; Tintore M, 2000). The clinical concept of DIS was maintained in case of a multifocal attack or according to findings on the neurological examination (ie, optic neuritis plus extensor plantar response). In cases in which MRI findings are not characteristic and do not fulfil the Barkhof-Tintore criteria, authors established an alternative criterion based on presence of at least 2 lesions on T2-weighted images on MRI plus OCB or increase of IgG in CSF (McDonald WI, 2001; Polman CH, 2005). Considerations regarding spinal cord MRI in the diagnosis were made: one spinal cord lesion could be equivalent to an infratentorial lesion to fulfil the Barkhof criteria. The McDonald criteria were revised again in 2010 to incorporate new evidence and to simplify their application whilst preserving sensitivity and specificity (Swanton JK, 2007; Rovira A, 2009; Montalban X, 2010). The criteria proposed by the MAGNIMS (MAGNetic resonance Imaging in Multiple Sclerosis) group (Swanton JK, 2007) (**Table II-1**) were incorporated in the 2010 revised version of the McDonald criteria (Polman CH, 2011). In this proposal, DIS is demonstrated by presence of one or more T2 lesions in at least two of the four typical MS regions: periventricular, juxtacortical, infratentorial or spinal cord or by the development of a further clinical attack implicating a different nervous system site (**Table II-2**). Symptomatic lesions of the brainstem or spinal cord are excluded and do not contribute to lesion count.

8.2. DIT. In the 2001 and 2005 criteria, if a new MRI was performed three months after the CIS and a Gd-enhancing lesion in an asymptomatic region was detected, DIT was fulfilled.

Table II-1. DIS on MRI according to the Barkhof-Tintoré and MAGNIMS criteria.

DIS criteria on MRI	
Barkhof-Tintoré	**MAGNIMS**
At least three of the following: 1. One Gd-enhancing lesion or nine T2 hyperintense lesions if there is no Gd-enhancing lesion 2. At least three periventricular lesions 3. At least one juxtacortical lesion 4. At least one infratentorial lesion	At least one T2 lesion in at least two of the following areas: • Periventricular • Juxtacortical • Infratentorial • Spinal cord
Note: In 2001, one spinal cord lesion can be substituted for one brain lesion. In 2005, individual spinal cord lesions can be included in the total lesion count.	Note: In the case of brainstem or spinal cord syndromes, the symptomatic lesions are excluded and do not contribute to lesion count.

Abbreviations: DIS: dissemination in space; MRI: magnetic resonance imaging; MAGNIMS: MAGNetic resonance Imaging in Multiple Sclerosis group; Gd: gadolinium.

When analysing T2-weighted images, detection of at least one new lesion, if the baseline scan had been performed at least 30 days after symptom onset, could also be used to demonstrate DIT (**Table II-2**). Hence, MRI now allowed an earlier diagnosis. As with DIS, later studies proposed some alternatives to establish DIT (Swanton JK, 2007; Rovira A, 2009; Montalban X, 2010). Since these studies demonstrated that specificity was preserved, the 2010 criteria also incorporated a simplified version of DIT. Simultaneous presence of asymptomatic Gd-enhancing and non-enhancing lesions at any time, or a new T2 or Gd-enhancing lesion on follow-up MRI, irrespective of its timing with reference to a baseline scan, or a second clinical attack fulfilled DIT (**Table II-2**). Thus, the main advantage of the 2010 criteria is the possibility of establishing DIS and DIT at the time of the CIS.

Table II-2. Evolution of the McDonald criteria for RRMS and CIS.

	McDonald criteria		
	2001	**2005**	**2010**
DIS	At least three of the following: One Gd-enhancing lesion or nine T2 hyperintense lesions At least three periventricular lesions At least one juxtacortical lesion At least one infratentorial lesion Or: At least two T2 lesions plus positive OCB or increased IgG index	At least three of the following: One Gd-enhancing lesion or nine T2 hyperintense lesions At least three periventricular lesions At least one juxtacortical lesion At least one infratentorial lesion Or: At least two T2 lesions plus positive OCB or increased IgG index	At least one T2 lesion in at least two of the following areas: Periventricular Juxtacortical Infratentorial Spinal cord Symptomatic lesions of the brainstem or spinal cord are excluded and do not contribute to lesion count.
DIT	At least one asymptomatic Gd-enhancing lesion at least three months after the CIS Or: At least one new T2 lesion on a follow-up MRI performed at least three months after the CIS	At least one asymptomatic Gd-enhancing lesion at least three months after the CIS Or: At least one new T2 lesion at any time compared with a reference scan done at least 30 days after the CIS	Simultaneous presence of asymptomatic Gd-enhancing and non-enhancing lesions at any time Or: A new T2 and/or Gd-enhancing lesion(s) on follow-up MRI with reference to a baseline scan, irrespective of its timing
Sensitivity*	47.1% (36.1-58.2)	60.0% (48.8-70.5)	71.8% (61.0-81.0)
Specificity*	91.1% (84.6-95.5)	87.8% (80.7-93.0)	87.0% (79.7-94.2)
Accuracy*	73.1% (66.5-79.0)	76.4% (70.1-82.0)	80.8% (74.8-85.9)

*Shown with 95% confidence intervals.
Abbreviations: RRMS: relapsing remitting multiple sclerosis; CIS: clinically isolated syndrome; DIS: dissemination in space; Gd: gadolinium; OCB: oligoclonal bands; DIT: dissemination in time; MRI: magnetic resonance imaging.

9. Differential diagnosis

Most diagnostic difficulties arise in patients who have atypical presentations or a first episode that can include differentials like vascular events, infections or neoplasms, or who live in areas of low prevalence for MS. Furthermore, a common error is to over-interpret the presence of multiple T2 hyperintense lesions on MRI as equivalent to MS lesions in the absence of suggestive clinical symptoms. Diseases that can produce such lesions include inflammatory diseases like systemic lupus erythematosus, Sjögren's disease, Behçet's disease, Susac's syndrome, and sarcoidosis; and infections like syphilis and retroviral diseases. All of these can present with or without a relapsing-remitting course (Miller DH, 2008). The differential diagnosis should also include mitochondrial diseases such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). At the time of the CIS, red flags should arise the suspicion of alternative diagnoses, such as progressive optic neuropathy, complete external ophthalmoplegia, a sharp sensory level to all modalities, and localized spinal pain or encephalopathy. These can be classified as minor, intermediate or major red flags, according to how definitely they point to a non-MS disease (Miller DH, 2008). Exploring the possibilities of an alternative etiology before accepting the diagnosis of MS is crucial (Carmosino MJ, 2005; Solomon AJ, 2012). Above all, there are at least a couple of demyelinating diseases that can lead to difficulties in the differential diagnosis at first presentation:

9.1. Acute disseminated encephalomyelitis (ADEM). Since ADEM occurs more frequently in children and young adults with an incidence of 0.4 per 100000 per year, most available information arises from studies in those populations (Leake JA, 2004). Patients usually present with encephalopathy, headache, fever, and focal neurological signs and symptoms (Tenembaum SN, 2013). The history of most patients includes previous infection or vaccination (Tenembaum S, 2002). Coincidental development of demyelinating polyradiculoneuropathies can occur (Aimoto Y, 1996). MRI shows multiple lesions, many of which can exceed 1-2 cm, and Gd enhancement occurs in 14.0-30.0% of cases (Verhey LH, 2011; Mikaeloff Y, 2007; Leake JA, 2004; Tenembaum S, 2002). Lesions in the thalamus and basal ganglia are more

typical of ADEM than MS (Verhey LH, 2011). MRI characteristics that better distinguish between ADEM and MS are the absence of hypointense lesions or of two or more periventricular lesions (Callen DJ, 2009). Extensive lesions can be seen on spinal cord MRI (Rostasy K, 2009). Serum NMO-IgG should be negative, antibodies against the myelin oligodendrocyte glycoprotein (anti-MOG) can be present but are usually transient, and CSF OCB are rarely observed (DiPauli F, 2011; Franciotta D, 2008; Alper G, 2009). Criteria for ADEM are not well validated. It was historically considered a monophasic disease, but repeated episodes occur in approximately 30.0% of cases (Krupp LB, 2007). For this reason, Krupp and collaborators originally suggested any fluctuations of initially present symptoms within three months after the initial manifestation during steroid taper or 30 days after steroid discontinuation to be part of the same episode (monophasic ADEM). If symptoms similar to the initial ones occurred at least three months after onset and at least one month after the last dose of steroids, this was considered recurrent ADEM. If new symptoms or new lesions on MRI appeared in the same timeframe, the term multiphasic ADEM was used (Krupp LB, 2007). However, multiphasic ADEM was considered controversial since it could also be regarded as atypical MS, especially in children (Zettl UK, 2012), and distinguishing ADEM from a CIS is of clinical interest due to therapeutic implications. Besides, about 20.0-30.0% of patients who meet the ADEM criteria will later be diagnosed as MS, especially adults, in whom ADEM is less common (Hartung HP, 2001; Miller DH, 2008). Therefore, a change has recently been proposed in the diagnostic criteria for ADEM in children, mostly by eliminating the term recurrent ADEM and modifying the definition of multiphasic ADEM as follows: two episodes consistent with ADEM separated by three months but not followed by any further events. The second event can involve new or prior neurological symptoms, signs, and MRI findings (Krupp LB, 2013). High-dose steroid treatment is recommended, with plasma exchange or intravenous immune globulin as alternatives in refractory cases (Leake JA, 2004). Of note, when paediatric ADEM is followed by subsequent events of demyelination leading to a diagnosis of MS, MS onset is considered at the time of the ADEM. However, this would not be considered a CIS since the

definition for the paediatric population is: a monofocal or polyfocal clinical CNS event with presumed inflammatory demyelinating cause, absence of a prior clinical history of CNS demyelinating disease, no encephalopathy that cannot be explained by fever, and no diagnosis of MS based on MRI features according to the 2010 McDonald criteria (Polman CH, 2011; Krupp LB, 2013).

9.2. Neuromyelitis optica spectrum disorders (NMOSD). NMO is an inflammatory autoimmune disease of the CNS with predominant involvement of the optic nerves and the spinal cord. It has a later age of onset (35-47 years) and females are affected in 80.0-100.0% of cases. This disease, however, is infrequent and affects non-Caucasian populations more often (O'Riordan JI, 1996; Wingerchuck DM, 1999; Collongues N, 2010). Up to 90.0% of cases are recurrent (Wingerchuk DM, 2007; Collongues N, 2010) and relapses are characteristically severe in nature. Classically, these can involve only the optic nerves or the spinal cord and can occur at the same time or sequentially. Spinal cord MRI is characterized by a longitudinally extensive transverse myelitis (LETM) longer than 3 vertebral segments and is usually central, although depending on the timing of the MRI, this characteristic finding might not be observed (O'Riordan JI, 1996; Wingerchuck DM, 1999; Nakamura M, 2008). Brain lesions can be observed in approximately 60.0% of cases and MS-like lesions in 10.0-16.0% (Pittock SJ, 2006a; Wingerchuk DM, 2015). CSF findings, particularly during attacks, include pleocytosis comprised mostly by neutrophils, but eosinophils have been reported in 14.0-79.0%. A pleocytosis >50 cells/µl occurs in 46.0-75.0% of cases, and OCB can be present in 0-37.0% (O'Riordan JI, 1996; Wingerchuck DM, 1999; De Seze J, 2002; Bergamaschi R, 2004). A set of diagnostic criteria was proposed in 1999 (Wingerchuk DM, 1999); however, difficulties differentiating NMO from MS, particularly in early stages of disease, still existed (Rubiera M, 2006). After histopathological evidence pointed towards an antibody-mediated disease (Lucchinetti C, 2002), a serum IgG autoantibody called NMO-IgG was identified and thought to be a specific marker for NMO. NMO-IgG antibodies bind selectively to the aquaporin 4 (AQP4) water channel, a plasma membrane protein expressed in astrocytic end foot processes at the BBB (Lennon VA, 2004; Lennon VA, 2005). Besides, it was

observed that brain lesions usually occurred at areas of high AQP4 expression, and although most were asymptomatic, encephalopathy or hypothalamic syndromes could be present (Poppe AY, 2005, Pittock SJ 2006a, Pittock SJ 2006b). Afterwards, revised diagnostic criteria for NMO were published in 2006 and required optic neuritis, myelitis, and at least two of three supportive criteria: a contiguous spinal cord lesion on MRI extending over three or more vertebral segments, initial brain MRI not meeting usual diagnostic criteria for MS or seropositivity for NMO-IgG (Wingerchuck DM, 2006). Nevertheless, a multicentre study in France showed that, in 90.0% of cases, the association of optic neuritis and LETM with radiological findings was enough to fulfil the criteria (Collongues N, 2010). Furthermore, there were different detection methods for NMO-IgG and, although all had a specificity of 91.0-100.0%, sensitivity varied from 54.0 to 91.0% (Waters P, 2008). Ever since, new detection methods or combinations of methods were developed, increasing sensitivity to around 75.0% (Waters PJ, 2012; Saiz A, 2007; Höftberger R, 2013). However, up to 30.0% of patients are NMO-IgG negative. Therefore, search for other auto-antibodies has ensued. Some of the most studied have been the anti-MOG antibodies that, although more common in children, have been found in 4.0% of adults with demyelinating diseases (Reindl M, 2013). A study showed that patients with NMO, LETM or optic neuritis with negative NMO-IgG antibodies could have anti-MOG antibodies (Mader S, 2011). Besides, although information is still preliminary, patients with positive anti-MOG antibodies appear to have a more benign clinical course and, even if spinal cord lesions can involve the entire cord, including the conus medullaris, lesions tend to disappear after the event (Kitley J, 2012; Kitley J, 2014a; Sato DK, 2014; Höftberger R, 2015). Additionally, in the context of NMO-IgG seropositivity, a number of manifestations other than classical NMO have been identified and called NMOSD: incomplete forms with only LETM or optic neuritis, associations with other autoimmune diseases that can be systemic or organ-specific as well as with other auto-antibodies, presence of symptomatic or asymptomatic brain lesions, and Asian optico-spinal MS (Sellner J, 2010). Differential diagnosis of MS with NMO and NMOSD can thus be very complex, particularly at the time of

the CIS (Boiko A, 2002). Furthermore, this differential diagnosis is of utmost importance in Latin America and Asia, where a lower MS prevalence and a wider ethnic diversity coexist (Lana-Peixoto MA, 2008). In the 2010 McDonald criteria, it is recommended to investigate presence of NMO-IgG antibodies with a sensitive assay only in patients suspected of having NMO or NMOSD, especially in patients of Latin American or Asian ascent, due to the higher prevalence of NMO in these populations (Polman CH, 2011). All these findings and controversies (Arrambide G, 2014) led to the proposal of new diagnostic criteria in 2015, unifying NMO and NMOSD into the latter term and suggesting different criteria according to NMO-IgG status, which are more stringent in seronegative cases (Wingerchuk DM, 2015). The criteria acknowledge area postrema syndromes and other symptomatic cerebral symptoms as chore characteristics together with optic neuritis and LETM, introduce the concept of DIS in NMOSD, and consider optico-spinal MS a superseded term after evaluating that most cases might be NMOSD.

Another important issue in this differential diagnosis is preventive treatment as it differs from therapy for CIS and MS. There is now at least an ongoing phase 3 trial evaluating the safety and efficacy of complement inhibitor eculizumab in NMO (http://clinicaltrials.gov/show/NCT01892345). Azathioprine and rituximab have been recommended as first line therapy, with mycophenolate mofetil, mitoxantrone, and other medications as second line treatments (Sellner J, 2010; Costanzi C, 2011; Bedi GS, 2011; Jacob A, 2009). Differentiating NMO from MS is also important because observational evidence suggests IFNβ treatment may be harmful when used to treat patients with NMOSD (Papeix C, 2007; Shimizu J, 2010). Similar observations have been reported with natalizumab and fingolimod (Kleiter I, 2012; Kitley J, 2014b; Min JH, 2012).

A comparison between typical characteristics of MS, ADEM, and NMOSD is shown in **Table II-3**.

10. Treatment

Different treatments exist for demyelinating attacks, for modifying the disease course, and for symptoms. The focus in this section is on DMT. Randomized

controlled trials in CIS have shown that early treatment can delay conversion to CDMS, although there is little evidence regarding its influence on delaying disability progression. IFNβ and glatiramer acetate have been approved for their use in CIS (evidence grade 2A).

Table II-3. Comparison between MS, ADEM, and NMOSD.

Characteristics		MS	ADEM	NMOSD
Demographic and clinical	Age	30	<10	39
	Female:Male	2.3-1	1:1	9:1
	Typical presentation	Optic neuritis Brainstem syndromes Myelitis	Encephalopathy Seizures Focal signs and symptoms of neurological involvement Headache	Optic neuritis Brainstem syndromes Myelitis Dyencephalic syndromes Area postrema syndromes
	Clinical course	85% relapsing-remitting; evolution to secondary progressive 15% progressive from onset with/without activity	70% monophasic 30% multiphasic	80-90% recurrent 10-20% monophasic
Lab tests	CSF pleocytosis	Rare	++	+/++
	OCB	+++	+	+
	NMO-IgG	Rare	Extremely rare	+++
	Anti-MOG	Rare	++	Extremely rare
MRI	Brain	Periventricular, infratentorial, T1-hypointensities, Gd+/-	Big, diffuse cerebral lesions, deep gray matter involvement	Long ON/chiasmal, periaqueductal, dyencephalic or diffuse cerebral lesions
	Spinal cord	Short, peripheral lesions	LETM if myelitis	LETM, central lesions

Abbreviations: MS: multiple sclerosis; ADEM: acute disseminated encephalomyelitis; NMOSD: neuromyelitis optica spectrum disorders; CSF: cerebrospinal fluid; OCB: oligoclonal bands; MOG: myelin oligodendrocyte glycoprotein; Gd: gadolinium; ON: optic nerve; LETM: longitudinally extensive transverse myelitis.

10.1. IFNβ. IFNs are a family of secreted proteins involved in the immune reaction against viral infections, in regulation of cell proliferation, and in immune response modulation (Goodbourn S, 2000). There are two types of IFN: type I (α and β), secreted by leukocytes and fibroblasts, and type II IFN (γ), secreted by natural killer cells and T cells. The exact mechanism by which IFNβ is beneficial in MS has not been defined (Goodin DS, 2013). There are several clinical trials that have investigated the efficacy of IFNβ in CIS. The CHAMPS trial enrolled 383 CIS patients with radiological evidence of subclinical

demyelination (Jacobs LD, 2000). During three years of follow-up, patients treated with weekly intramuscular injections of IFNβ1a 30 μg had a significantly lower probability of evolving to CDMS than those who received placebo (cumulative probability 35.0 vs 50.0%). Patients enrolled in the CHAMPS trial were offered intramuscular IFNβ1a in the CHAMPIONS open label extension study (Kinkel RP, 2006). Those initially assigned to IFNβ were considered the immediate treatment group, whereas those assigned to placebo were considered the delayed treatment group. At five years, the immediate treatment group continued to have a lower risk of developing CDMS compared to the delayed treatment group [adjusted hazard ratio (aHR) 0.57, 95% CI 0.38-0.86]. The ETOMS clinical trial enrolled 308 patients and followed them for two years (Comi G, 2001). Significantly fewer patients treated with weekly subcutaneous IFNβ1a 22 μg developed CDMS in comparison to patients in the placebo arm (34.0 vs 45.0%). The time at which 30.0% of patients converted to CDMS was significantly longer in the IFNβ group compared to placebo (569 days vs 252). The number of T2-weighted lesions on MRI and the increase in lesion burden were significantly lower with IFNβ treatment. The BENEFIT clinical trial assigned 292 CIS patients to subcutaneous IFNβ2b 250 μg every other day and 176 patients to placebo (Kappos L, 2006). At two years, significantly fewer patients treated with IFNβ had converted to CDMS than those in the placebo group [28.0 and 45.0%, hazard ratio (HR) 0.5, 95% CI 0.36-0.70]. Similar results were found for the other primary outcome as defined by the McDonald criteria (69.0 and 85.0%, HR 0.54, 95% CI 0.43-0.67). Active treatment was also associated with significant reductions in the cumulative number of new active lesions and change in T2 lesion volume on brain MRI. In two follow-up blind studies, patients initially assigned to IFNβ were compared with those initially assigned to placebo with the option of starting IFNβ after a diagnosis of CDMS or after two years (Kappos L, 2007; Kappos L, 2009). Early treatment was associated with a statistically significant reduction in the risk of developing CDMS at three years (absolute risk reduction 14.0%) and at five years (absolute risk reduction 11.0%). Finally, the REFLEX clinical trial evaluated 517 CIS patients with at least two clinically silent T2 lesions on brain

MRI. At two years, the probability of diagnosing MS according to the McDonald criteria was significantly lower with subcutaneous IFNβ1a 44 µg dosed either three times a week or once a week (63.0 and 76.0% vs 86.0% for placebo) (Comi G, 2012). Both doses of IFNβ also significantly reduced conversion to CDMS (21.0 and 22.0% versus 38.0% with placebo). The effectiveness of all IFNβ for CIS patients was analysed in a meta-analysis with a total of 1160 patients (639 treated and 521 on placebo) (Clerico M, 2008). The probability of converting to CDMS was significantly lower with IFNβ treatment compared to placebo both at one year [pooled odds ratio (OR) 0.53, 95% CI 0.40-0.71] and at two years of follow-up (pooled OR 0.52, 95% CI 0.38-0.70).

10.2. Glatiramer acetate. Glatiramer acetate is a synthetic polypeptide initially developed in an attempt to exaggerate the development of EAE. Instead, it was found to confer protection to the animals (Teitelbaum D, 1971). Its mechanism of action is unknown (Goodin DS, 2013). In the PreCISe clinical trial, 481 CIS patients were enrolled (Comi G, 2009). Treatment with glatiramer acetate 20 mg subcutaneously daily significantly reduced the risk of conversion to CDMS (HR 0.55, 95% CI 0.40-0.77), prolonged time to CDMS for 25.0% of patients (722 days vs 336 for placebo), and reduced the frequency of conversion to CDMS (25.0% vs 43.0% with placebo).

10.3. Teriflunomide. This is an oral drug approved for its use in RRMS. It inhibits dihydroorotate dehydrogenase, an enzyme in the pyrimidine synthesis for DNA replication. Teriflunomide inhibits proliferation and activation of fast dividing cells and thus interferes with inflammation (Gold R, 2011). Given that resting immune cells use the salvage pathway for pyrimidine use, they do not rely on the *de novo* synthesis as activated lymphocytes, which are thought to be affected by this drug (Gold R, 2011; Claussen MC, 2012). Results from a clinical trial of teriflunomide in CIS (TOPIC) were recently published: this is a phase 3 randomized, placebo-controlled study comparing the efficacy of teriflunomide 7 and 14 mg vs placebo in CIS patients. The primary endpoint was the rate of conversion to CDMS (Miller AE, 2014). In total, 618 patients were included. Compared to placebo, teriflunomide significantly reduced the risk of CDMS both at the 14 mg [HR 0.574, (95% CI 0.379-0.869), p = 0.0087]

and at the 7 mg dose [HR 0.628, (95% CI 0.416-0.949), p = 0.0271]. Both doses also reduced the risk of developing new lesions on follow-up MRI.

10.4. Other. Although they have been approved for treatment of RRMS, dimethyl fumarate or alemtuzumab could be considered in CIS patients fulfilling the radiological 2010 McDonald criteria. Dimethyl fumarate is the methyl ester of fumaric acid. Combined with other fumaric acid esters, it was licensed in Germany as treatment for psoriasis (Mrowietz U, 2007). The CONFIRM trial assigned over 1400 RRMS patients to treatment with dimethyl fumarate at 480 mg daily in two doses, 720 mg daily in three doses, glatiramer acetate or placebo in a 1:1:1:1 ratio (Fox RJ, 2012). Compared to placebo, the annualized relapse rate at two years was lower in the three treated groups compared to placebo (0.22, 0.20, and 0.29 vs 0.40, respectively). The number of new or enlarging lesions on MRI was also significantly reduced in all treated groups compared to placebo, observing a trend towards significance in lowering the rate of disability accumulation. The DEFINE trial assigned 1200 RRMS patients to dimethyl fumarate 480 mg, 720 mg or placebo (Gold R, 2012). Treatment with dimethyl fumarate resulted in reductions in the annualized relapse rate at two years compared to placebo (0.17 and 0.19 vs 0.36, respectively), in the proportion of patients with disability accumulation (16.0 and 18.0% vs 27.0%), and in the number of new T2 lesions on MRI. Alemtuzumab is a humanized monoclonal antibody that causes depletion of CD52 T and B cells with varying times of recovery (Coles AJ, 2013). The CARE-MS I trial evaluated over 550 RRMS patients with no prior DMT (Cohen, JA, 2012), assigned to alemtuzumab or IFNβ1a 44 mcg in a 2:1 ratio. Alemtuzumab was administered intravenously at 12 mg daily for five days the first years and for three days at 12 months. At two years, alemtuzumab significantly reduced the annualized relapse rate compared to IFNβ (0.18 vs 0.39), with no significant effect on sustained disability accumulation. Regarding imaging findings, there was no difference between groups for median change in T2 lesion volume, although the group assigned to alemtuzumab had significantly fewer new or enlarging T2 lesions as well as fewer Gd-enhancing lesions. The CARE-MS II trial included almost 800 RRMS patients with at least one relapse while on treatment with IFNβ or

glatiramer acetate (Coles AJ, 2012). They were allocated in a 1:2 ratio to IFNβ1a 44 mcg or alemtuzumab 12 mg. Alemtuzumab significantly reduced the annualized relapse rate at two years (0.26 vs 0.52). A significantly lower rate of disability accumulation was observed in the group treated with alemtuzumab. MRI findings were similar to the CARE-MS I trial.

A summary of the possible treatment indications focused on CIS is shown in **Figure II-8** (adapted from Montalban X, 2014).

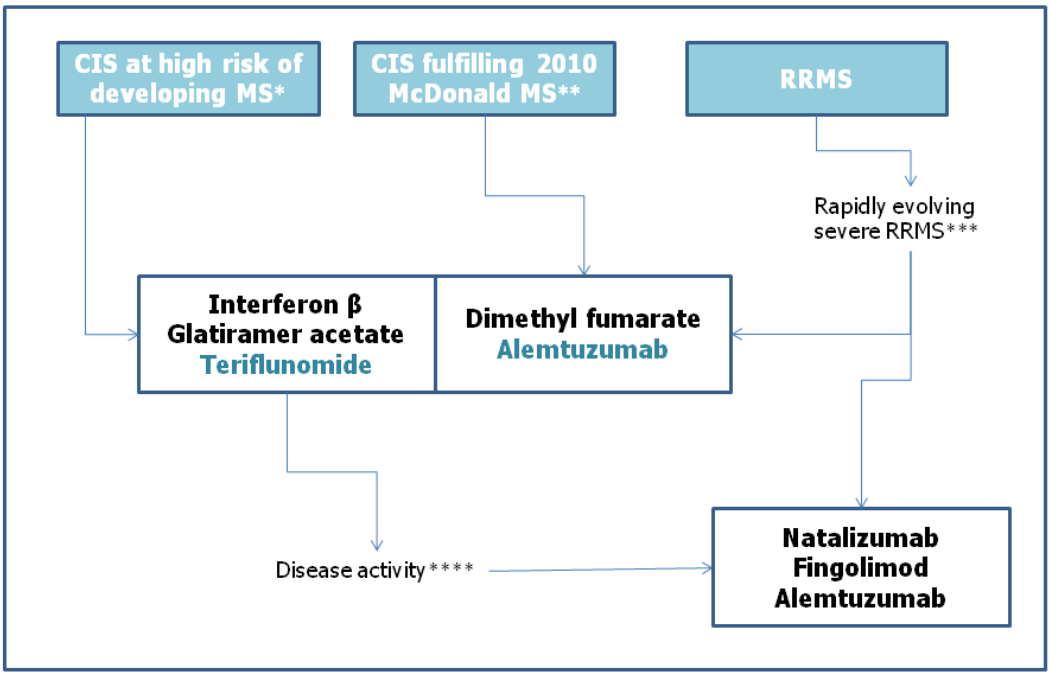


Figure II-8. Current indications for disease-modifying treatment in CIS and RRMS in Europe.
*9 T2 lesions and at least 1 new T2 or 1 Gd+ lesion on a follow-up scan.
**Not all MS, according to 2010 McDonald, have 9 T2 lesions.
***Two or more disabling relapses in one year with evidence of increasing lesions on two consecutive MRI scans.
****At least one relapse in the previous year whilst on therapy and evidence of active lesions on a brain MRI scan.
Abbreviations: CIS: clinically isolated syndrome; MS: multiple sclerosis; RRMS: relapsing-remitting multiple sclerosis.

11. Baseline prognostic factors for CDMS and disability progression at the time of the CIS

Accurately identifying which CIS patients will present a second demyelinating episode and, above all, determining the degree of disability they could develop over the medium- to long-term is considered crucial for a more individualized treatment. A number of baseline characteristics at the time of the CIS, with

varying degrees of predictive power over conversion and disability, can be assessed in everyday clinical practice (Arrambide G and Sastre-Garriga J, 2014).

11.1. Demographic and clinical factors. Whereas data on conversion to CDMS is more robust, information about demographic and clinical risk factors for disability progression at the time of CIS is weak; most of the available knowledge derives from long-term follow-up studies in patients who already have MS, in which absolute disability milestones such as time to secondary progression or to expanded disability status scale (EDSS) scores of 4.0, 6.0 or 7.0 are used.

11.1.1. Age and gender: Several studies have consistently shown that a younger age at the time of the CIS increases the risk of conversion to CDMS (Polman C, 2008; Mowry EM, 2009; Nilsson P, 2005). As for disability progression, it is generally accepted that a younger age at disease onset correlates with a younger age at the time of reaching disability milestones, although disease progression is slower (Tremlett H, 2006; Confavreux C 2003; Confavreux C, 2006). Regarding gender, no consensus about its influence on conversion to CDMS or on disability progression exists due to contradictory information in several studies (Dobson R, 2012; Mowry EM, 2009; Confavreux C, 2003; Confavreux C, 2006; Tremlett H, 2006).

11.1.2. CIS topography: Some studies have shown that fewer CIS patients presenting with optic neuritis convert to CDMS or that it takes a longer period for a second demyelinating episode to occur in comparison to CIS affecting other topographies (Miller D, 2005; Nilsson P, 2005). However, these results were obtained without taking possible confounding factors in consideration. For instance, when adjusting for CSF findings or presence of lesions on brain MRI, the risk of conversion to CDMS was very similar for all CIS topographies (Nilsson P, 2005; Tintore M, 2005). A small retrospective study showed that in CIS of the brainstem or cerebellum, the presence of facial sensory symptoms predicts a lower risk of conversion to CDMS in comparison to gait disturbances or diplopia (Sastre-Garriga J, 2010). Regarding disability, cerebellar symptoms have been associated with a worse prognosis (Miller DH, 2012). Although not in all studies, attacks with sphincter dysfunction have also been associated with a

worse outcome (Langer-Gould A, 2006). As for disability, median intervals from disease onset to reach the abovementioned EDSS milestones are longer for optic neuritis in comparison with other CIS topographies (Confavreux C, 2003); however, the presence of lesions on brain MRI was not taken into account and no symptom at onset predicted disability progression to an EDSS ≥6.0 in a later study (Tremlett H, 2006).

11.1.3. Monofocal vs multifocal CIS: Whether this is a prognostic factor for conversion remains somewhat controversial due to contradictory findings in different clinical trial subanalyses and observational studies. It may seem that the risk of converting to CDMS is probably higher in cases with more typical, monofocal presentations with a brain MRI suggestive of MS, although further studies might be needed to obtain more conclusive results (Comi G, 2001; Mowry EM, 2009; Polman C, 2008; O'Connor P, 2009; Nielsen JM, 2009). The role of multifocal CIS on disability progression appears to be better elucidated: involvement of multiple functional systems at onset has been associated with a higher probability of reaching disability milestones (Degenhardt A, 2009).

11.1.4. CIS severity and recovery: Results from previous studies do not appear to support CIS severity and recovery as prognostic markers of conversion to CDMS (Eriksson M, 2003; Mowry EM, 2009). On the other hand, time to reach disability milestones has been reported to be longer in patients with full recovery after a CIS (Eriksson M, 2003; Confavreux C, 2003). Besides, incomplete recovery from the CIS was found to be a strong predictor of future disability in a meta-analysis [hazard ratio (HR) 1.3-3.3] (Langer-Gould A, 2006).

11.2. Conventional brain MRI. To date, MRI remains the most reliable prognostic marker in MS according to the results of several studies in CIS. Presence of at least one lesion suggestive of demyelination or of at least 1-2 Barkhof criteria on baseline brain MRI scans confers an increased risk of developing CDMS and of disability progression over time in comparison with patients with no lesions or with 0 Barkhof criteria. This risk increases according to number of T2 lesions or of Barkhof and Swanton criteria (Fisniku LK, 2008; Optic Neuritis Study Group, 2008; Tintore M, 2006; Jacobs LD, 2000; Young J, 2009). Lesion topography on MRI is also predictive of conversion to CDMS.

Brainstem lesions alone or in combination with cerebellar lesions increase the probabilities of converting to CDMS and of reaching an EDSS ≥3.0 in comparison with patients with no infratentorial lesions. Furthermore, presence of infratentorial lesions independently increased three-fold the risk of reaching an EDSS ≥3.0 in patients with nine or more lesions on baseline MRI (Minneboo A, 2004; Tintore M, 2010).

11.3. Conventional spinal cord MRI. Even though it has been included as one of the possible topographies used to determine DIS in the 2010 revised McDonald criteria for the diagnosis of MS (Polman CH, 2011) and despite being commonly performed in the clinical setting in some centres, the role of spinal cord MRI to predict conversion to CDMS remains somewhat controversial, partly because it is a technically challenging study due to the size of the spinal cord and to motion artefacts (Agosta F, 2007). Specifically in CIS, some studies conclude that other than performing a spinal cord MRI when the presenting symptom is myelitis, a spinal cord MRI could also be considered when brain MRI findings demonstrate focal lesions not fulfilling criteria for DIS (Thorpe JW, 1996; Lycklama G, 2003; Rovira A and Tintore M, 2014). Results regarding spinal cord MRI and fulfilment of the McDonald criteria remain somewhat contradictory, probably due to the different versions of the criteria that have been used and to the differing baseline characteristics of the studied populations. A study of 115 patients with optic neuritis showed that 27.0% of them had spinal cord lesions, but when the 2001 McDonald criteria were applied, the number of patients fulfilling the criteria for DIS was only increased from 41 to 44 when adding the spinal cord lesions; and even though 12.0% of patients with normal MRI had spinal cord lesions, these were not taken into account since a normal brain MRI does not comply with DIS criteria (Dalton CM, 2003). A preliminary study from our group supports these findings, indicating that a spinal cord MRI is not always necessary since it adds limited information to the brain MRI (Rovira A, 2010). On the other hand, in a study that included 21 CIS patients, spinal cord lesions were observed at baseline in 67.0% of cases, and when applying the 2001 and 2005 McDonald criteria, the authors concluded that adding the lesions seen on spinal cord MRI slightly improved the

number of patients fulfilling DIS; nevertheless, this means that only two more patients had DIS when applying the 2005 vs the 2001 criteria in a group comprised by the CIS and early MS patients (Jacobi C, 2008). In the same line, a more recent study in non-spinal cord CIS patients showed that when adding spinal cord findings to brain MRI to diagnose one additional patient (2010 DIS and DIT), the number of cases needed to scan was 7 (Sombekke MH, 2013). The authors then recommended performing a spinal cord MRI in patients with non-spinal cord CIS who do not fulfil McDonald brain MRI criteria. As for evolution to CDMS, in a study that included 75 CIS patients, the multivariate analysis showed that the risk of conversion to CDMS increased more than three times in cases with one spinal cord lesion (HR 3.5, 95%CI 2.1-6.9) and 6 times with two or more spinal cord lesions (HR 5.9, 95%CI 3.2-10.9) (Patrucco L, 2012). Similarly, in 42 non-spinal cord CIS patients not fulfilling the 2010 McDonald criteria when exclusively assessing brain MRI, presence of one spinal cord lesion was associated with a higher risk of conversion to CDMS (OR 14.4; 95% CI 2.6-80.0) and shorter time to conversion to CDMS (HR 51.4; 95% CI 5.5-476.3] (Sombekke MH, 2013). As for disease progression, there is barely any evidence suggesting the usefulness of spinal cord MRI to predict disability progression in CIS.

11.4. IgG OCB. Presence of OCB in CSF is a predictive factor of conversion to CDMS independently of MRI (Tintore M, 2008b; Dobson R, 2013b). Presence of OCB was used as a DIS criterion together with at least two T2 lesions suggestive of MS on MRI (McDonald WI, 2001; Polman CH, 2005). A study showed that this DIS criterion yielded a diagnostic accuracy of 70.0% due to an increase in both specificity and negative predictive value (Zipoli V, 2009). However, it is no longer valid in the 2010 revisions to the McDonald criteria in the case of RRMS (Polman CH, 2011) since, although it confirms the inflammatory nature of the condition, it does not have any relation with the concept of DIS. But independently of its role in the diagnostic criteria, OCB determination is useful in the everyday practice in cases with atypical clinical presentations, or in patients with a CIS and a baseline MRI showing no lesions or lesions that do not comply with the current DIS criteria (Polman CH, 2011).

As for disability progression in CIS, a large study showed that presence of OCB does not predict reaching an EDSS ≥3.0 (Tintore M, 2008b). However, a recent meta-analysis concluded that presence of OCB is associated with reaching specific disability outcomes at follow-up compared with negative OCB determination (Dobson R, 2013b). An important limitation of this study is that presence of OCB was always considered as the unique prognostic factor.

11.5. Multimodal evoked potentials. A study showed that abnormal multimodal evoked potentials individually did not modify the risk of conversion to CDMS or of disability progression, but concomitant presence of three abnormal multimodal evoked potentials increased the risk of moderate disability by reaching an EDSS of 3.0 or more independently of baseline MRI; however, as the number of patients with three abnormal evoked potentials at baseline in this study was low [n=19 (8.0%)], their usefulness was rendered as limited (Pelayo R, 2010). A small study evaluated the role of motor evoked potentials to determine the risk of conversion to CDMS, and found that the contralateral silent period may be a parameter that might help identify patients who will develop a second demyelinating attack with a positive predictive value of 75.0% (Pallix-Guyot M, 2011). However, the practical usefulness of multimodal evoked potentials in predicting conversion to CDMS and disability progression seems to be limited.

Nevertheless, although most of the aforementioned studies are based on large multicentre cohorts, they have limitations such as a lack of standardized protocols, a retrospective nature or lack of systematic MRI or OCB data collection, often resulting in failure to include all these variables in multivariate models. A recent study overcame some of these limitations and was able to stratify risk factors for CDMS and disability progression in low (clinical and demographic), medium (OCB), and high (brain MRI) impact prognostic factors (Tintore M, 2015) (**Tables II-4** and **II-5**).

11.6. Combinations of prognostic factors. Although the 2010 McDonald criteria (Polman CH, 2011) have a higher sensitivity and similar specificity to the 2005 version (Polman CH, 2005), a significant proportion of CIS patients does not fulfil DIS and DIT at baseline and they are still at risk of developing MS. In

this sense, Ruet and collaborators analyzed a cohort of 114 spinal cord CIS patients and observed that age of onset ≤40 years, presence of OCB, and ≥3 periventricular lesions on brain MRI were independent predictive factors for MS (Ruet A, 2011).

Table II-4. Effect of baseline characteristics on conversion to CDMS.

CDMS	aHR	95% CI
Gender		
Male	1	
Female	1.0	0.8-1.2
Age		
40 to 49	1	
30 to 39	1.4	1.0-2.0
20 to 29	1.8	1.3-2.5
0 to 19	1.9	1.2-3.2
CIS topography		
Other	1	
Optic neuritis	0.9	0.7-1.2
OCB		
Absent	1	
Present	1.3	1.0-1.8
T2 lesions on baseline brain MRI		
0	1	
1 to 3	5.1	2.9-8.9
4 to 9	7.5	4.3-13.1
≥10	11.3	6.7-19.3
DMT		
After 2nd attack	1	
Before 2nd attack	0.9	0.6-1.2

Abbreviations: CDMS: clinically definite multiple sclerosis; aHR: adjusted hazard ratio; CI: confidence interval; CIS: clinically isolated syndrome; OCB: oligoclonal bands; MRI: magnetic resonance imaging; DMT: disease-modifying treatment.

Additionally, one interesting finding was that combinations of ≥2 predictive factors indicated conversion to MS with better accuracy than the 2005 and 2010 DIS criteria (78.2% vs 62.3% and 50.0%, respectively). Nevertheless, study

limitations included its retrospective nature and focus on spinal cord CIS. Besides, an updated comparison with the 2010 revisions of the McDonald criteria also including DIT would be necessary, since with these criteria the diagnosis of MS can be done with one single MRI.

Table II-5. Effect of baseline characteristics on progression to EDSS ≥3.0.

EDSS ≥3.0	aHR	95% CI
Gender		
Male	1	
Female	0.8	0.5-1.2
Age		
40 to 49	1	
30 to 39	0.7	0.4-1.2
20 to 29	0.6	0.3-1.0
0 to 19	1.0	0.4-2.1
CIS topography		
Other	1	
Optic neuritis	0.6	0.4-1.0
OCB		
Absent	1	
Present	2.0	1.2-3.6
T2 lesions on baseline brain MRI		
0	1	
1 to 3	0.9	0.3-2.1
4 to 9	1.2	0.5-2.8
≥10	2.9	1.4-6.0
DMT		
After 2nd attack	1	
Before 2nd attack	0.5	0.3-0.9

Abbreviations: EDSS: Expanded Disability Status Scale; aHR: adjusted hazard ratio; CI: confidence interval; CIS: clinically isolated syndrome; OCB: oligoclonal bands; MRI: magnetic resonance imaging; DMT: disease-modifying treatment.

12. Search for other markers of disease evolution at the time of the CIS

As previously mentioned, MRI remains the most reliable prognostic marker in MS. However, a diagnostic and prognostic marker specific for MS is still lacking and, at the time of disease onset, it is not possible to predict exactly which patients will evolve to CDMS or develop disability, rendering it necessary to perform a long-term follow-up and to repeat the MRI studies in some cases. Therefore, a search for specific, prognostic biological markers in CSF or serum is still deemed necessary. Biological markers in CIS that could be useful in the daily clinical practice should allow an early identification of patients who will convert to MS, be a differential diagnosis tool, act as prognostic factors for disease activity and disability accumulation, aid to better understand the pathogenesis of the disease in terms of inflammation and neurodegeneration or be markers of treatment response (**Figure II-9**).

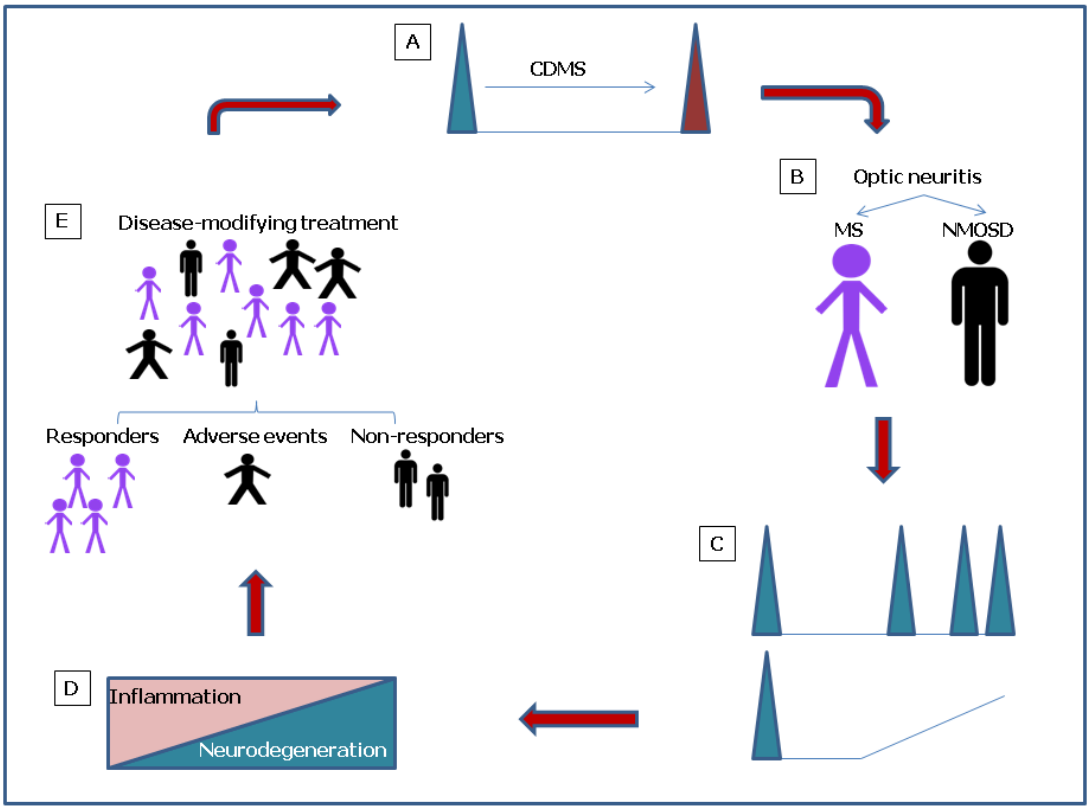


Figure II-9. Characteristics of useful biological markers in the daily clinical practice.
A: Prediction of conversion to CDMS after a CIS; B: biomarkers a differential diagnosis tool; C: prognostic factors for disease activity and disability accumulation; D: biomarkers of disease pathogenesis; E: biomarkers of treatment response.
Abbreviations: CDMS: clinically definite multiple sclerosis; MS: multiple sclerosis; NMOSD: neurmyelitis optica spectrum disorders.

They can be categorised in four groups: predictive, diagnostic, disease activity, and treatment-response markers, although a given marker can be useful for more than one category (Comabella M, 2014). In recent years, many potential CSF and serum markers for MS have been identified and several of them show initial promising results.

12.1. Neurofilaments. Neurofilaments are type IV intermediate filaments specific for neurons, are the main component of the myelinated axonal cytoskeleton, and are released into the CSF when axonal damage occurs, a finding observed in neurodegenerative diseases and stroke (Kuhle J, 2010; Petzold A, 2005a; Nylén K, 2006; Petzold A, 2007; Scherling CS, 2013). Neurofilaments are heteropolymers composed of three subunits: a light (NfL), a medium, and a heavy (NfH) chain. NfL is considered the most abundant subunit, being also the smallest and most soluble, whereas NfH is considered the most resistant to CSF proteases in its phosphorylated form (Petzold A, 2005a).

NfL levels have been found to be significantly higher in CIS patients who evolve to CDMS in comparison to those who remain as CIS (Teunissen CE, 2009), but this finding was not confirmed in later studies (Khalil M, 2013; Fialová L, 2013; Avsar T, 2012). Regarding disability, except for one, most studies have shown significant correlations of NfL levels with EDSS, but the analyses have included CIS and different MS subtypes, with the strongest correlations observed in progressive MS forms (Malmeström C, 2003; Teunissen CE, 2009; Norgren N, 2004; Kuhle J, 2013a; Semra YK, 2002). Khalil and collaborators evaluated CIS patients exclusively and found a correlation coefficient of r_s=0.324, $p<0.05$, but no separate results for CIS patients who converted to CDMS and those who remained as CIS were reported (Khalil M, 2013). It has been observed that progression to SPMS is more likely in patients with higher NfL levels according to different cut-off values, but this study was done with RRMS patients (Salzer J, 2010). As for NfL and MRI, correlations with T2 lesion number and Gd-enhancing lesions on baseline brain MRI have been reported in mixed CIS and MS cohorts (Teunissen CE, 2009; Burman J, 2014; Villar LM, 2015). However,

another study showed no correlations with Gd-enhancing lesions on baseline MRI, although it was carried out specifically in patients with acute optic neuritis (Modvig S, 2013). Similarly, a study of CIS patients with baseline and follow-up brain MRI at one year did not show any correlations between NfL levels and T2 lesion volume at baseline and at one year (Khalil M, 2013). As for neurodegeneration on brain MRI, a previous study did not find any correlations between NfL levels and baseline normalized volumes of whole brain, grey matter, white matter, cortical grey matter, and ventricular CSF (Khalil M, 2013). Additionally, NfL levels have been shown to decrease after natalizumab treatment, suggesting that NfL could be a marker of treatment response, although this implies performing more than one lumbar puncture (Gunnarsson M, 2011; Kuhle J, 2013b). In this sense, a new immunoassay to detect NfL in serum was recently developed and tested in patients with spinal cord injury, detecting higher levels in these cases in comparison with healthy controls (Kuhle J, 2014).

Regarding NfH, higher levels have been observed at an older age and in neurological disorders with axonal damage (Kuhle J, 2010). In MS, higher levels have been observed in progressive phases of the disease and during relapses (Brettschneider J, 2006; Teunissen CE, 2009). Correlations between NfH levels and EDSS have been reported, although in groups of patients with different MS phenotypes, including CIS (Teunissen CE, 2009; Kuhle J, 2011). As for MRI, a small study of CIS patients compared to controls demonstrated a correlation of NfH levels with brain volume change at one year ($r=0.518$, $p<0.01$), but not with change in T2 lesion load (Teunissen CE, 2012). Another study assessed the differences in NfH levels between CIS patients who evolve to CDMS and those who remained as CIS, finding lower levels in the converter group, although time of follow-up was limited. (Brettschneider J, 2006). NfH have also been tested as treatment response in patients with natalizumab, but NfL appear to be better biomarker in this setting (Kuhle J, 2013b).

12.2. Glial fibrillary acidic protein (GFAP). GFAP is the main intermediate filament found in astrocytes. High levels in CSF have been reported in neurological conditions like Alzheimer's disease and NMOSD (Jesse S, 2009;

Uzawa A, 2013; Storoni M, 2011; Takano R, 2008). GFAP CSF levels have been found to be higher in cohorts of patients with mixed MS phenotypes in comparison to normal controls, whilst higher levels also correlate with increasing disability in MS or in later phases of relapses (Norgren N, 2004; Malmeström C, 2003; Petzold A, 2002; Burman J, 2014). Another study showed that GFAP levels were significantly higher in CIS patients who converted to CDMS in comparison to those who did not when analysed together with other markers, but such significant difference was lost when analysing only GFAP (Avsar T, 2012).

12.3. Neurofascin. Neurofascin is a cell adhesion molecule of the L1 family. It is part of a protein complex that forms the paranodal junction that connects the myelin loop to the axon (Derfuss T, 2010). Presence of anti-neurofascin antibodies has been detected in MS. These antibodies recognize the extracellular domain of neurofascin 186, a neuronal protein found in myelinated fibers at the Ranvier node (Howell OW, 2006), and neurofascin 155, which is an oligodendrocyte-specific isoform (Maier O, 2005). Anti-neurofascin antibodies have been shown to be present in 10.0-30.0% of patients with MS (Mathey EK, 2007; Kawamura N, 2013). Specific studies in CIS have not been reported, nor has an assay directed to identify neurofascin instead of anti-neurofascin antibodies in MS.

12.4. Semaphorin 3A. Semaphorins (sema) comprise a family of secreted and transmembrane proteins. Secreted sema3A and 3F act as repulsive and attractive axonal guidance cues, respectively, and as chemotactic factors for oligodendroglial cells during development of the nervous system (Tsai HH, 2002). These are up-regulated in the MS brain (Williams A, 2007b). However, most studies have been performed in animal models or in brain tissue, with no evaluation of sema3A and 3F levels in the CSF of CIS patients (Syet YA, 2011; Piaton G, 2011). Furthermore, it is important to note that, given that the pathogenesis of MS involves both the immune system and the CNS and that sema3A seems to both terminate the immune response and inhibit the capacity of olygodendrocyte precursor cell migration towards the lesions, it is complicated to elucidate whether changes in sema3A expression would be

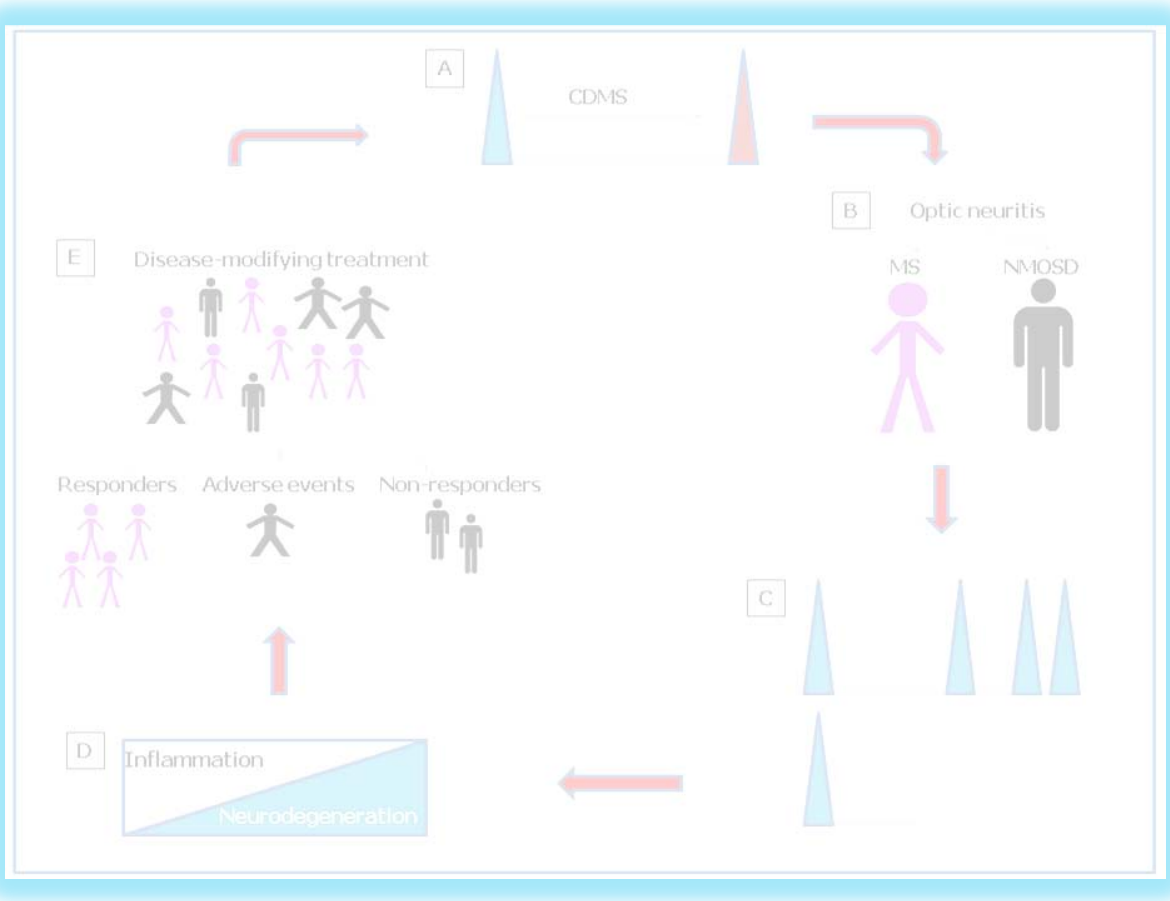
beneficial or damaging in MS (Eixarch H, 2013). More recent data suggest sema3A may create a negative environment for tissue regeneration in MS lesions (Costa C, 2015).

12.5. Fetuin A. Fetuin A is a glycoprotein involved in calcium metabolism and is a TNF synthesis inhibitor. Decreased serum levels have been reported in systemic infections and rheumatoid arthritis (Lebreton JP, 1979; Sato H, 2007). In MS, low CSF fetuin A levels were related to conversion to CDMS in CIS patients, although in later studies this result was not as clear (Tumani H, 2009; Ottervald J, 2010; Harris VK, 2010). Fetuin A has more recently been explored as a biomarker of treatment response in MS patients receiving natalizumab, observing that CSF levels decreased after treatment (Harris VK, 2013).

12.6. Anti-glycan antibodies. With the exception of neurofilaments and chitinase 3-like 1, all the aforementioned markers have been studied exclusively in CSF (Eikelenboom MJ, 2003; Petzold A, 2004; Fialová L, 2013a; Fialová L, 2013b; Kuhle J, 2014;). Given that an ideal biomarker should be non-invasive, it would be relevant to study potential markers that can be detected in serum (Teunissen CE, 2005). In this sense, antibodies against glycans have been detected in serum of patients with autoimmune diseases, incuding MS (van Kooyk Y, 2008; Menge T, 2005; Lolli F, 2005a; Lolli F, 2005b). Glycans consist of a large number of monosaccharides linked glycosidically. They cover the cell surface and are a major component of the extracellular matrix (Dove A, 2001; van Kooyk Y, 2008). In CIS, as part of the BENEFIT study, several antibodies directed against the glucose monosaccharides were analysed, including P63, [a polymer based on Glc(α1-3)Glc(α) and Glc(α 1-6)Glc(α)], alpha-ramose, alpha-N-acetyl glucose, and P64 [a polymer based on Glc(α 1-4)Glc(α) and Glc(α 1-6)Glc(α)]. Only P63 normalized to age predicted time to CDMS and was included as part of a dichotomic classification rule (positive vs negative) that identified patients at higher risk of conversion to CDMS in the first two years after the CIS, named gMS-Classifier2 (Freedman MS, 2009; Freedman M, 2010). However, this study requires validation and it was not assessed whether gMS-Classifier2 is an independent predictor of conversion to CDMS. On the

other hand, P63 was not a useful marker for disease progression according to the EDSS (Freedman MS, 2009; Freedman M, 2010; Freedman M, 2012).

12.7. Other. Many other biomarkers have been studied in MS. Among them, potentially useful proteins in the clinical practice include presence of IgM OCB, which appears to increase the risk of conversion to CDMS and is associated with an aggressive disease course (Villar LM, 2005; Ferraro D, 2013; Magraner MJ, 2012; Durante L, 2012), and chitinase 3-like 1, as higher CSF levels are independent predictors of conversion to CDMS (Comabella M, 2010; Cantó E, 2015). These two proteins have been discussed widely in other studies and are beyond the scope of the present work.



III. HYPOTHESES

III. Hypotheses

We started out with the following general hypothesis: biomarkers can contribute to the differential diagnosis and to better assess prognosis in patients presenting with a CIS.

Given that the search for diagnostic or prognostic markers in CIS is a very broad subject, in this thesis project we focused on inconclusive areas regarding markers already in use in the clinical practice and on further exploring and, if possible, validating, some of the potential markers of disease evolution that are still under research (**Figure III-1**).

1. We wanted to assess NMO-IgG as a biomarker of differential diagnosis: despite the availability of diagnostic criteria for both MS and NMO, an overlap between these two diseases exists, particularly in early stages. For this reason, we wanted to explore whether NMO-IgG determination at the time of presentation as CIS can differentiate between patients with relapsing optic neuritis or myelitis who will eventually develop MS from patients who will develop a classical NMO. The hypothesis is that NMO-IgG is negative in CIS patients with an NMO phenotype.
2. We decided to assess combinations of risk factors as prognostic markers of evolution to MS. Knowing that certain epidemiological, biological, and MRI data at baseline are predictors of CDMS, and as not all CIS patients fulfil the 2010 McDonald criteria at baseline, we wanted to study the risk of evolving to MS in those with combinations of ≥2 pre-established predictive factors (age ≤40 years, presence of OCB or ≥3 periventricular lesions on MRI) who do not fulfil the 2010 McDonald criteria for MS at baseline. The hypothesis is that these patients still have an increased risk of evolving to CDMS or of fulfilling McDonald MS over time.
3. We wanted to evaluate the role of spinal cord MRI as a diagnostic and prognostic marker of MS. Given the contradictory reports regarding the usefulness of spinal cord MRI for the diagnosis of MS in non-spinal cord CIS patients, we decided to assess its value in fulfilling the 2010 McDonald criteria when adding its findings to brain MRI in non-spinal

cord CIS. The hypothesis is that spinal cord MRI does help identify additional non-spinal cord CIS patients who fulfil 2010 DIS and DIT. Besides, we also decided to evaluate the role of spinal cord lesions as prognostic markers of evolution to MS and disability accumulation after a CIS, with the hypothesis that presence of at least one spinal cord lesion confers greater probabilities of reaching such outcomes.

4. As for serum markers under research, we wanted to study gMS-Classifier2 in CIS. We decided not to evaluate prognosis on disability progression as P63, which is part of the gMS-Classifier2, has not demonstrated its usefulness in this topic. Therefore, we focused on the need to validate previous findings regarding conversion of CIS patients who were gMS-Classifier2 positive, and on whether this classification rule is an independent biomarker of conversion to CDMS. The hypothesis is that gMS-Classifier2 differentiates CIS patients who develop CDMS early in the disease course from those who do not.
5. Regarding markers under research in CSF, this topic in itself is so wide that we decided to focus on proteins that have been identified as possible markers of tissue damage reflecting inflammation or neurodegeneration in MS, and that can be quantified with readily available immunoassays, but that still lack a proper validation of their role as markers of conversion to CDMS and of disability progression at the time of the CIS. The hypothesis is that CSF levels of these proteins differ in patients who remain as CIS from those who develop CDMS, and that they can also predict which patients will have disability progression.

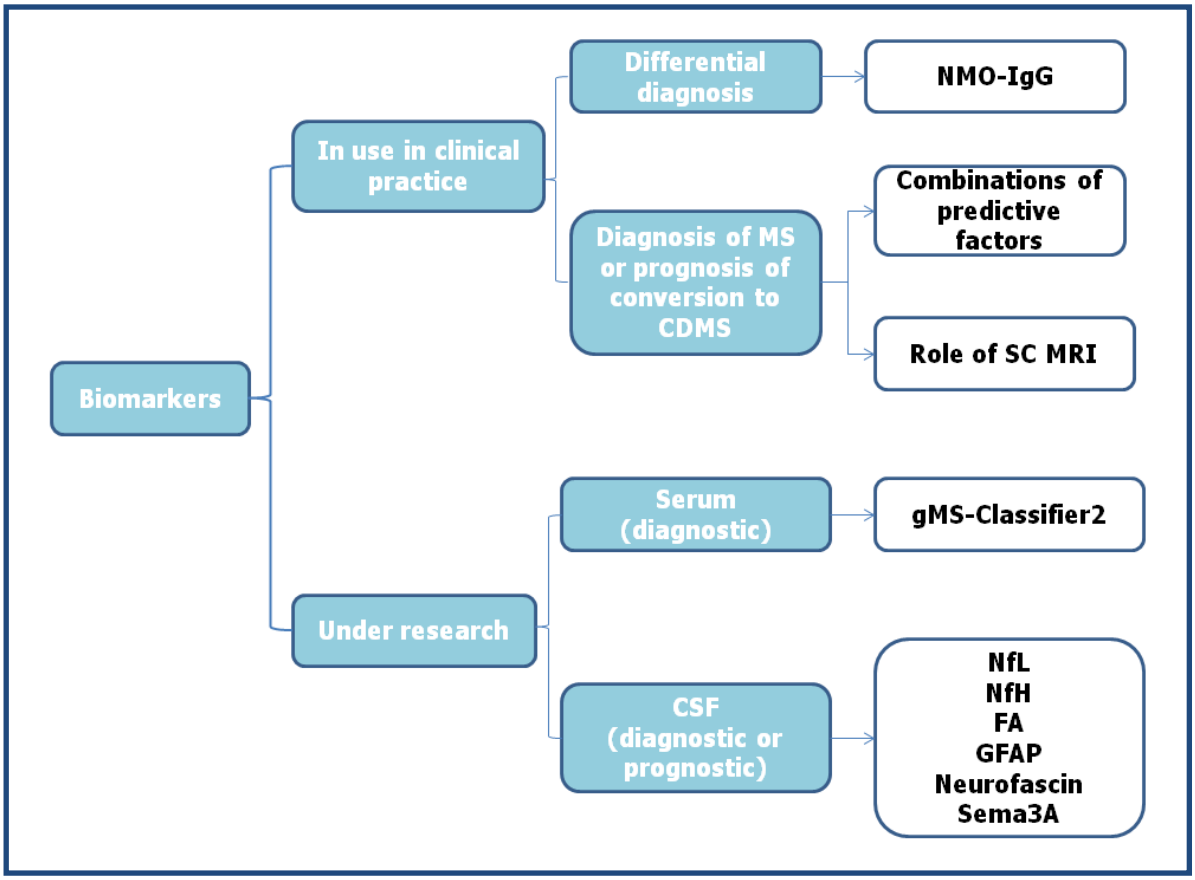
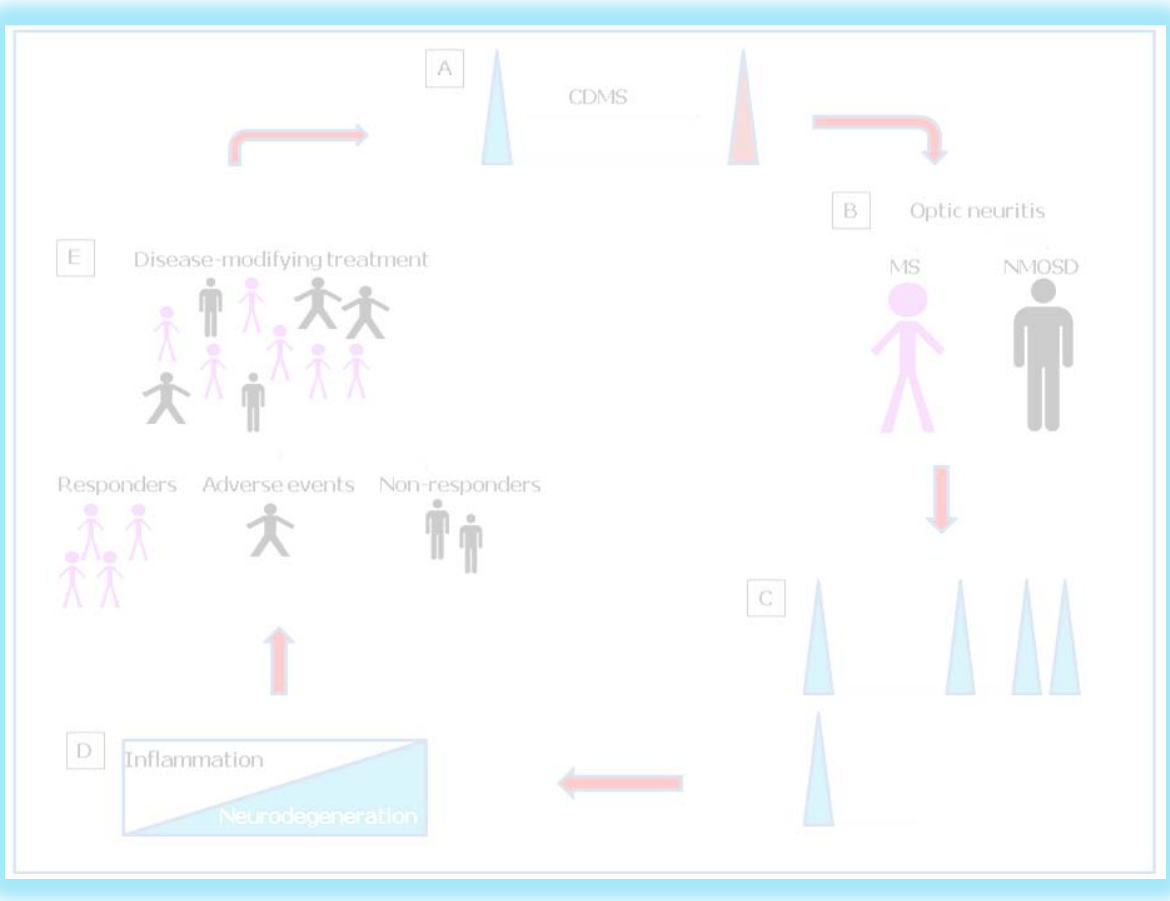


Figure III-1. Classification of markers under study in this project.
Abbreviations: NMO: neuromyelitis optica; MS: multiple sclerosis; CDMS: clinically definite multiple sclerosis; SC: spinal cord; MRI: magnetic resonance imaging; CSF: cerebrospinal fluid; NfL: neurofilament light subunit; NfH: neurofilament heavy subunit; FA: fetuin A; GFAP: glial fibrillary acidic protein; Sema3A: semaphorin 3A.

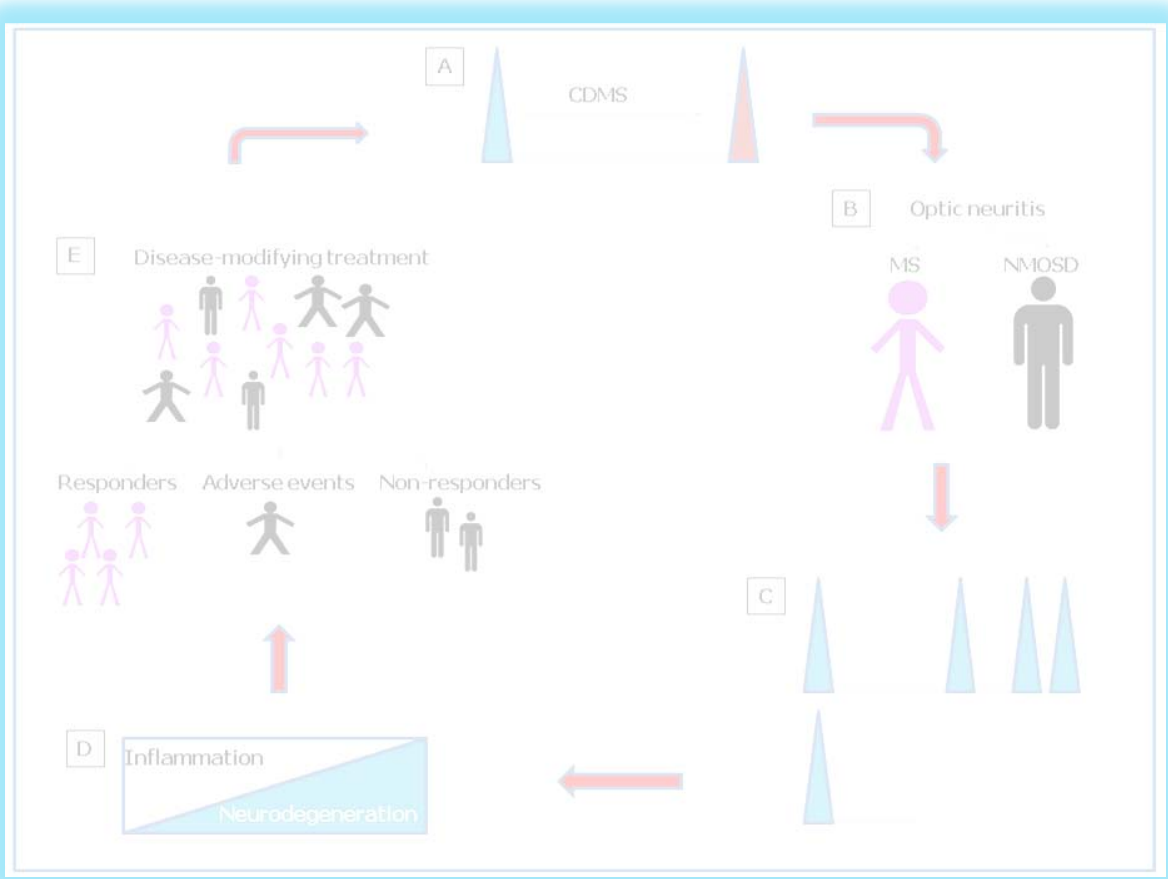


IV. OBJECTIVES

IV. Objectives

The objectives of this thesis are:

1. To assess the value of NMO-IgG status in a cohort of patients regarded as having a CIS at the time of presentation, who develop an NMO phenotype consisting of sequential or relapsing optic neuritis and myelitis.
2. To assess the added value of presence of ≥2 predictive factors for MS in patients not fulfilling the 2010 DIS criteria in a large prospective cohort of CIS patients, with separate evaluations for CDMS and 2010 McDonald MS.
3. To analyse the added value of spinal cord MRI in the fulfilment of the 2010 DIS and DIT criteria in non-spinal cord CIS patients, and to evaluate the prognostic value of spinal cord lesions for evolution to MS and disability accumulation.
4. To analyse the predictive value of gMS-Classifier2 determination in serum for early conversion to CDMS in a large cohort of CIS patients, and to determine whether gMS-Classifier2 is an independent predictor of conversion to CDMS.
5. To determine the value of selected biological markers in CSF as prognostic factors for conversion to CDMS and for disability progression in patients with CIS. Potential markers selected after a Pubmed search were: NfL, NfH, FA, GFAP, neurofascin, and sema3A.



V. MATERIALS AND METHODS

V. Materials and Methods

1. Study cohort description

All studies were based on a prospective open cohort of CIS patients with the following characteristics (**Figure V-1**):

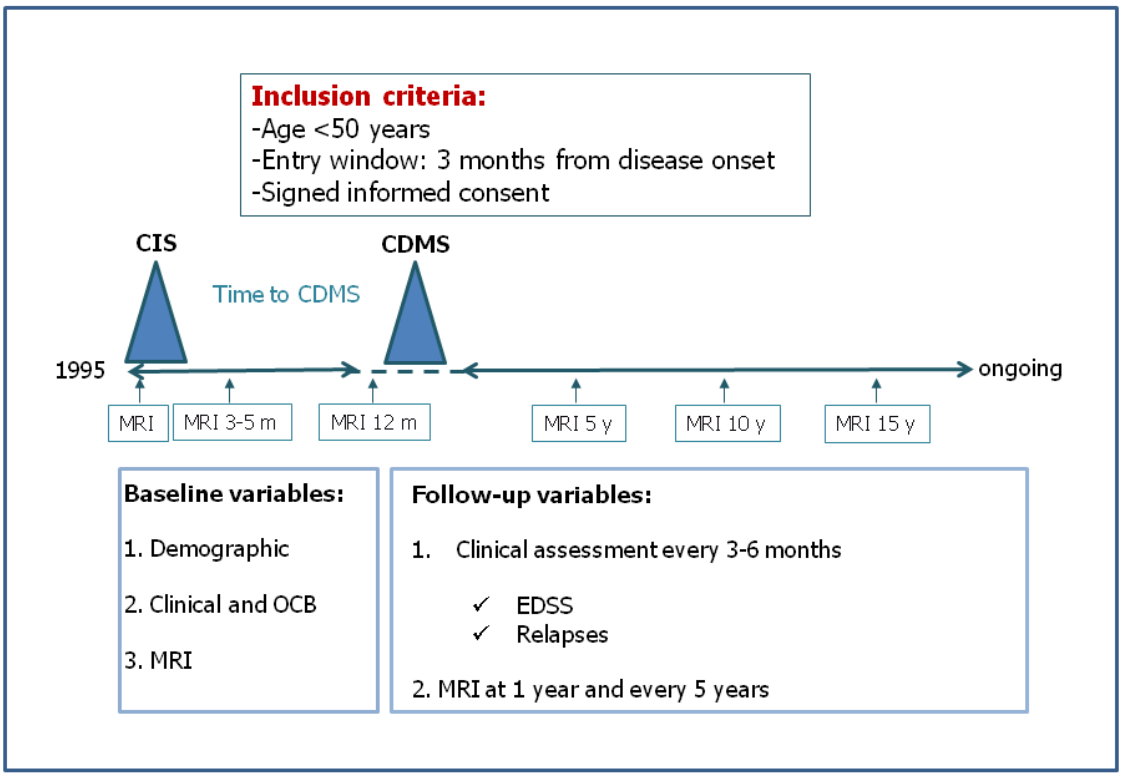


Figure V-1. Graph depicting the CIS cohort. Adapted from Tintore M, 2015.
Abbreviations: CIS: clinically isolated syndrome; CDMS: clinically definite multiple sclerosis; MRI: magnetic resonance imaging; m: months; y: years; OCB: oligoclonal bands; EDSS: expanded disability status scale.

1.1. Clinical data. Patients <50 years of age with a CIS suggestive of CNS demyelination and not attributable to other diseases were included if they were first seen within three months of disease onset. At baseline, the demographic data, previous history of neurologic abnormalities, the CIS topography, steroid use, and disability according to the EDSS score were recorded. Clinical follow-up was performed every 3-6 months, assessing for relapses and disability progression according to the EDSS measured during stability periods. Additionally, patients were evaluated in case of new symptoms or of worsening.

CDMS was diagnosed when a second attack occurred with a new neurological abnormality confirmed by examination (Poser CM, 1983). Disability accumulation was defined as either a sustained increase in EDSS of 1.0 point in one year or an EDSS ≥3.0. Time of follow-up was defined as the difference between the dates of the last visit and of the CIS.

1.2. Biological samples. Venous blood and CSF samples were collected simultaneously within three months of disease onset. CSF samples were centrifuged for 5 minutes at 1500 rpm to remove cells. Serum was prepared centrifuging the clotted blood for 10 minutes at 3000 rpm. Samples were then used for routine analysis that included biochemistry and determination of IgG OCB by agarose isoelectric focusing combined with immunoblotting (Andersson M, 1994). Remnant serum and CSF samples were aliquoted and stored at -80 °C.

1.3. MRI acquisition and analysis. Baseline brain MRI was performed within 3-5 months of disease onset and follow-up scans, at one year and every five years thereafter. Before 2001, diagnostic MRI scans performed <3 months after the CIS were also included, although the image acquisition was heterogeneous. Follow-up scans were performed at least 30 days after the last dose of steroids in case a given patient received such therapy. Baseline spinal cord MRI was routinely performed since 2007; they were previously performed only in case of CIS of the spinal cord. Scans were obtained at 3.0 Tesla (Trio, Siemens Medical Solutions, Erlangen, Germany) since 2010 and at 1.5 Tesla (Avanto or Symphony, Siemens Medical Solutions, Erlangen, Germany) beforehand, always with a standard head coil. The following conventional sequences of the brain were performed: dual echo T2-weighted fast spin-echo, transverse and sagittal T2-weighted FLAIR, and transverse T1-weighted spin-echo. The transverse T1-weighted sequence was repeated after Gd injection (0.2 mmol/Kg) in patients with focal white matter lesions suggestive of MS on T2-weighted sequences. Spinal cord sequences included sagittal dual echo T2-weighted fast spin-echo, sagittal short-tau inversion-recovery (STIR) and, in patients with brain Gd T1-weighted sequences or symptomatic spinal cord lesions, a Gd-enhanced sagittal

T1-weighted. Additional transverse T2-weighted fast spin-echo sequences were obtained to confirm signal abnormalities detected on sagittal views. For all brain sequences, 44–46 contiguous axial sections were acquired with a 3 mm section thickness. Spinal cord sequences were acquired with a contiguous 3 mm section thickness. MRI scans were assessed by neuroradiologists blinded to clinical follow-up. White matter lesions were defined as areas of increased signal intensity of at least 3 mm in diameter on T2-weighted images. MRI scans were considered abnormal if at least one lesion suggestive of demyelination was observed on the T2-weighted images. The number and location of lesions on T2-weighted images, number of Gd-enhancing lesions, and number of new T2 lesions were scored.

Additionally, volumetric analysis on brain MRI was performed since 2001. MS white matter lesion masks were created by a trained operator by delineating the white matter lesions on proton density sequences, with T2-weighted images as reference. Over time, lesion masks have been semi-automatically processed using different software packages with results that were not directly comparable. For this reason, since 2010, all previously acquired images as well as the newly obtained ones were re-assessed using the semi-automated Jim medical image display package (Xinapse Systems, Ltd., Colchester, UK). Similarly, brain parenchymal fraction (BPF) is currently being re-calculated using the Structural Image Evaluation, using Normalization, of Atrophy (SIENA) software, part of FSL (FMRIB Software Library) (Smith SM, 2004): single-time-point ("cross-sectional") analysis was performed with SIENAx (Smith SM, 2001; Smith SM, 2002), which estimates total brain tissue volume from a single image, normalized for skull size. It calls a series of FSL programs: It first strips non-brain tissue, and then uses the brain and skull images to estimate the scaling between the subject's image and standard space. It then runs tissue segmentation to estimate the volume of brain tissue, and multiplies this by the estimated scaling factor, to reduce head-size-related variability between subjects.

All sub-studies received approval from the local ethical committees and the included patients in each sub-study signed a written informed consent.

2. First objective: to assess the value of NMO-IgG status in a cohort of patients regarded as having a CIS at the time of presentation

2.1. Patients. Three different groups of patients were selected:

a. *Group 1:* patients selected from the abovementioned CIS cohort with a first demyelinating event involving the optic nerve or the spinal cord, and at least a second demyelinating event affecting either topography (NMO phenotype).
b. *Group 2:* patients selected from the CIS cohort with a first demyelinating event involving either the brainstem or the brain hemispheres, and at least one relapse affecting any topography (negative control group).
c. *Group 3:* patients selected from a retrospective NMO cohort at the Multiple Sclerosis Centre of Catalonia. Diagnosis was made according to the 2006 revised diagnostic criteria for NMO (Wingerchuk DM, 2006).

2.2. NMO-IgG assay. In all groups, the analysis was performed if a minimum of one 200 µL serum aliquot was available. Serum NMO-IgG was determined by indirect immunofluorescence (Lennon VA, 2004):

a. *Tissue:* mouse cerebellum, neocortex, hippocampus, kidney, and liver were obtained and frozen into OCT blocks using liquid nitrogen.
b. *Immunohistochemistry:* cryostat sectioning at 7 µm and placement of tissue on slides was performed, letting the preparation dry. Then, the slides were washed with phosphate buffered saline (PBS) and post-fixed for one hour with a 4% paraformaldehyde solution. The preparation was then washed with PBS and blocked with 10% goat serum in PBS for one hour. Patient sera were diluted at 1:60 in blocking solution to reduce interference from any coexisting non-organ-specific autoantibodies. The rabbit anti-rat AQP4 antibody (Chemicon AB3594) was used as a positive control at a 1:200 dilution, whereas for negative controls, the blocking solution was used. All samples (serum samples, positive and negative controls) were incubated overnight at 4ºC. Afterwards, they were equilibrated at room temperature for one hour and washed three times with PBS. Fluorescein isothiocyanate

(FITC) goat anti-human IgG (Southern Biotechnology Associates, Birmingham, AL, USA) diluted at 1:100 in PBS was used as secondary antibody and incubated for one hour at room temperature. For the positive control, biotinylated goat anti-rabbit IgG (Dako Corporation, Carpinteria, CA, USA) 1:200 in PBS was used, and goat anti-human IgG FITC served as negative control. The preparations were then washed three times with PBS. Only for positive controls, avidin-FITC at a 1:200 dilution in PBS was incubated for one hour at room temperature and afterwards it was washed three times with PBS. The preparations were then mounted using the Vestashield Mounting Medium (Vector Laboratories, Ltd., Peterborough, UK). Samples were then stored at 4ºC away from light sources. The slides were visualized using a Leica DMR microscope (Leica Microsystems, Wetzler, Germany).

This technique was previously validated in our laboratory with a sensitivity of 50.0%–60.0% and a specificity >90.0%, consistent with published data (Lennon VA, 2004; Saiz A, 2007). The result was considered positive when staining was observed in the pia mater, capillaries or in the kidney's distal collecting tubes.

2.3. Statistical analysis. Descriptive statistics were evaluated for baseline demographic and clinical characteristics in each group and Fisher exact tests for group comparisons were performed. Statistical analyses were performed at the 0.05 level of significance using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, Illinois, USA) version 18.0.

3. Second objective: to assess the added value of presence of ≥2 predictive factors for MS in patients not fulfilling the 2010 DIS criteria

3.1. Patients. Due to technical changes in MRI protocols in 2001, patients seen for the first time between 2001 and 2011 were selected. The assessed demographic, clinical, and biological variables were age, gender, CIS topography, OCB, and DMT. DMT was proposed to patients who had a second attack within three years of disease onset or to CIS patients fulfilling 3-4

Barkhof-Tintoré criteria (Barkhof F, 1997; Tintore M, 2000), according to the Catalan Institute of Health guidelines.

3.2. MRI. The 2010 McDonald criteria were applied: presence of ≥1 T2 lesion in ≥2 of the four MS-characteristic areas provided evidence of DIS in any MRI, excluding the symptomatic lesion in brainstem or spinal cord CIS. DIT was fulfilled by simultaneous presence of asymptomatic Gd-enhancing and non-enhancing lesions on any MRI, or when new T2 lesions appeared on follow-up scans (Polman CH, 2011).

3.3. Predictive factors. According to a previous publication by Ruet and collaborators, patients were classified according to age (≤40 or >40), OCB (present or absent), and periventricular lesions (≥3 or <3). A cut-off of ≥2 predictive factors was applied after observing similar outcomes for combinations of either two or three predictive factors (Ruet A, 2011).

3.4. Outcomes. CDMS was diagnosed after a second attack with a new neurological abnormality confirmed by neurological examination (Poser CM, 1983). The 2010 McDonald criteria were met by demonstration of DIS and DIT on baseline or follow-up MRIs. Patients with a second clinical attack also fulfilled the criteria (Polman CH, 2011).

3.5. Statistical analysis. Nonparametric descriptive statistics were performed. Results are shown as mean and standard deviation (SD) for continuous variables and as percentages for categorical variables. Uni- and multivariate Cox proportional hazard regressions were performed to assess the risk of CDMS in patients not fulfilling DIS at baseline was evaluated for ≥2 predictive factors and in the following combinations: age≤40 and ≥3 periventricular lesions, age≤40 and OCB, and ≥3 periventricular lesions and OCB. For comparison, the risk was also estimated for the 2010 diagnostic criteria as follows: DIS only, DIT only, and DIS + DIT compared to No DIS + No DIT on baseline MRI, all assessed together in one multivariate analysis to estimate their independent effects. A similar analysis for 2010 McDonald MS was done evaluating DIS and DIT unadjusted for each other and the combinations of predictive factors. CIS topography, gender, and DMT before MS diagnosis were considered potential covariates. As DMT was the only term with

a significant effect, it was included in the Cox model to obtain an aHR for prediction of CDMS or McDonald MS. Since treatment is not a baseline static characteristic, DMT was defined as a time-dependent variable. Finally, the performance of the predictive factors in the previously described combinations and of 2010 DIS and DIT on baseline MRI was assessed with CDMS as the outcome at two years of follow-up. Sensitivity, specificity, and accuracy, all with exact binomial 95% confidence intervals (CI), were calculated.

Statistical analyses were performed at the 0.05 level of significance using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, Illinois, USA) version 19.0.

4. Third objective: to analyse the added value of spinal cord MRI in the fulfilment of the 2010 DIS and DIT criteria in non-spinal cord CIS patients, and to evaluate the prognostic value of spinal cord lesions for evolution to MS and disability accumulation

4.1. Patients. From the CIS cohort, 100 patients with brain and spinal cord MRI at 3.0 Tesla and 107 with MRI at 1.5 Tesla were identified. Baseline demographic and clinical characteristics were compared (**Table V-1**). There were no significant differences other than in CIS topography, mostly due to a higher number of CIS of the optic nerve in the 3.0 Tesla group [43 (43.0%) for 3.0 Tesla and 27 (25.2%) for 1.5 Tesla, p=0.038 for all comparisons]. Furthermore, lesion detection on spinal cord MRI was similar between 3.0 and 1.5 Tesla for the entire CIS cohort, for spinal cord CIS cases, and for non-spinal cord CIS patients (**Table V-2**). Therefore, both CIS groups were merged (n=207) for the analysis.

4.2. Statistical Analysis. Descriptive statistics were performed on the demographic and clinical variables.

4.2.1. Spinal cord MRI and diagnosis of MS. Of 207 patients, 64 (30.9%) had a CIS of the spinal cord. Since an MRI of that topography would almost invariably be performed in such cases, we focused on the 143 (69.1%) with non-spinal cord CIS to evaluate the added value of spinal cord MRI using the

following approach: the proportion of patients fulfilling the 2010 DIS criteria (Polman CH, 2011) was determined, first with an alternative criterion in which only the brain MRI is assessed to evaluate the presence of ≥2 of 3 DIS criteria, and then with both brain and spinal cord MRI to evaluate the presence of ≥2 of 4 DIS criteria, to finally determine how many patients fulfilled radiological DIS and DIT in each case (**Figure V-2**).

Table V-1. Comparison of baseline variables: 1.5T vs 3.0T.

Clinical and demographical variables	SC 1.5T n=107	SC 3.0T n=100	p
Females: n (%)	71 (66.4)	60 (60.0)	0.388
Age: Mean (SD)	31.6 (8.1)	33.6 (8.0)	0.060
CIS topography: n (%) ON BS SC Other	 27 (25.2) 29 (27.1) 40 (37.4) 11 (10.3)	 43 (43.0) 22 (22.0) 24 (24.0) 11 (11.0)	0.038
Positive OCB*: n (%)	50 (55.6)	39 (45.9)	0.228
Baseline number of lesions on T2WI: n (%) 0 1-3 4-9 ≥10	 31 (29.5) 15 (14.0) 16 (15.2) 45 (42.1)	 41 (41.0) 8 (8.0) 18 (18.0) 33 (33.0)	0.155
Abnormal SC MRI: n (%)	54 (50.5)	39 (39.0)	0.124

*OCB: n=176, 90 in the 1.5 T and 86 in the 3.0 T groups.
Abbreviations: SC: spinal cord; MRI: magnetic resonance imaging; T: Tesla; CIS: clinically isolated syndrome; SD: standard deviation; ON: optic neuritis; BS: brainstem; OCB: oligoclonal bands; T2WI: T2-weighted images.

Table V-2. Detection of spinal cord lesions on MRI at 1.5 and 3.0 T.

	SC 1.5T	SC 3.0T	p
Total cohort*	54 of 107 (50.5)	39 of 100 (39.0)	0.124
SC-CIS*	33 of 40 (82.5)	17 of 24 (70.8)	0.353
Non-SC CIS*	21 of 67 (31.3)	22 of 76 (28.9)	0.855

* Number of patients (%) with SC lesions.
Abbreviations: SC: spinal cord; MRI: magnetic resonance imaging; T: Tesla; CIS: clinically isolated syndrome.

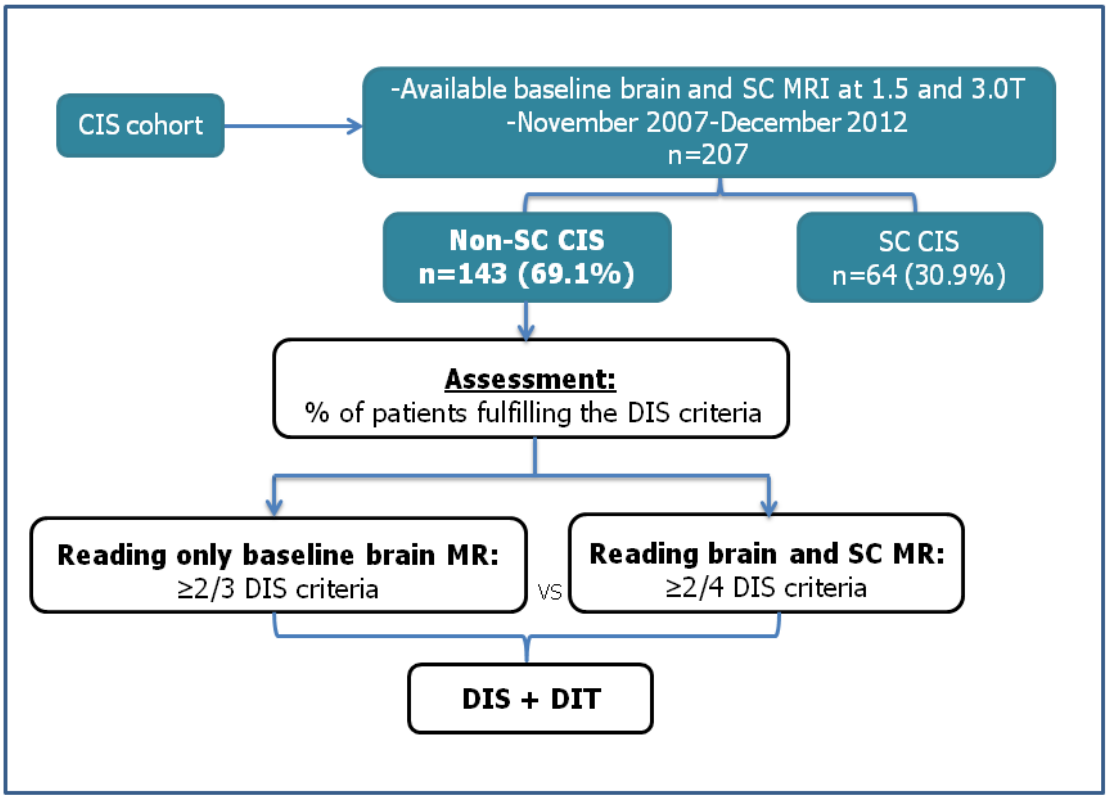


Figure V-2. Diagnostic value of spinal cord MRI: selection of patients (green) and MRI analysis (white). DIS and DIT are assessed according to the 2010 McDonald criteria (Polman CH, 2011).
Abbreviations: CIS: clinically isolated syndrome; SC: spinal cord; MRI: magnetic resonance imaging; DIS: dissemination in space; DIT: dissemination in time.

Afterwards, the number needed to scan to diagnose one additional MS case was calculated. The number needed to scan was determined based on the number needed to treat as follows: number needed to scan=1/absolute risk increase (re-named absolute diagnostic capacity increase), where absolute diagnostic capacity increase is the percentage change between the control group's event rate and the experimental group's event rate. The relative risk increase (renamed relative diagnostic capacity increase) was calculated dividing the absolute diagnostic capacity increase by the experimental group´s event rate.

4.2.2. Prognostic value of spinal cord lesions. Kaplan-Meyer analyses were performed and HRs and aHRs were calculated in the following groups

according to presence of spinal cord lesions with CDMS and clinical or radiological 2010 McDonald MS (Polman CH, 2011) as the outcomes:

- All cases (symptomatic spinal cord lesion accounted and not accounted for total lesion count)
- Non-spinal cord CIS cases
- Non-spinal cord CIS + pathological baseline brain MRI
- Non-spinal cord CIS + pathological baseline brain MRI not fulfilling DIS and DIT

To assess EDSS ≥3.0 as the outcome, all symptomatic and asymptomatic spinal cord lesions were taken into account. HR were calculated according to presence of spinal cord lesions in all cases (n=207) and in the following sub-groups: spinal cord (n=64) and non-spinal cord CIS (n=143).

In all multivariate survival analyses, potential covariates were age, gender, CIS topography, OCB, T2 lesion number on brain MRI, magnet field strength, and DMT (before CDMS, McDonald MS or EDSS ≥3.0) as a time dependent variable. A "missing" category was added for patients with no OCB determination.

Except when assessing EDSS ≥3.0 as the outcome, these analyses were also performed according to number of spinal cord lesions: 0, 1 or ≥2.

4.2.3. Performance of spinal cord lesions. Finally, the performance of spinal cord lesions detected on baseline MRI was assessed as a dichotomic variable with CDMS, 2010 McDonald MS, and EDSS ≥3.0 as the outcomes at two years in the previously mentioned patient groups. Sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV), all with exact binomial 95% confidence intervals (CI), were calculated.

Statistical tests were performed on the 0.05 level of significance using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) version 20.0.

5. Fourth objective: to analyse the predictive value of gMS-Classifier2 determination in serum for early conversion to CDMS in a large cohort of CIS patients, and to determine whether gMS-Classifier2 is an independent predictor of conversion to CDMS

5.1. Patients. Cases were selected from the CIS cohort based on the following eligibility criteria: consecutive patients older than 18 years of age first evaluated between 1995 and 2007, with a minimum of two available 200 µL stored serum aliquots that had not undergone previous thawing.

5.2. gMS-Classifier2. Serum samples and assessment:

a. *Serum samples handling:* De-identified and coded serum samples obtained at the time of enrolment in the department-wide sample repository were shipped to Glycominds, Ltd. (Modi'in, Israel) for analysis. Clinical data were not shared with collaborators at Glycominds until after the results of the serological analysis had been returned. Serum samples were thawed according to the following protocol to prevent IgM precipitation:
   - Samples were allowed to reach room temperature
   - Samples were incubated at 37ºC for 2 hours
   - Samples were vortexed to homogeneity

   IgM antibody measurement is stable if these conditions are met for no more than two freeze-thaw cycles.

b. *Assay:* Levels of anti-P63 IgM antibodies were measured in IgG-depleted serum samples by enzyme immunoassay (EIA) in duplicate in the following manner: first, microtiter 96-well plates were prepared by immobilizing P63 to the solid surface through a covalent link at its reducing end using an oligomer of 1,8-diamino-3,6-dioxaoctan (Sigma, St. Louis, MO) as a linker (Schwarz M, 2003). Second, IgG was depleted from the samples using rheumatoid factor removal reagent (Chemicon, Australia, Cat. RFRR) according to the manufacturer's instructions. Following IgG removal, serum samples (using a dilution of 1:600) were dispensed into microtiter 96-wells in duplicate, incubated for 180 minutes at 4ºC, and washed with wash buffer. Then, bound antibodies were labeled with horseradish peroxidase (HRP)-conjugated goat anti-human IgM antibody, washed, and 3, 39, 5, 59-

tetramethylbenzidine substrate was added for detection. After 30 minutes, the enzymatic reaction was stopped by adding 1.0% sulphuric acid solution, and the optical density (OD) was read at 450 nm using a Victor 1420 plate reader (Wallac, Turku, Finland). Each plate included a 5-point calibration curve. Anti-P63 serum levels were reported in arbitrary enzyme-linked immunosorbent assay (ELISA) units (EU). gMS-Classifier2 units were calculated according to the following algorithm: [1.171 - 0.082 x age in years at the time of blood collection]+[0.015 x anti-P63 (EU)]. The gMS-Classifier2 was considered positive when the number of units was equal to or greater than 0.289 (Freedman M, 2010; Yarden J, 2010).

5.3. Outcome. A diagnosis of conversion to CDMS was made when new symptoms occurred after an interval of at least one month and only when other diagnoses had been excluded (Poser CM, 1983).

5.4. Statistical analysis. Parametric and nonparametric comparative statistics were performed depending on the normality of the distributions of the continuous variables. Fisher's exact test was performed to compare categorical variables. Kaplan-Meier analysis was used to estimate cumulative survival probabilities and to construct survival plots. To assess whether gMS-Classifier2 can independently predict time to CDMS, a multivariate analysis using Cox proportional hazard regression was performed for both gMS-Classifier2 status (positive or negative) and continuous values. Baseline MRI parameters such as number of Barkhof criteria, number of T2 lesions (0, 1–9, >9 lesions) and OCB were considered as potentially relevant covariates. Age was already included in the gMS-Classifier2 algorithm as a covariate; the role of gender and CIS topography as possible covariates was also evaluated. Time to event analysis was performed primarily at two years; it was additionally assessed at five years and total time of follow-up to evaluate the length of time during which the biomarker could be useful. To assess the clinical utility of gMS-Classifier2, a Hosmer and Lemeshow goodness-of-fit test was performed as a calibration measure for two models: one with number of Barkhof criteria, OCB and gMS-Classifier2; and one for number of T2 lesions, OCB and gMS-Classifier2. As

discrimination measures, two receiver operating characteristic (ROC) curve analyses were made:

a. One model using number of Barkhof criteria, OCB and gMS-Classifier2 continuous units and compared with a model without OCB.
b. The second ROC curve analysis compared one model using number of T2 lesions, OCB and gMS-Classifier2 continuous units versus another in which OCB were excluded.

Statistical tests were performed at the 0.05 level of significance using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) version 17.0.

6. Fifth objective: to determine the value of selected biological markers in CSF as prognostic factors for conversion to CDMS and for disability progression in patients with CIS

This study was divided into a screening and a replication phase.

6.1. Screening phase.

6.1.1. Patients. Two opposite groups of patients with a follow-up of at least two years were selected from the Vall d'Hebron cohort to screen for differences in biomarker levels:

- *CIS-CDMS (n=33-38):* patients with 3-4 Barkhof-Tintore criteria on baseline MRI and presence of OCB who had converted to CDMS.
- *CIS-CIS (n=33-39):* patients with 0 Barkhof-Tintore criteria on baseline MRI and absence of OCB who had remained as CIS.

Besides, two control groups were selected: the group with other inflammatory neurological diseases included 34 patients with the following diagnoses: Guillain-Barré Syndrome (n=7), chronic inflammatory demyelinating neuropathy (n=11), viral encephalitis (n=7), bacterial meningitis (n=4), systemic lupus erythematosus with central nervous system (CNS) involvement (n=1), neurosarcoidosis (n=1), neurosyphilis (n=2), and primary vasculitis of the CNS (n=1). The group with non-inflammatory neurological diseases included 24 patients distributed as follows: stroke/transient ischemic attack (n=6), primary headache (n=8), Alzheimer's Disease (n=2), Parkinson's Disease (n=1), non-

inflammatory neuropathies (n=2), spinocerebellar ataxia (n=1), and amyotrophic lateral sclerosis (n=4).

According to availability, samples from the same patients were used for each biomarker determination.

6.1.2. Biological markers. Potential biomarkers that could either be indicators of pathogenic processes or that could predict conversion to CDMS were selected after an initial Pubmed search in 2008 that was regularly updated throughout the duration of the study and that included the following terms: "biological markers" or "biomarkers" and "multiple sclerosis" or "clinically isolated syndromes". The following proteins were selected: fetuin A, GFAP, NfH, NfL, neurofascin, and sema3A.

Except for NfH, levels of the selected biomarkers were determined using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' recommendations: fetuin A (Biovendor, Brno, Czech Republic, Catalogue Number RD191037100R), NfL (Umandiagnostics AB, Umeå, Sweden, Catalogue Number UD51001), GFAP (Abnova, Taipei City, Taiwan, Catalogue Number KA0024); neurofascin (USCN Life Science Inc., Wuhan, China, Catalogue Number SEL939Hu); and sema3A (USCN Life Science Inc., Wuhan, China, Catalogue Number SEL917Hu). Assays were optimized using varying dilutions of CSF measured in duplicates and read in an ELx800 Absorbance Reader (BioTek Instruments, Inc., Winooski, VT, USA).

As for NfH, CSF levels were determined using an *electrochemiluminescence* based solid-phase sandwich immunoassay (Kuhle J, 2010) at the University Hospital Basel (Basel, Switzerland), selected among different assays for its higher sensitivity (Teunissen CE, 2009; Shaw G, 2005; Petzold A, 2003). Clinical data were not shared with collaborators at University Hospital Basel until after the results of the CSF analysis had been returned.

The following were the reagents used:

- *Antibodies:* capture monoclonal antibody SMI 35 (Covance, Emeryville, CA), secondary (detector) polyclonal rabbit anti-$NfH^{SMI\ 35}$ antibody

(Sigma-Aldrich, Saint Lousi, MO), and the indicator polyclonal Sulfo-TAG labeled goat anti-rabbit antibody (ruthenylated) (MSD, Gaithersburg, MD).

- *Chemicals:* barbitone, bovine serum albumin (BSA), ethylenediaminetetraacetic disodium salt (EDTA), sodium chloride, PBS, Tris base, and Tween 20 were of analytical grade (Sigma-Aldrich, Saint Louis, MO).
- *Standards:* Bovine NfH was obtained from USBiological (United States Biological, Swampscott, MA). Standards were diluted in Tris buffered saline (TBS) containing 1% BSA, 0.1% Tween 20, and 0.06 mM EDTA, pH 7.5 and ranged from 0 to 2500 pg/mL. The standards were stored at -20ºC.

The analytical procedure was as follows:

- The 96-well plates (Multy-Array® plates, Meso Scale Discovery, Gaithersburg, MD) include integrated screen-printed carbon ink electrodes on the bottom of the wells. Coating was done overnight with 25 µL of capture antibody diluted 1/2500 in PBS (pH 7.4).
- All following incubation steps were done with vigorous shaking (800 rpm) and were preceded by three wash steps with 200 µL of TBS containing 0.1% Tween 20 (pH 7.5) per well.
- Unspecific binding sites were blocked with 25 µL of TBS containing 3% BSA per well for one hour.
- After washing, 25 µL of TBS containing 1% BSA, 0.1% Tween 20, and 50 mM Barbitone were added as sample diluent to each well
- 25 µL of standard, control or CSF sample was then added in duplicate and the plate incubated at room temperature for one hour.
- After washing, 25 µL of the secondary antibody diluted 1/2000 in TBS containing 1% BSA, 0.1% Tween 20, and 50 mM barbitone was added to each well and the plate was incubated for two hours at room temperature

- After washing Sulfo-TAG labeled goat anti-rabbit antibody diluted 1/2000 in TBS containing 1% BSA and 0.1% Tween 20 was added and incubated for one hour at room temperature.
- Following a final wash, 150 µL of enhanced chemiluminescence (ECL) read buffer (MSD, Rockville, MA, USA) diluted 1:2 with distilled water was added and the ECL signal, detected by photodetectors, was measured using the SECTOR Imager 2400 plate reader (MSD, Rockville, MA, USA).
- A four-parameter weighted logistic fit curve was generated, sample concentrations extrapolated and analysed using the Discovery Workbench 3.0 software (MSD, Rockville, MA, USA).

6.2. Replication phase in unselected CIS patients

6.2.1. Patients. For the replication phase, a second cohort recruited at the Neurology Department of Ramon y Cajal University Hospital in Madrid was also assessed. Inclusion and follow-up criteria were similar to those of the Vall d'Hebron cohort and OCB determination followed the same method as previously described. As for imaging studies, baseline brain MRI was performed within the first three months after disease onset, whereas baseline spinal cord MRI was performed only in cases of myelitis. Follow-up MRI scans were performed 12 months after the CIS. MRI acquisition followed the same protocol as described for the Vall d'Hebron cohort. The scans were obtained on a 1.5 Tesla magnet. The number and location of lesions on T2-weighted images, number of Gd-enhancing lesions, and number of new T2 lesions were scored. T2 lesion volume and BPF were not estimated in this cohort.

This phase had the following, less stringent selection criteria: any combination of clinical, MRI or CSF findings on consecutive patients with availabe CSF samples from the Vall d'Hebron and Ramon y Cajal cohorts (n=155).

6.2.2. Biological markers. Biomarkers that showed significant differences between extreme groups in the screening phase were evaluated in a replication phase to establish their added value as independent predictors of conversion to

MS. Only NfL levels showed significant differences. Patients from Vall d'Hebron were selected from a different time range (years 2009-2011) than those from the NfL screening phase due to sample availability. NfL determination was performed in each hospital separately using the abovementioned ELISA kit.

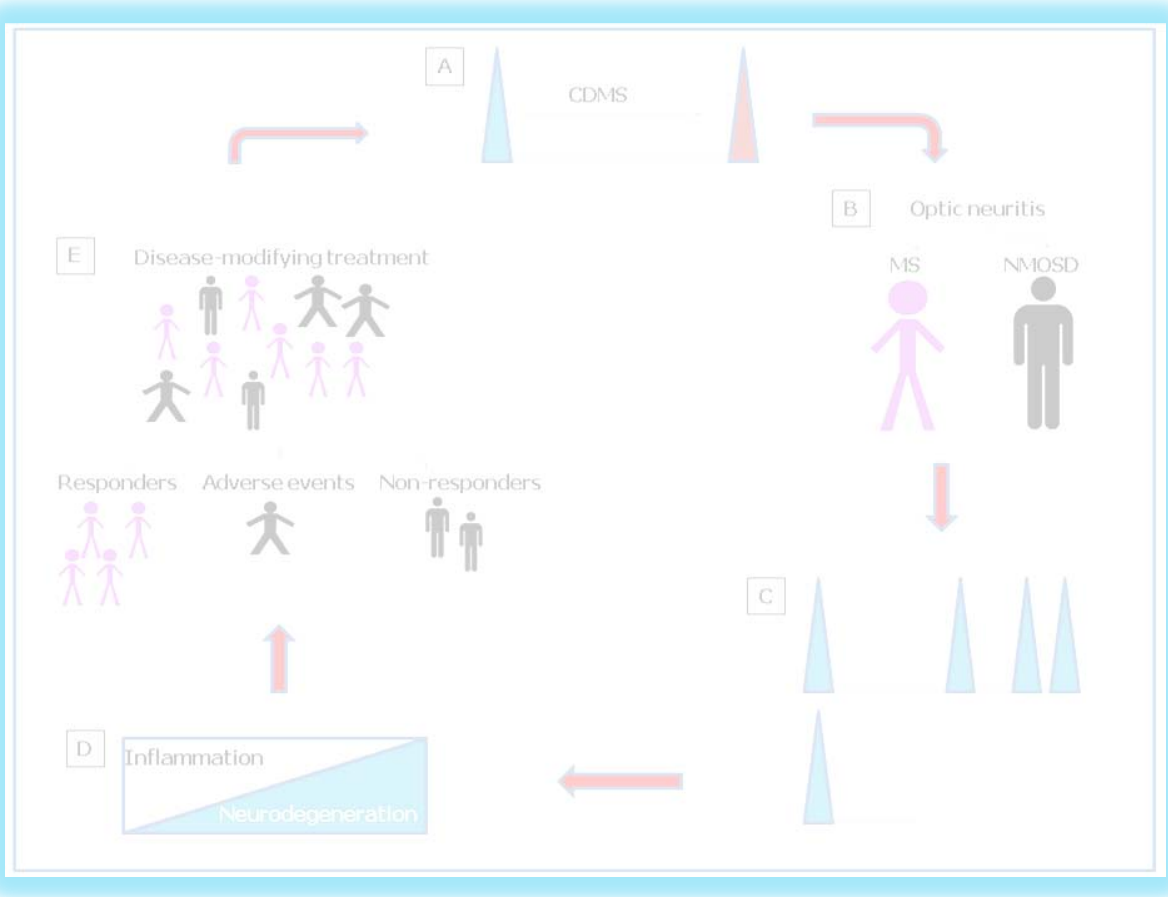
6.3. Statistical analysis. Parametric and non-parametric descriptive statistics were performed depending on the normality of the distributions of the continuous variables in both the screening and the replication phase.

6.3.1. Screening phase. Sample size for each biomarker was calculated for 80% power at the 0.05 level of significance based on previous data and it was fulfilled in every case. When necessary, biomarker levels were log-transformed for a more normal distribution in subsequent analyses. CIS groups were compared in terms of conversion to CDMS and of disability accumulation using generalized linear models for group comparisons, with the Bonferroni correction for multiple comparisons when additionally evaluating the control groups. Data are reported as the estimated mean (95% CI). Age, gender, CIS topography, time from CIS to lumbar puncture, frozen sample storage time, and DMT before CDMS were considered potential covariates. Additionally, Spearman correlations with EDSS at baseline and at one to five years of follow-up were assessed. Correlations were calculated with the Spearman test for MRI inflammatory parameters at baseline, one, and five years (T2 lesion number and volume, number of Gd-enhancing lesions, and number of new T2 lesions when applicable), whereas partial correlations were assessed for MRI neurodegeneration parameters at one and five years of follow-up [BPF percentage change (BPFΔ) adjusted for age and baseline Gd-enhancing lesions]. BPFΔ estimated by SIENAx was calculated subtracting the baseline from the one- and five-year follow-up estimates and dividing by the baseline values, then multiplying by 100. PBVC estimated by SIENA was also assessed for changes from baseline to one and five years. Finally, Spearman correlations between NfL and NfH levels were also assessed in a subgroup of 42 patients in which both biomarkers were evaluated.

6.3.2. Replication phase. Primary endpoints were conversion to CDMS and 2010 McDonald MS according to NfL status (positive/negative) based on a cut-

off value of 900 ng/L obtained from a control group with non-inflammatory or neurodegenerative diseases by calculating the mean + 3 standard deviations of the control values (Villar LM, 2014). To evaluate whether NfL levels independently predict time to MS, uni- and multivariate analyses using the Cox proportional hazard regression were performed for NfL levels as a continuous or dichotomic (positive-negative) variable and evaluated with OCB and T2 lesion number on baseline MR. DMT as a time-dependent variable and hospital where NfL levels were determined were considered as covariates. Generalized linear models were used to compare NfL levels in terms of disability accumulation (EDSS ≥3.0). Spearman correlations or generalized linear models were used to determine the association between NfL levels, T2 lesion number and volume, new T2 lesion number and volume when applicable, and Gd-enhancing lesions at baseline and one year. Results for NfL levels according to semi-quantitative number of T2 lesions are displayed in box-and-whisker plots.

All statistical tests were performed on the 0.05 level of significance. The IBM SPSS Statistics (SPSS Inc., Chicago, IL, USA), version 20.0 was used. Graphs were also prepared using Graph Pad Prism 5.02 for Windows (GraphPad Software, San Diego, CA).


A
CDMS
B
Optic neuritis
MS
NMOSD
C
D
Inflammation
Neurodegeneration
E
Disease-modifying treatment
Responders
Adverse events
Non-responders

VI. RESULTS

VI. Results

1. First objective: to assess the value of NMO-IgG status in a cohort of patients regarded as having a CIS at the time of presentation

Between 1995 and 2008, 822 patients were included in the CIS cohort. Of them, 97 fulfilled the group 1 (NMO phenotype) criteria and had available serum samples and 51 were included in group 2 (negative controls). Fourteen patients were identified for group 3 (retrospective NMO positive control group), of which 9 had serum samples available. The results of NMO-IgG detection and group characteristics are summarized in **Table VI-1** and a characteristic NMO-IgG antibody determination is shown in **Figure VI-1**. Mean (SD) follow-up was 7.1 (3.1) years for group 1 (NMO phenotype), 7.4 (3.1) for group 2 (negative control group), and 4.5 (2.2) for group 3 (NMO group). Only 3 patients (3.1%) in group 1 (NMO phenotype) were positive for NMO-IgG in comparison to 4 (44.5%) of the control cases (group 3) fulfilling the 2006 diagnostic criteria for NMO. Two patients (3.9%) in group 2 (negative control group) were positive for NMO-IgG. After obtaining the abovementioned proportions of NMO-IgG positivity in groups 1 and 2, no significant difference was found between these results (p=0.640). The characteristics of all CIS patients who were positive for NMO-IgG are shown in **Table VI-2**; only one of them developed NMO during follow-up.

Table VI-1. Demographic characteristics and results of NMO-IgG determination in each studied group*.

	Group 1 NMO phenotype	Group 2 Negative controls	Group 3 NMO
n	97	51	9
Females (%)	67 (69.1)	31 (61.0)	8 (89.0)
Median age in years (range)	28 (13-48)	25 (17-47)	36 (22-49)
Mean (SD) follow-up in years	7.1 (3.1)	7.4 (3.1)	4.5 (2.2)
NMO-IgG+ patients (%)	3 (3.1)	2 (3.9)	4 (44.5)

* Fisher exact test for group comparisons and no significant differences were found between groups 1 and 2 NMO IgG positivity percentages (p=0.640).
Abbreviations: IgG: immunoglobulin G; NMO: neuromyelitis optica; SD: standard deviation.

Table VI-2. Clinical and paraclinical features of positive NMO-IgG patients in groups 1 and 2.

	Group 1: NMO phenotype			Group 2: negative controls	
Patient, gender	1, female	2, female	3, female	4, male	5, female
Topography of first event	Optic nerve	Spinal cord	Optic nerve	Brainstem	Brainstem
OCB	Positive	Positive	Positive	Positive	Positive
Barkhof criteria at baseline	3	3	0	1	1
Baseline spinal cord MRI	1 lesion <1 vertebral segment	NA	Not done	Normal	NA
Topography of second attack	Spinal cord	Spinal cord	Optic nerve and spinal cord	Spinal cord	Multifocal
Total follow-up in years	11.51	12.12	5.50	3.80	13.33
Follow-up brain MRIs	DIS + DIT, typical MS lesions	DIS + DIT, typical MS lesions	Normal	DIT, no DIS	DIS + DIT, typical MS lesions
Follow-up spinal cord MRIs	Not done	Not done	First: 1 cervical lesion extending 1 vertebral segment. Second : 1 cervical lesion from bulbo-medullary junction to C7 and 1 thoracic lesion extending 1 vertebral segment	Normal	Not done
Treatment	IFN β-1b; natalizumab	IFN β-1a IM; glatiramer acetate	IFN β-1b; mitoxantrone; plasmapheresis	No DMT	Glatiramer acetate
Evolution	Prototypical MS	Prototypical MS	NMO	Prototypical MS	Prototypical MS
Last EDSS	5.5	2.5	9.5	1.0	2.5

Mean (SD) follow-up time for these 5 cases was 9.3 (4.3) years.
As of 2011, none of these patients had been lost to follow-up.

Abbreviations: DIS: dissemination in space; DIT: dissemination in time; DMT: disease-modifying treatment; EDSS: Expanded Disability Status Scale; IFN: interferon; IgG: immunoglobulin G; MS: multiple sclerosis; NA: not available (spinal cord MRI performed in another center that was not retrieved); NMO: neuromyelitis optica; OCB: oligoclonal bands.

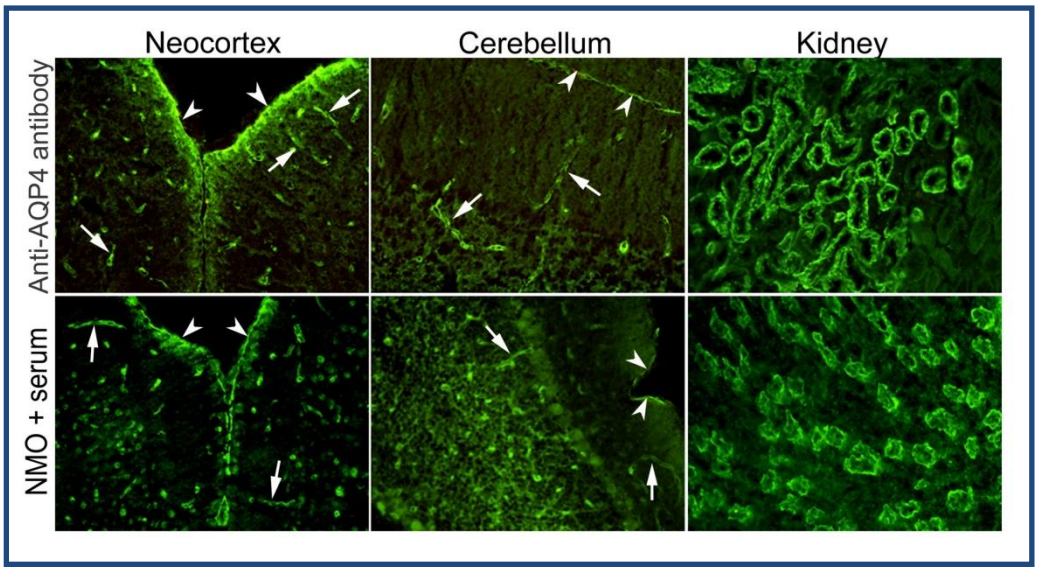


Figure VI-1. NMO-IgG antibody positive immunohistochemistry. Photo courtesy of Carme Costa. Abbreviations: NMO: neuromyelitis optica; AQP4: aquaporin 4.

2. Second objective: to assess the added value of presence of ≥2 predictive factors for MS in patients not fulfilling the 2010 DIS criteria

2.1. Baseline demographic and clinical characteristics.

Study population. A total of 652 patients with a mean follow-up of 47.5 (36.0) months were included. Mean age was 32.0 (8.2) years, and the female:male ratio was 2:1 (**Table VI-3**).

CSF. Of 492 patients (75.5%) with lumbar puncture, 272 (55.3%) had positive OCB. Among those, 125 (46.0%) developed CDMS and 146 (53.7%) McDonald MS compared to 49 (22.3%) and 54 (24.5%) with negative OCB, respectively. Compared to these 492 patients, the 160 patients who did not undergo the procedure were more likely to have optic neuritis [lumbar puncture n=156 (31.7%) versus no lumbar puncture n=76 (47.5%), p=0.001] and a normal

MRI [lumbar puncture n=141/408 (34.6%) versus no lumbar puncture n=46/97 (47.4%), p=0.020].

Table VI-3. Demographic and clinical findings in patients selected from the CIS cohort.

	652 patients	401 patients followed for ≥2 years
Age at onset in years	32.0 (8.2)	31.7 (8.1)
Gender -Male -Female	 220 (33.7) 432 (66.3)	 125 (31.2) 276 (68.8)
CIS topography -Optic nerve -Brainstem -Spinal cord -Other	 232 (35.6) 180 (27.6) 165 (25.3) 75 (11.5)	 127 (31.7) 115 (28.7) 110 (27.4) 49 (12.2)
Follow-up in months	47.5 (36.0)	70.6 (26.4)
CDMS	201 (30.8)	176 (43.9)
2010 McDonald MS	237 (36.3)	202 (50.4)

Abbreviations: CIS: clinically isolated syndrome; CDMS: clinically definite multiple sclerosis; MS: multiple sclerosis.
Data are the mean (SD) for age and follow-up or number (percentage) of patients otherwise.

MRI. At the time of the analysis, brain MRI was available in 505 patients (77.5%) and it was abnormal in 318 (63.0%). Among these, 156 (49.1%) developed CDMS and 190 (59.7%) fulfilled the 2010 McDonald criteria. Of the 187 (37.0%) with normal brain MRI, 22 (11.8%) developed CDMS and 24 (12.8%) fulfilled the McDonald criteria.

Spinal cord MRI was performed in 154 (23.6%) patients and was abnormal in 79 (51.3%). Among these, 30 (38.0%) had CDMS and 37 (46.8%) fulfilled McDonald MS. Of the 75 (48.7%) patients with a normal spinal cord MRI, three (4.0%) developed CDMS and eight (10.7%) fulfilled McDonald MS. However, 60 (75.9%) patients with spinal cord lesions also had an abnormal brain MRI, of which 36 (60.0%) had a spinal cord syndrome.

DMT. Altogether, 236 patients (36.2%) were on DMT: 136 (57.6%) before CDMS and 87 (36.9%) before McDonald MS.

2.2. Predicting MS after a CIS.

Having ≥2 predictive factors or the periventricular lesions plus presence of OCB combination resulted in similar aHRs for CDMS as DIS only or DIT only, whereas the estimated aHR for DIS+DIT was higher (**Table VI-4**). The risk of McDonald MS when having ≥2 predictive factors was higher than for CDMS, although it was even higher in cases fulfilling DIS or DIT on baseline MRI (**Table VI-5**).

2.3. Predicting MS in the first two years after a CIS. Of 652 patients, 401 (61.5%) were followed for ≥2 years. Sensitivity, specificity, and accuracy of DIS, DIT, DIS + DIT, and predictive factors for CDMS are shown in **Table VI-4**. Performance of DIS, DIT, and predictive factors for McDonald MS is shown in **Table VI-5**.

Table VI-4. Risk assessment and performance of the 2010 McDonald criteria and the predictive factors by Ruet and collaborators (Ruet A, 2011) for predicting conversion to CDMS in CIS patients.

CDMS						
	Cox regression models in the entire study population		**Performance in patients followed for ≥2 years**			
	n (%)	aHR (95% CI)	n (%)	Sensitivity	Specificity	Accuracy
				(95% CI)		
2010 DIS and DIT criteria according to baseline MRI findings						
DIS only	459 (70.4)	3.8 (2.5-5.8)[a]	360 (89.8)	69.6 (61.8-76.7)	67.3 (60.4-73.7)	68.3 (63.3-73.1)
DIT only	459 (70.4)	4.2 (1.9-9.2)[a]	339 (84.5)	42.3 (34.2-50.6)	87.9 (82.4-92.2)	67.8 (62.6-72.8)
DIS + DIT	459 (70.4)	8.6 (5.4-13.8)[a]	344 (85.8)	36.4 (28.8-44.6)	90.2 (85.1-94.0)	66.6 (61.3-71.5)
Predictive factors in cases not fulfilling DIS						
≥2 predictive factors	266 (40.8)	3.7 (2.1–6.7)[a]	184 (45.9)	60.4 (45.3-74.2)	73.5 (65.3–80.7)	70.1 (62.9-77.0)
Age ≤40 and ≥3 PV lesions	266 (40.8)	5.5 (1.6–19.2)[b]	184 (45.9)	31.3 (18.7-46.3)	88.2 (81.6–93.1)	73.4 (66.4-79.6)
Age ≤40 and +OCB	212 (32.5)	2.7 (0.8–9.0)	184 (45.9)	54.2 (39.2-68.6)	77.9 (70.0–84.6)	71.7 (64.6-78.1)
≥3 PV lesions and +OCB	212 (32.5)	3.6 (1.7–7.9)[b]	184 (45.9)	25.0 (13.7-39.6)	89.7 (83.3–94.3)	72.8 (65.8-79.1)

The value of *n* represents the size of the sample for each variable of interest.
[a] $p < 0.001$; [b] $p < 0.01$.
Abbreviations: CDMS: clinically definite multiple sclerosis; aHR: adjusted hazard ratio; CI: confidence interval; DIS: dissemination in space; DIT: dissemination in time; MRI: magnetic resonance imaging; PV: periventricular; OCB: oligoclonal bands.

Table VI-5. Risk assessment and performance of the 2010 McDonald criteria and the predictive factors by Ruet and collaborators (Ruet A, 2011) for predicting fulfilment of 2010 McDonald MS in CIS patients.

2010 McDonald						
	Cox regression models in the entire study population		**Performance in patients followed for ≥2 years**			
	n (%)	aHR (95% CI)	n (%)	Sensitivity	Specificity	Accuracy
				(95% CI)		
2010 DIS and DIT criteria according to baseline MRI findings						
DIS only	495 (75.9)	5.1 (3.7-7.0)[a]	360 (89.8)	72.3 (65.2-78.6)	75.6 (68.5-81.7)	73.9 (69.0-78.4)
DIT only	459 (70.4)	10.7 (7.9-14.5)[a]	339 (84.5)	47.1 (39.5-54.8)	97.6 (93.9-99.3)	71.7 (66.6-76.4)
Predictive factors in cases not fulfilling DIS						
≥2 predictive factors	266 (40.8)	3.3 (1.9–5.8)[a]	184 (45.9)	60.8 (46.1-74.2)	74.4 (66.2–81.6)	70.7 (63.5-77.1)
Age ≤40 and ≥3 PV lesions	266 (40.8)	4.6 (1.3–16.2)[c]	184 (45.9)	29.4 (18.5-43.8)	88.0 (81.2–93.0)	71.8 (64.6-78.1)
Age ≤40 and +OCB	212 (32.5)	2.7 (0.8–9.1)	184 (45.9)	54.9 (40.3-68.9)	79.0 (71.0–85.5)	72.3 (65.2-78.6)
≥3 PV lesions and +OCB	212 (32.5)	2.9 (1.3–6.2)[b]	184 (45.9)	23.5 (12.8-37.5)	89.5 (83.0–94.1)	71.2 (64.1-77.6)

The value of *n* represents the size of the sample for each variable of interest.
[a] $p < 0.001$; [b] $p < 0.01$; [c] $p < 0.05$.
Abbreviations: aHR: adjusted hazard ratio; CDMS: clinically definite multiple sclerosis; CI: confidence interval; PV, periventricular; OCB: oligoclonal bands.

3. Third objective: to analyse the added value of spinal cord MRI in the fulfilment of the 2010 DIS and DIT criteria in non-spinal cord CIS patients, and to evaluate the prognostic value of spinal cord lesions for evolution to MS and disability accumulation

3.1. Baseline demographic and clinical characteristics. Results are shown in **Table VI-6.**

Table VI-6. Baseline demographic and clinical characteristics.

Demographic and clinical variables	n=207
Females: n (%)	131 (63.3)
Age: mean (SD)	32.6 (8.1)
CIS topography: n (%) ON BS SC Other	 70 (33.8) 51 (24.6) 64 (30.9) 22 (10.6)
Positive OCB*: n (%)	89 (50.9)
Number of lesions on T2WI: n (%) 0 1-3 4-9 ≥10	 72 (34.8) 23 (11.1) 34 (16.4) 78 (37.7)
Abnormal SC MRI: n (%)	93 (44.9)
Total time of follow-up: mean (SD)	35.7 (15.8)

*n=175.
[a]p <0.001; [b]p <0.01; [c]p <0.05.
Abbreviations: SD: standard deviation; CIS: clinically isolated syndrome; ON: optic nerve; BS: brainstem; SC: spinal cord; OCB: oligoclonal bands; T2WI: T2-weighted images; MRI: magnetic resonance imaging.

Of the 207 patients, two thirds were female with a mean (SD) age of 32.6 (8.1) years. As for CIS topography, 64 (30.9%) had a myelitis, 70 (33.8%) an optic neuritis, 51 (24.6%) a brainstem syndrome, and 22 (10.6%) a multifocal or hemispheric CIS. One hundred and seventy-five patients had OCB determination, of which 89 (50.9%) were positive. Regarding baseline brain MRI, 135 (65.2%) had at least one lesion. The spinal cord MRI demonstrated the presence of at least one lesion in 93 (44.9%) patients [50 (78.1%) spinal cord versus 43 (30.1%) non-spinal cord CIS, p<0.0001].

The mean (SD) follow-up time was 35.7 (15.8) months during which 61 (29.5%) patients converted to CDMS, 93 (44.9%) fulfilled the 2010 McDonald criteria, and 13 (6.3%) reached an EDSS ≥3.0.

3.2. Diagnostic value of spinal cord MRI in CIS. In general, when assessing both brain and spinal cord MRI, the absolute diagnostic capacity increased from 2.8% to 6.0% as the CIS groups became more specific. In turn, the number needed to scan decreased from 36 to 17. The relative diagnostic capacity increase was close to 15.0% in the CIS groups in which it could be calculated (**Table VI-7**).

Table VI-7. Added value of spinal cord MRI: number needed to scan to diagnose one additional MS patient.

Groups of patients	Patients fulfilling DIS and DIT: n (%)		ADI	NNS	RDI
Non-SC CIS n=143	Brain	23 (16.1)	2.8	36	14.8
	Brain + SC	27 (18.9)			
Non-SC CIS with pathological brain MRI n=90	Brain	23 (25.6)	4.4	23	14.7
	Brain + SC	27 (30.0)			
Non-SC CIS with pathological brain MRI not fulfilling DIS + DIT n=67	Brain + SC	4 (6.0)	6.0	17	NA

Abbreviations: DIS: dissemination in space; DIT: dissemination in time; ADI: absolute diagnostic capacity increase; NNS: number needed to scan; RDI: relative diagnostic capacity increase; SC: spinal cord; CIS: clinically isolated syndrome; MRI: magnetic resonance imaging; NA: not applicable.

3.3. Prognostic value of spinal cord lesions. In all groups, more patients with spinal cord lesions converted to CDMS than those with no lesions (**Figure VI-2**).

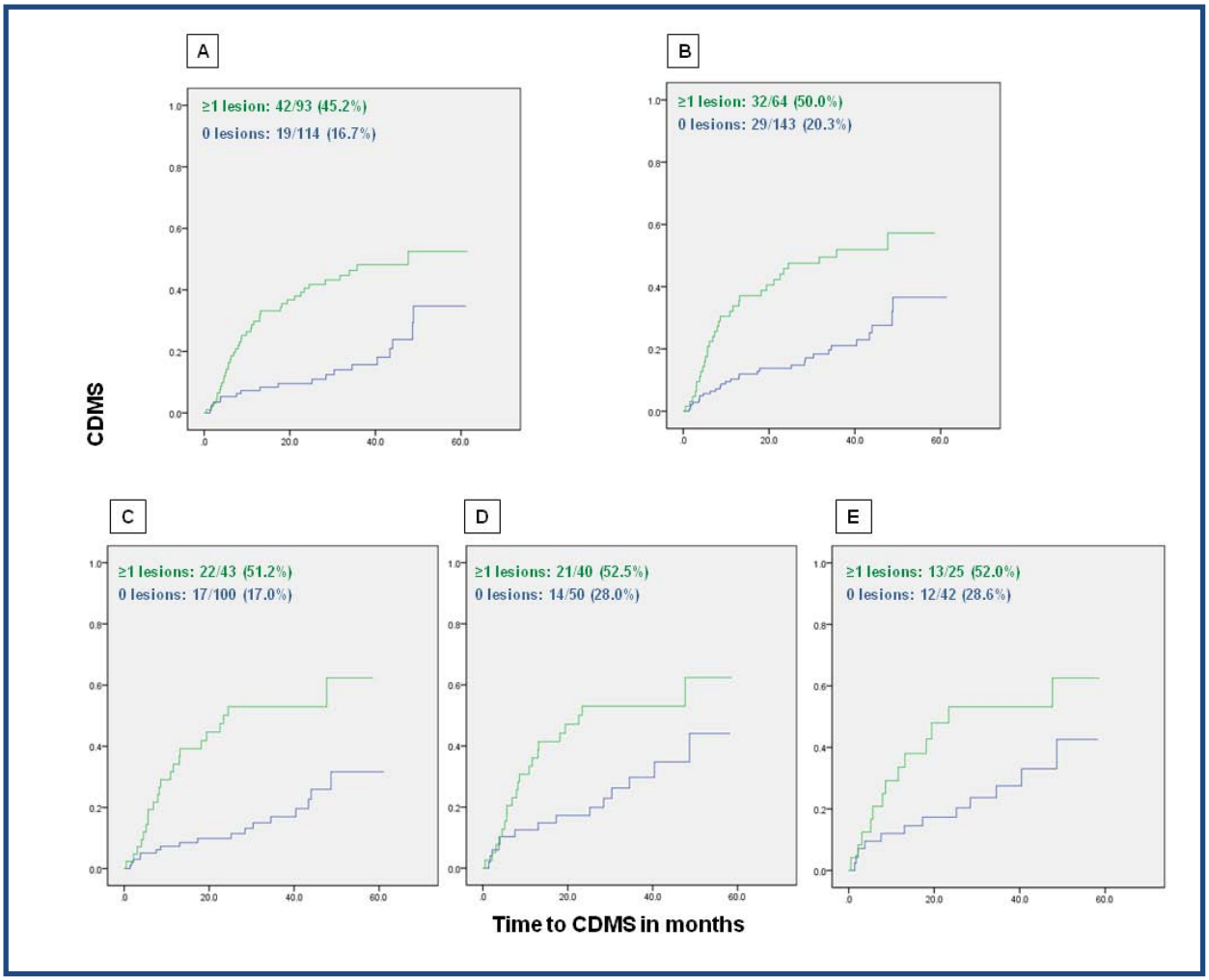


Figure VI-2. Survival curves showing patients with 0 or ≥1 spinal cord lesions who convert to CDMS over time.
A: All patients, symptomatic lesion accounted for (n=207); B: All patients, symptomatic lesion unaccounted for (n=207); C: Non-SC CIS (n=143); D: Non-SC CIS with pathological baseline MRI (n=90); E: Non-SC CIS with pathological baseline MRI not fulfilling DIS and DIT (n=67). Green lines represent patients with at least one lesion demonstrated on SC MRI; blue lines represent patients with no SC lesions. Abbreviations: CDMS: clinically definite multiple sclerosis; SC: spinal cord; CIS: clinically isolated syndrome; MRI: magnetic resonance imaging; DIS: dissemination in space; DIT: dissemination in time.

Spinal cord lesions increased the risk of CDMS, independently of brain lesion number, presence of OCB or CIS topography, when evaluating all 207 cases counting the symptomatic lesion, observing a trend towards significance in other groups (**Table VI-8**).

When evaluating fulfilment of the 2010 McDonald MS criteria in the five groups, more patients with spinal cord lesions fulfilled the criteria than those with no lesions (**Figure VI-3**).

Table VI-8. Adjusted hazard ratios and performance for presence of spinal cord lesions in the five groups of patients assessing CDMS as the outcome.

Patients	CDMS									
	n	aHR	95% CI	p	n	Sensitivity	Specificity	Accuracy	PPV	NPV
All (symptomatic lesion accounted for):										
No SC lesions	114	1			160	69.5	59.4	63.1	50.0	76.9
SC lesions	93	1.9	1.0-3.6	0.041		(56.1-80.9)	(49.2-69.1)	(55.1-70.6)	(38.8-61.3)	(66.0-85.7)
All (symptomatic lesion unaccounted for):										
No SC lesions	143	1			160	52.5	73.3	65.6	53.5	72.6
SC lesions	64	1.5	0.9-2.7	0.140		(39.1-65.7)	(63.5-81.6)	(57.7-72.9)	(39.9-66.6)	(62.8-80.9)
Non-SC CIS:										
No SC lesions	100	1			105	56.8	76.5	69.5	56.8	76.5
SC lesions	43	1.9	0.9-4.0	0.080		(39.5-72.9)	(64.6-85.9)	(59.8-78.1)	(39.5-72.9)	(64.6-85.9)
Non-SC CIS with pathological brain MRI:										
No SC lesions	50	1			74	60.6	63.4	62.2	57.1	66.7
SC lesions	40	1.8	0.8-3.8	0.129		(42.1-77.1)	(46.9-77.9)	(50.1-73.2)	(39.4-73.7)	(49.8-80.9)
Non-SC CIS with pathological brain MRI not fulfilling DIS + DIT										
No SC lesions	42	1			55	50.0	74.2	63.6	60.0	65.7
SC lesions	25	2.0	0.8-5.1	0.142		(29.1-70.9)	(55.4-88.1)	(49.6-76.2)	(36.1-80.9)	(47.8-80.9)

Performance is shown with 95% CI.
Abbreviations: CDMS: clinically definite multiple sclerosis; aHR: adjusted hazard ratio; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value; SC: spinal cord; CIS: clinically isolated syndrome; MRI: magnetic resonance imaging; DIS: dissemination in space; DIT: dissemination in time.

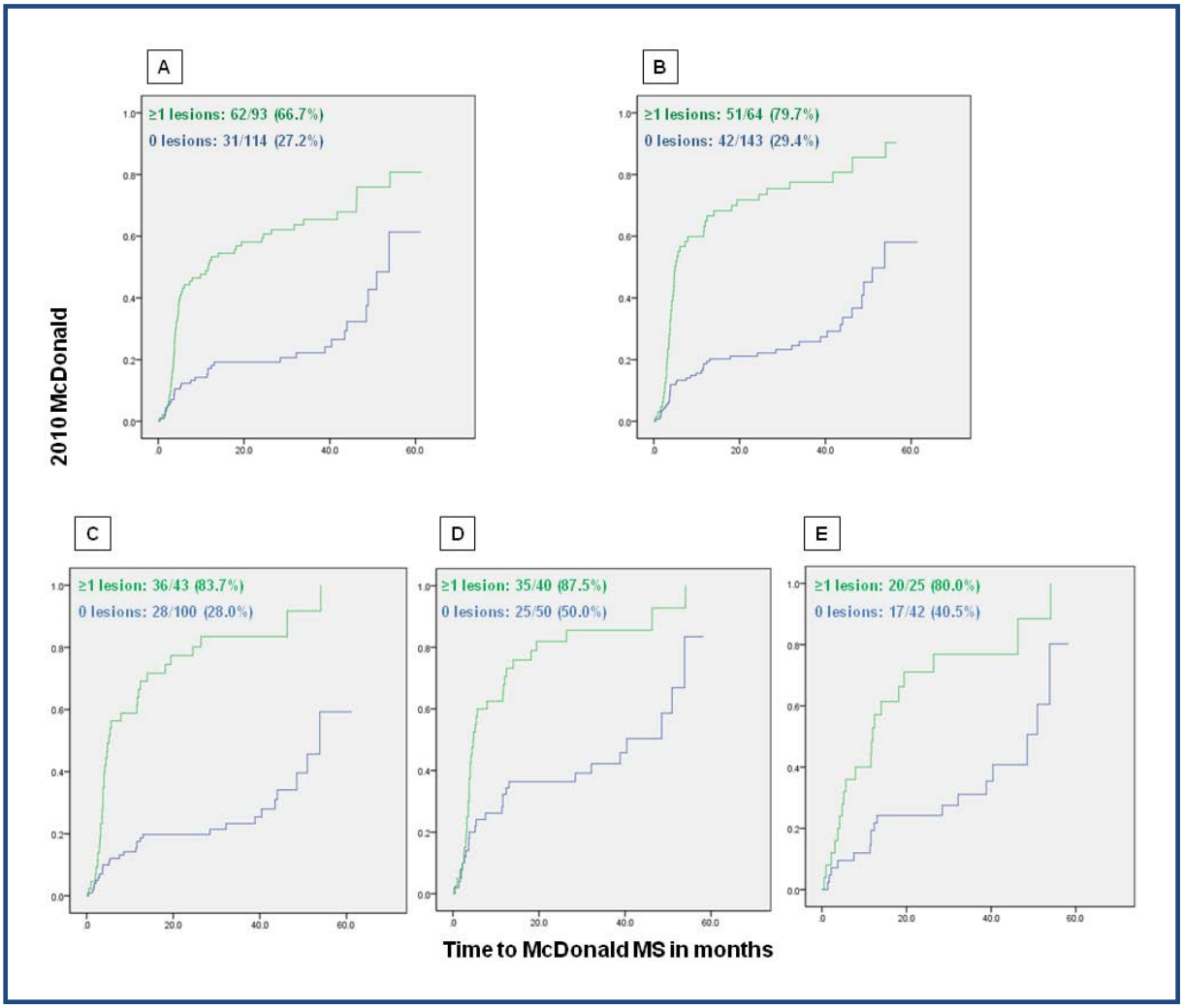


Figure VI-3. Survival curves showing patients with 0 or ≥1 spinal cord lesions who fulfil the 2010 McDonald criteria over time.
A: All patients, symptomatic lesion accounted for (n=207); B: All patients, symptomatic lesion unaccounted for (n=207); C: Non-SC CIS (n=143); D: Non-SC CIS with pathological baseline MRI (n=90); E: Non-SC CIS with pathological baseline MRI not fulfilling DIS and DIT (n=67). Green lines represent patients with at least one lesion demonstrated on SC MRI; blue lines represent patients with no SC lesions. Abbreviations: MS: multiple sclerosis; SC: spinal cord; CIS: clinically isolated syndrome; MRI: magnetic resonance imaging; DIS: dissemination in space; DIT: dissemination in time.

The aHR were significant in all groups, except for patients not fulfilling DIS and DIT on brain MRI, observing a trend towards significance (**Table VI-9**). If considering spinal cord lesion number, the HR for both CDMS and 2010 McDonald MS was higher with ascending number of lesions in all groups.

Table VI-9. Adjusted hazard ratios and performance for presence of spinal cord lesions in the five groups of patients assessing 2010 McDonald MS as the outcome.

Patients					2010 McDonald					
	n	aHR	95% CI	p	n	Sensitivity	Specificity	Accuracy	PPV	NPV
All (symptomatic lesion accounted for):										
No SC lesions	114	1			165	67.8	69.3	68.5	72.6	64.2
SC lesions	93	2.0	1.2-3.2	0.008		(57.1-77.3)	(57.6-79.5)	(60.8-75.5)	(61.8-81.8)	(52.8-74.6)
All (symptomatic lesion unaccounted for):										
No SC lesions	143	1			165	55.6	86.7	69.7	83.3	61.9
SC lesions	64	1.9	1.2-3.0	0.007		(44.7-66.0)	(76.8-93.4)	(62.1-76.6)	(71.5-91.7)	(51.9-71.2)
Non-SC CIS:										
No SC lesions	100	1			112	57.1	91.8	72.3	90.0	62.5
SC lesions	43	1.9	1.1-3.4	0.024		(44.1-69.5)	(80.4-97.7)	(63.1-80.4)	(76.3-97.2)	(50.3-73.6)
Non-SC CIS with pathological brain MRI:										
No SC lesions	50	1			82	58.3	86.4	65.9	92.1	43.2
SC lesions	40	1.8	1.0-3.2	0.043		(44.9-70.9)	(65.1-97.1)	(54.6-77.9)	(78.6-98.3)	(28.4-59.0)
Non-SC CIS with pathological brain MRI not fulfilling DIS + DIT										
No SC lesions	42	1			59	54.1	86.4	66.1	87.0	52.8
SC lesions	25	2.0	0.9-4.4	0.078		(36.9-70.5)	(65.1-97.1)	(52.6-77.9)	(66.4-92.7)	(35.5-69.6)

Performance is shown with 95% CI.
Abbreviations: CDMS: clinically definite multiple sclerosis; aHR: adjusted hazard ratio; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value; SC: spinal cord; CIS: clinically isolated syndrome; MRI: magnetic resonance imaging; DIS: dissemination in space: DIT: dissemination in time.

The aHRs showed that patients with ≥2 spinal cord lesions had a two-fold increase in risk which was more clearly demonstrated in the case of McDonald MS as the outcome, regardless of how the symptomatic lesion was assessed (**Table VI-10**).

As for disability, 6 of 64 (9.4%) spinal cord CIS patients and 7 of 143 (4.9%) non-spinal cord CIS reached an EDSS ≥3.0 (p=0.352). The presence of spinal cord lesions was associated with an EDSS ≥3.0 in 11/93 (11.8%) compared to 2/114 (1.8%) patients without spinal cord lesions (p=0.003). The aHR for reaching an EDSS ≥3.0 showed a trend towards significance in all patients, and were especially significant in non-SC CIS (**Figure VI-4** and **Table VI-11**).

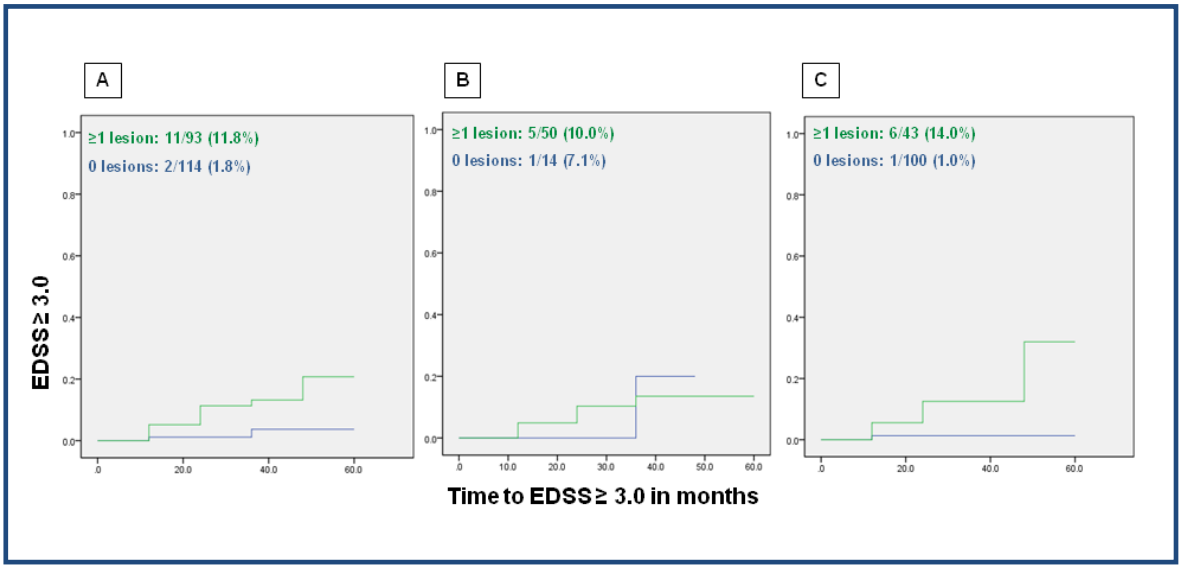


Figure VI-4. Survival curves showing patients with 0 or ≥1 spinal cord lesions who reach an EDSS ≥3.0 over time.
A: All patients (n=207). B: SC CIS (n=64). C: Non-SC CIS (n=143). Green lines represent patients with at least one lesion demonstrated on SC MRI; blue lines represent patients with no SC lesions.
Abbreviations: EDSS: expanded disability status scale; SC: spinal cord; CIS: clinically isolated syndrome.

3.4. Performance of spinal cord lesions. When evaluating CDMS as the outcome, presence of spinal cord lesions showed a good specificity and NPV. In the case of 2010 McDonald MS, both specificity and PPV were very high. In contrast, the presence of spinal cord lesions was highly sensitive and had a very good NPV for reaching an EDSS ≥3.0 (**Tables VI-8, VI-9,** and **VI-11**).

Table VI-10. HR and aHR for number of spinal cord lesions in the five groups of patients assessing CDMS and 2010 McDonald MS as the outcome.

			CDMS						2010 McDonald					
	Lesion number	**n**	**HR**	**95% CI**	**p**	**aHR**	**95% CI**	**p**	**HR**	**95% CI**	**p**	**aHR**	**95% CI**	**p**
All (symptomatic lesion accounted for)	0	114	1			1			1			1		
	1	46	2.4	1.3-4.7	0.008	2.1	0.99-4.3	0.054	2.2	1.3-3.7	0.005	1.7	0.96-3.2	0.067
	≥2	47	3.6	2.0-6.5	<0.0001	1.8	0.9-3.7	0.081	4.9	3.0-7.9	<0.0001	2.2	1.2-3.9	0.007
All (symptomatic lesion unaccounted for)	0	143	1			1			1			1		
	1	17	2.4	1.0-5.4	0.040	1.8	0.7-4.6	0.192	3.6	1.9-6.8	<0.0001	1.8	0.9-3.7	0.081
	≥2	47	2.9	1.7-5.0	<0.0001	1.5	0.8-2.7	0.218	4.5	2.9-7.1	<0.0001	1.9	1.1-3.2	0.014
Non-SC CIS	0	100	1			1			1			1		
	1	17	2.9	1.2-6.9	0.019	1.9	0.7-5.0	0.215	4.0	2.1-7.8	<0.0001	1.6	0.8-3.5	0.217
	≥2	26	4.3	2.1-8.5	<0.0001	2.0	0.9-4.5	0.113	6.8	3.8-12.2	<0.0001	2.2	1.1-3.2	0.024
Non-SC CIS with pathological brain MRI	0	50	1			1			1			1		
	1	14	1.7	0.7-4.5	0.264	1.7	0.6-4.9	0.311	2.3	1.2-4.6	0.018	1.5	0.7-3.2	0.355
	≥2	26	2.5	1.2-5.1	0.015	1.8	0.8-4.2	0.154	3.4	1.9-6.1	<0.0001	2.1	1.1-4.1	0.033
Non-SC CIS with pathological brain MRI not fulfilling DIS + DIT	0	42	1			1			1			1		
	1	11	1.4	0.5-4.4	0.534	1.5	0.4-5.7	0.521	2.9	1.3-6.7	0.010	1.7	0.6-4.8	0.346
	≥2	14	3.0	1.3-7.2	0.014	2.4	0.8-7.0	0.118	3.4	1.5-7.4	0.003	2.4	0.9-6.1	0.080

Abbreviations: CDMS: clinically definite multiple sclerosis; HR: hazard ratio; aHR: adjusted hazard ratio; CI: confidence interval; SC: spinal cord; CIS: clinically isolated syndrome; MRI: magnetic resonance imaging; DIS: dissemination in space; DIT: dissemination in time.

Table VI-11. aHR and performance of presence of spinal cord lesions in the three groups of patients assessing EDSS ≥3.0 as the outcome.

Patients	EDSS ≥3.0									
	n	aHR	95% CI	p	n	Sensitivity	Specificity	Accuracy	PPV	NPV
All:										
No SC lesions	114	1			157	84.6	51.4	54.1	13.6	97.4
SC lesions	93	5.7	0.9-36.0	0.067		(54.6-98.1)	(42.9-59.8)	(46.0-62.1)	(7.0-23.0)	(90.8-99.7)
SC CIS:										
No SC lesions	14	1			53	83.3	17.0	24.5	11.4	88.9
SC lesions	50	0.5	0.04-7.9	0.647		(35.9-99.6)	(7.7-30.8)	(13.8-38.3)	(3.8-24.6)	(51.8-99.7)
Non-SC CIS:										
No SC lesions	100	1			104	85.7	68.0	69.2	16.2	98.5
SC lesions	43	36.2	1.5-880.4	0.028		(42.1-99.6)	(57.8-77.2)	(59.4-77.9)	(6.2-32.0)	(92.0-99.9)

Abbreviations: CDMS: clinically definite multiple sclerosis; EDSS: expanded disability status scale, SC: spinal cord; CIS: clinically isolated syndrome; MRI: magnetic resonance imaging; DIS: dissemination in space; DIT: dissemination in time.

4. Fourth objective: to analyse the predictive value of gMS-Classifier2 determination in serum for early conversion to CDMS in a large cohort of CIS patients, and to determine whether gMS-Classifier2 is an independent predictor of conversion to CDMS

4.1. Baseline demographic and clinical characteristics.

Between 1995 and 2007, 723 patients were included in the CIS cohort. gMS Classifier2 units were determined in a subgroup of 249 (34.0%) patients that met the present study's selection criteria. The screened cohort was similar to the non-screened cohort in age, follow-up time, and proportion of both positive OCB and baseline number of Barkhof criteria. There were differences in the proportion of females and the topography of disease presentation (**Table VI-10**). In comparison to the mean follow-up displayed in the table, median (range) time of follow-up was 68.7 (0.53-177.0) in the screened group and 63.2 (0.30-171.2) in the non-screened group, p=0.002.

When comparing the demographic variables between patients with positive and negative gMS-Classifier2 status, including median time of follow-up, there was a difference in the proportion of females and in the distribution of CIS topography, but since the gMS-Classifier2 HR estimate was not substantially modified when gender or topography were included in the model, it was not considered necessary to adjust the results for these clinical variables (gMS-Classifier2 status, multivariate analysis with gender: HR 1.6, 95% CI 1.0-2.7; with topography: HR 1.6, 95% CI 1.0-2.6. gMS-Classifier2 continuous variable, analysis with gender: HR 1.2, 95% CI 1.0-1.5; with topography: HR 1.2, 95% CI 1.0-1.5, assessing Barkhof criteria in all cases). There was also a difference in the median time of follow-up; however, as it was of approximately 5 months between groups, it was not considered relevant when the total follow-up time was of up to 14 years.

4.2. gMS-Classifier2 status at specified time points and conversion to CDMS. The median value of gMS-Classifier2 was -0.24 units (range -2.3 to 5.5 units) and seventy-five patients (30.1%) were positive for gMS-Classifier2. The

median time to CDMS was 37.8 months for gMSClassifier2-positive patients (95% CI 10.4–65.3 months) and 83.9 months (95% CI 57.5–110.5) for gMS-Classifier2-negative patients. gMS-Classifier2 predicted conversion to CDMS within two years (HR 1.8, 95%CI 1.1–2.9; p=0.013) and within five years of follow-up (HR 1.5, 95% CI 1.0–2.4; p=0.033) but not for total follow-up time, although a trend was observed (HR 1.4, 95% CI 1.0–2.2; p=0.060) (**Figure VI-5**).

Table VI-10. Demographic, clinical and MRI characteristics of screened and non-screened patients: gMS-Classifier2 serum assay.

Group characteristics (1995-2007)	Screened CIS cohort (n= 249)	Non-screened CIS cohort (n= 474)	p
Mean age in years (SD)	31.6 (7.9)	31.1 (7.9)	0.455
Females: n (%)	187 (75.1)	315 (66.5)	0.017
Mean follow-up in months (SD)	75.3 (41.9)	65.6 (44.9)	0.005
Topography: n (%) -Optic nerve -Brainstem -Spinal cord -Other	 106 (42.6) 51 (20.5) 65 (26.1) 27 (10.8)	 154 (32.5) 144 (30.4) 122 (25.7) 54 (11.4)	 0.014
Positive OCB: n (%)*	152 (64.4)	181 (60.1)	0.311
Barkhof criteria on baseline MRI: n (%)** 0 1-2 3-4	 88 (35.5) 56 (22.6) 104 (41.9)	 173 (38.6) 104 (23.2) 171 (38.2)	 0.601

*The total number of patients with available cerebrospinal fluid for OCB determination was 236 for the screened CIS cohort and 301 for the non-screened CIS cohort. Percentages in the table correspond to these figures.
**The total number of patients with available baseline MRI for Barkhof criteria determination was 248 for the screened CIS cohort and 448 for the non-screened CIS group. Percentages in the table correspond to these numbers.
Abbreviations: CIS: clinically isolated syndrome; SD: standard deviation; OCB: oligoclonal bands; MRI: magnetic resonance imaging.

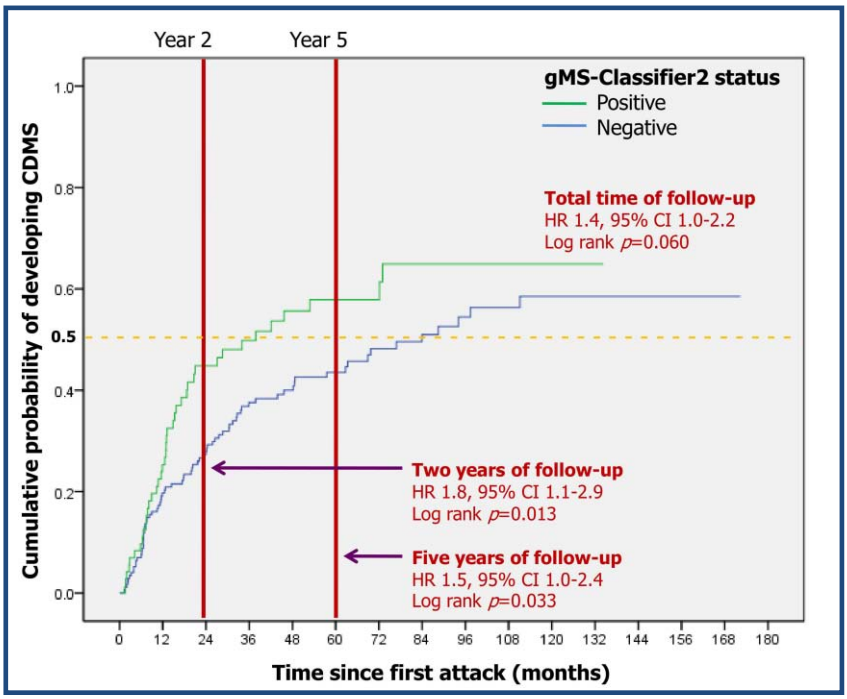


Figure VI-5. Time to reach CDMS based on gMS-Classifier2 status. Dotted line: median time of follow-up. Abbreviations: HR = hazard ratio; CI = confidence interval.

Table VI-11 shows the proportion of CDMS patients that were positive and negative for gMSClassifier2 at 2 years, 5 years, and total time of follow-up.

Table VI-11. gMS-Classifier2 status and number of patients converting to CDMS at specified time points.

Specified time points	CDMS in positive patients (75)	CDMS in negative patients (174)	p
	n (%)		
Two years	31 (41.3)	45 (25.9)	0.017
Five years	38 (50.7)	67 (38.5)	0.093
Total time of follow-up (up to 14 years)	40 (53.3)	77 (44.3)	0.214

Abbreviations: CDMS = clinically definite multiple sclerosis.

4.3. Predictive value of gMS-Classifier2.

Predictive value of gMS-Classifier2 status for conversion to CDMS. In a univariate analysis, gMS-Classifier2 status (positive/negative), Barkhof criteria, number of T2 lesions and presence of OCB were predictors for early conversion to CDMS at two years of follow-up (**Table VI-12**).

In the multivariate analyses at two years of follow-up, gMSClassifier2 status remained significant when tested with baseline number of Barkhof criteria (aHR 1.8, 95% CI 1.1–2.8, p=0.014) or number of T2 lesions (aHR 1.7, 95% CI 1.1–2.7, p=0.020). When combining gMS-Classifier2 status and OCB, the former's significance was lost (aHR 1.5, 95% CI 0.9–2.4, p=0.095). When combining gMS-Classifier2 status with OCB and either Barkhof criteria or number of T2 lesions the HR were non-significant (aHR 1.5, 95% CI 0.9–2.5, p=0.081 and aHR 1.5, 95% CI 0.9–2.4, p=0.100, respectively) (**Table VI-13**).

When adding treatment to these models, there were no statistically significant changes in the aHR of gMS-Classifier2 (gMS-Classifier2 status, multivariate analysis with treatment: aHR 1.5, 95% CI 0.9-2.4, assessing Barkhof criteria in all cases).

Predictive value of gMS-Classifier2 continuous unit values for conversion to CDMS. In the univariate analysis, gMS-Classifier2 continuous units, Barkhof criteria, number of T2 lesions and presence of OCB were predictors for early conversion to CDMS at two and five years of follow-up (**Table VI-12**). In the multivariate analyses at two years of follow-up, gMSClassifier2 continuous units remained significant when tested with number of Barkhof criteria (aHR 1.3, 95% CI 1.1–1.5, p=0.003) or number of T2 lesions (aHR 1.3, 95% CI 1.1–1.6, p=0.001). When combining gMS-Classifier2 continuous units and OCB, the former`s significance was lost (aHR 1.1, 95% CI 0.9–1.4, p=0.088), but when combining gMS-Classifier2 continuous units with OCB and either Barkhof criteria or number of T2 lesions the HR were once again statistically significant (aHR 1.2, 95% CI 1.0– 1.5, p=0.020 and aHR 1.3, 95% CI 1.1–1.5, p=0.008, respectively) (**Table VI-14**). When adding treatment to these models, there were no statistically significant changes in the aHR of gMS-Classifier2 (gMS-Classifier2 continuous variable: aHR 1.2, 95% CI 1.0-1.4, assessing Barkhof criteria).

Table VI-12. Univariate Cox proportional hazard regression for conversion to CDMS.

Univariate model	n	Two years of follow-up			Five years of follow-up			Total time of follow-up		
		HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
gMS-Classifier2 status (positive or negative)	249	1.8	1.1–2.8	0.017	1.5	1.0–2.3	0.034	1.4	1.0–2.1	0.061
gMS-Classifier2 continuous units*	249	1.2	1.0–1.4	0.027	1.1	1.0–1.3	0.038	1.1	1.0–1.3	0.117
1–2 Barkhof criteria**	56	3.9	1.6–9.4	0.002	4.3	2.1–8.6	<0.0001	5.3	2.7–10.6	<0.0001
3–4 Barkhof criteria	104	7.5	3.4–16.5	<0.0001	6.7	3.6–12.8	<0.0001	8.1	4.3–15.4	<0.0001
1–9 T2 lesions	76	5.6	1.7–18.9	0.005	10.0	3.1–32.6	<0.0001	11.5	3.6–37.4	<0.0001
≥10 T2 lesions	107	12.5	3.9–39.9	<0.0001	17.4	5.5–55.2	<0.0001	21.4	6.7–68.1	<0.0001
Positive OCB	152	3.7	1.9–7.3	<0.0001	3.1	1.8–5.3	<0.0001	2.9	1.8–4.8	<0.0001

*For continuous values, HR indicates how much the hazard (for CDMS) increases per unit increase in gMS-Classifier2. **Barkhof criteria and number of T2 lesions on baseline MRI.
Abbreviations: HR = hazard ratio, CI = confidence interval, OCB = oligoclonal bands.

Table VI-13. Multivariate Cox proportional hazard regression for conversion to CDMS according to gMS-Classifier2 status (positive or negative).

Multivariate models: gMS-Classifier2 status	Two years of follow-up			Five years of follow-up			Total time of follow-up		
	aHR	95% CI	p	aHR	95% CI	p	aHR	95% CI	p
gMS-Classifier2	1.8	1.1–2.8	0.014	1.6	1.1–2.4	0.019	1.5	1.0–2.2	0.031
1–2 Barkhof criteria	4.1	1.7–9.8	0.002	4.4	2.2–8.9	<0.0001	5.5	2.8–10.9	<0.0001
3–4 Barkhof criteria	7.5	3.4–16.5	<0.0001	6.9	3.6–13.1	<0.0001	8.3	4.4–15.7	<0.0001
gMS-Classifier2	1.7	1.1–2.7	0.020	1.5	1.0–2.3	0.044	1.4	1.0–2.1	0.074
1–9 T2 lesions	5.4	1.6–18.0	0.007	9.6	3.0–31.3	<0.0001	11.2	3.4–36.4	<0.0001
≥10 T2 lesions	12.2	3.8–39.1	<0.0001	17.2	5.4–54.8	<0.0001	21.4	6.7–67.9	<0.0001
gMS-Classifier2	1.5	0.9–2.4	0.095	1.3	0.9–2.0	0.217	1.2	0.8–1.8	0.307
Positive OCB	3.6	1.8–7.0	<0.0001	3.1	1.8–5.2	<0.0001	2.9	1.7–4.6	<0.0001
gMS-Classifier2	1.5	0.9–2.5	0.081	1.4	0.9–2.1	0.126	1.3	0.9–2.0	0.151
1–2 Barkhof criteria	3.1	1.2–7.6	0.015	3.5	1.7–7.3	0.001	4.4	2.2–9.0	<0.0001
3–4 Barkhof criteria	5.2	2.3–11.9	<0.0001	5.3	2.7–10.2	<0.0001	6.5	3.3–12.5	<0.0001
Positive OCB	2.2	1.1–4.5	0.022	1.9	1.1–3.4	0.014	1.8	1.1–2.9	0.023
gMS-Classifier2	1.5	0.9–2.4	0.100	1.3	0.9–2.0	0.204	1.2	0.8–1.9	0.267
1–9 T2 lesions	3.9	1.1–13.7	0.030	7.6	2.3–25.2	0.001	9.1	2.7–30.0	<0.0001
≥10 T2 lesions	8.2	2.5–27.1	0.001	13.1	4.0–42.7	<0.0001	16.5	5.1–53.3	<0.0001
Positive OCB	2.2	1.1–4.4	0.024	1.9	1.1–3.2	0.021	1.7	1.0–2.8	0.030

Abbreviations: HR = hazard ratio, CI = confidence interval, OCB = oligoclonal bands.

Predictive value of gMS-Classifier2 status and continuous unit values for conversion to CDMS at five years and total time of follow-up. Similar results were obtained at five years of follow-up in the uni- and multivariate analyses for gMS-Classifier2 status and continuous units (**Tables VI-12, VI-13,** and **VI-14**). At total time of follow-up, gMS-Classifier2 status and continuous units were not predictive of conversion to CDMS in the univariate analysis (**Table VI-12**), but when included in the multivariate models, gMS-Classifier2 continuous units remained independent predictors except when combined with OCB, whereas gMS-Classifier2 status yielded mostly negative results (**Tables VI-13** and **VI-14**).

Table VI-14. Multivariate Cox proportional hazard regression for conversion to CDMS according to gMS-Classifier2 continuous units.

Multivariate models: gMS-Classifier2 units	Two years of follow-up			Five years of follow-up			Total time of follow-up		
	aHR	95% CI	p	aHR	95% CI	p	aHR	95% CI	p
gMS-Classifier2	1.3	1.1–1.5	0.003	1.3	1.1–1.5	0.001	1.2	1.1–1.4	0.004
1–2 Barkhof criteria	4.5	1.8–10.9	0.001	4.9	2.4–10.0	<0.0001	6.0	3.0–12.0	<0.0001
3–4 Barkhof criteria	8.3	3.7–18.4	<0.0001	7.6	4.0–14.5	<0.0001	9.1	4.8–17.4	<0.0001
gMS-Classifier2	1.3	1.1–1.6	0.001	1.3	1.1–1.5	0.001	1.3	1.1–1.5	0.001
1–9 T2 lesions	6.0	1.8–20.5	0.004	10.7	3.3–34.8	<0.0001	12.4	3.8–40.3	<0.0001
≥10 T2 lesions	15.0	4.6–48.8	<0.0001	20.9	6.5–67.3	<0.0001	25.8	8.0–82.9	<0.0001
gMS-Classifier2	1.1	0.9–1.4	0.088	1.1	0.9–1.3	0.157	1.1	0.9–1.2	0.283
Positive OCB	3.4	1.9–7.2	<0.0001	3.1	1.8–5.2	<0.0001	2.9	1.8–4.7	<0.0001
gMS-Classifier2	1.2	1.0–1.5	0.020	1.2	1.0–1.4	0.015	1.2	1.0–1.4	0.020
1–2 Barkhof criteria	3.4	1.3–8.4	0.010	3.8	1.8–7.9	<0.0001	4.8	2.3–9.8	<0.0001
3–4 Barkhof criteria	5.7	2.5–13.1	<0.0001	5.8	2.9–11.3	<0.0001	7.1	3.6–13.8	<0.0001
Positive OCB	2.2	1.1–4.4	0.025	1.9	1.1–3.3	0.019	1.7	1.1–2.9	0.031
gMS-Classifier2	1.3	1.1–1.5	0.008	1.2	1.1–1.5	0.007	1.2	1.0–1.4	0.009
1–9 T2 lesions	4.4	1.3–15.2	0.020	8.3	2.5–27.6	0.001	9.9	3.0–32.9	<0.0001
≥10 T2 lesions	9.9	2.9–33.1	<0.0001	15.6	4.8–51.3	<0.0001	19.6	6.0–64.2	<0.0001
Positive OCB	2.2	1.1–4.3	0.029	1.8	1.1–3.1	0.031	1.7	1.0–2.7	0.044

For continuous values, HR indicates how much the hazard (for CDMS) increases per unit increase in gMS-Classifier2.
Abbreviations: HR = hazard ratio, CI = confidence interval, OCB = oligoclonal bands.

4.4. Discrimination measures: ROC curve analyses. To assess the clinical utility of gMS-Classifier2, the calibration measures for number of Barkhof criteria, OCB and gMSClassifier2 continuous units yielded a p value of 0.303; and the one performed for number of T2 lesions, OCB and gMSClassifier2 continuous units showed a p value of 0.664. When performing the ROC analyses as discrimination measures, in the model using number of Barkhof criteria, the ROC association statistics showed that when number of Barkhof criteria, OCB and gMS-Classifier2 were put together, the area under the curve (AUC) was 0.7786 (95% CI 0.7169–0.8403), in comparison with an AUC ROC of 0.7552 (95% CI 0.6945–0.8160) when not including gMS-Classifier2. Thus, the

AUC change (ΔAUC) ROC was 0.0233 (p=0.0788). But when the model excluded OCB, the results were the following: AUC ROC 0.7651 (95% CI 0.6990–0.8312) with gMS-Classifier2 versus AUC ROC 0.7236 (95% CI 0.6616–0.7856) without it, with a ΔAUC ROC of 0.0415, p=0.012. Similar findings were observed when using number of T2 lesions instead of number of Barkhof criteria: when adding OCB to the model, the AUCs were 0.7855 (95% CI 0.7255–0.8455) with gMS-Classifier2 and 0.7528 (95% CI 0.6927–0.8128) without it, leading to a ΔAUC of 0.0328, p=0.0515. When OCB were excluded from the model, the AUCs were 0.7733 (95% CI 0.7102–0.8364) and 0.7266 (95% CI 0.6669–0.7864), respectively, with a ΔAUC of 0.0467, p=0.009.

5. Fifth objective: to determine the value of selected biological markers in CSF as prognostic factors for conversion to CDMS and for disability progression in patients with CIS

Identification of NfL as a predictor of conversion to CDMS

5.1. Screening phase. At baseline, demographic and clinical characteristics were comparable between the CIS-CDMS and the CIS-CIS groups for each biomarker (**Table VI-15**) except for topography in the case of fetuin A, NfL, and NfH, with significant differences due to a higher number of optic neuritis cases in the CIS-CIS groups. The proportion of patients on DMT before CDMS varied between 8.8 and 17.7% for each biomarker. In comparison to both CIS groups, other inflammatory neurological diseases and other non-inflammatory neurological diseases controls were older (p<0.0001) and had a male predominance (p=0.005). Mean follow-up time was significantly longer for CIS-CDMS than for CIS-CIS groups tested for GFAP and NfH. Although there were differences in storage time between CIS-CDMS and CIS-CIS samples for NfH, this factor did not correlate with levels of this biomarker (r_S =-0.131, p=0.256). NfH levels correlated with age (r_S=0.230, p=0.044). Finally, no differences were found in time from CIS to lumbar puncture among groups.

Table VI-15. Demographic and clinical characteristics of CIS-CDMS and CIS-CIS groups evaluated for each biomarker. The screening phase was performed between 2010 and 2012.

	FA and NfL (2010)		GFAP (2011)		NfH (2012)	
	CIS-CDMS	CIS-CIS	CIS-CDMS	CIS-CIS	CIS-CDMS	CIS-CIS
n	35	33	33	33	38	39
Mean age in years (SD)	29.3 (7.3)	30.1 (9.7)	30.7 (8.0)	30.4 (8.2)	30.7 (7.5)	31.6 (8.2)
Females: n (%)	24 (68.6)	26 (78.8)	21 (63.6)	24 (72.7)	28 (73.7)	32 (82.1)
Mean follow-up in months (SD)*	78.8 (33.4)	57.9 (35.2)	79.1 (36.2)	43.7 (50.0)	104.3 (31.1)	74.3 (36.4)
CIS topography: n (%)**						
- Optic nerve	10 (28.6)	22 (66.7)	11 (33.3)	20 (60.6)	10 (26.3)	24 (61.5)
- Brainstem	9 (25.7)	5 (15.2)	7 (21.2)	7 (21.2)	8 (21.1)	4 (10.3)
- Spinal cord	11 (31.4)	4 (12.1)	10 (30.3)	4 (12.1)	14 (36.8)	3 (7.7)
- Other	5 (14.3)	2 (6.1)	5 (15.2)	2 (6.1)	6 (15.8)	8 (20.5)
Mean time from CIS to lumbar puncture in days (SD)	58.9 (53.5)	80.1 (72.3)	184.4 (534.1)	199.7 (303.6)	57.1 (52.5)	81.1 (169.2)
Frozen samples mean storage time in months (SD)¤	88.5 (34.1)	80.2 (33.5)	91.5 (34.5)	75.0 (44.3)	113.1 (33.7)	97.6 (40.5)

*FA and NfL: p=0.050; GFAP: p=0.002; NfH p<0.0001. **FA and NfL: p=0.017; NfH: p=0.002; GFAP p=0.099. ¤NfH: p=0.037.
Categorical variables: Chi^2 test. Continuous variables: Student´s T test for two groups, ANOVA for multiple comparisons.
Abbreviations: FA: fetuin A; NfL: neurofilament light subunit; GFAP: glial fibrillary acidic protein; NfH: neurofilament heavy subunit; CIS: clinically isolated syndrome; CDMS: clinically definite multiple sclerosis; SD: standard deviation.

Therefore, for each protein, the analyses were adjusted for age, gender and/or topography according to the aforementioned findings. DMT before CDMS was also evaluated as a covariate.

Fetuin A levels in CSF were similar (p=0.100 to 1.000) between groups [1417.0 (785.5-2048.6) ng/mL for CIS-CDMS, 1246.1 (586.4-1905.8) for CIS-CIS, 2843.8 (2001.2-3686.5) for other inflammatory neurological diseases, and 1505.7 (763.8-2247.6) for other non-inflammatory neurological diseases; **Figure VI-6, panel A**]. The intraplate coefficient of variation (CV) was not calculated due to the number of samples tested and plate space restrictions given the use of quality controls provided in the ELISA kit. The average interplate CV was 1.9%. Finally, no significant associations with EDSS (p=0.898 for EDSS 3.0) and MRI variables (p=0.058 to 0.951) were found.

Regarding GFAP, levels were similar between groups, concretely 1.37 (1.00-1.73) ng/mL for the CIS-CDMS group and 1.47 (1.08-1.85) for the CIS-CIS group (p=0.704). The average intra- and interplate CV were 5.8% and 38.1%, respectively. Due to the high interplate CV, GFAP ratios were calculated dividing the concentration value of each sample by the concentration value of the intraplate control sample tested in all plates. Ratios were similar between CIS-CDMS [1.29 (0.78-1.79)] and CIS-CIS [1.27 (0.74-1.80)] groups (p=0.963) (**Figure VI-6, panel B**). In addition, neither GFAP levels nor GFAP ratios correlated with EDSS progression (p=0.487 and 0.472 for EDSS 3.0, respectively) or MRI variables (p=0.059 to 0.980).

When NfH levels were assessed, no differences were found between CIS-CDMS [46.8 (39.1-54.6) pg/mL] and CIS-CIS [40.1 (32.7-47.5) pg/mL] (p=0.207) (**Figure VI-6, panel C**). All intra and interplate CVs were below 15% (low, medium, and high concentration control samples). Additionally, no correlations were found between NfH levels and EDSS (p=0.606 for EDSS 3.0) or MRI variables (p=0.091 to 0.997).

Regarding neurofascin, its ELISA detection range was 0.312-20 ng/mL with a 0.083 ng/mL minimum detection limit. As for the sema3A ELISA, the detection

range was 0.156-10 ng/mL and the minimum detection limit was 0.042 ng/mL. We could not detect neurofascin and sema3A in any CSF samples, probably because the concentration of both proteins in our samples was under the detection limit of the available commercial ELISA kits.

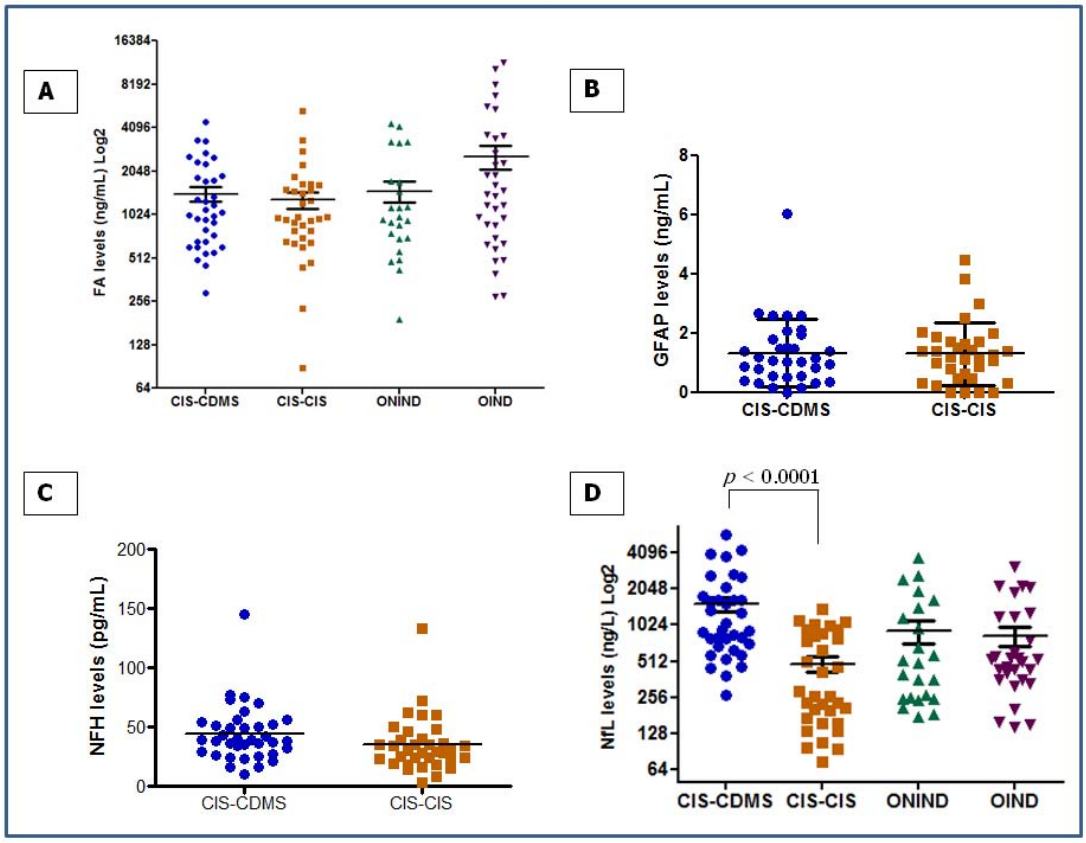


Figure VI-6. FA (panel A) and NfL (panel D) levels in CIS-CDMS, CIS-CIS, OIND and ONIND. Levels are also shown for GFAP (panel B) and NfH (panel C) levels in CIS-CDMS and CIS-CIS. Results were adjusted for age, gender or topography. The Bonferroni correction for multiple comparisons was applied in figures VI-6A and D.
Abbreviations: FA: fetuin A, CIS: clinically isolated syndrome; CDMS: clinically definite multiple sclerosis; ONIND: other non-inflammatory neurological diseases; OIND: other inflammatory neurological diseases; NfL: neurofilament light subunit; GFAP: glial fibrillary acidic protein; NfH: neurofilament heavy subunit.

Finally, NfL levels were quantified. A statistically significant difference between CIS-CDMS [1553.1 (1208.7-1897.5) ng/L] and CIS-CIS [499.0 (168.8-829.2) ng/L] was found ($p<0.0001$), as well as between CIS-CDMS and other inflammatory neurological diseases [752.4 (352.1-1152.6) ng/L, $p=0.048$]. NfL levels were 1020.3 (602.0-1438.6) ng/L in other non-inflammatory neurological

diseases (**Figure VI-6, panel D**). The average intra- and interplate CV were 9.7% and 0.5%, respectively. No correlations were found between NfL levels and EDSS at baseline and at years one through five of follow-up, nor were there any differences for sustained EDSS progression of one point (yes/no) or EDSS ≥3.0, even though in the latter case NfL levels were 1506.1 (645.2-2367.1) ng/L in the 15 patients with EDSS ≥3.0 and 906.6 (693.2-1120.1) ng/L if <3.0 (p=0.185). Significant correlations were found between NfL levels and inflammatory parameters on baseline MRI and follow-up scans at years one and five, except for Gd-enhancing lesions at five years (**Table VI-16**). As for neurodegenerative parameters, NfL levels and both BPFΔ and PBVC at five years showed the strongest correlations (**Table VI-16** and **Figure VI-7**).
Additionally, a correlation between NfL and NfH levels was observed (r_s=0.466, p=0.002).
In summary, NfL levels were significantly increased in patients converting to CDMS, correlating with MRI markers of inflammation and neurodegeneration.

5.2. Replication phase. After finding differences in NfL levels between the CIS groups, we next explored their role as a risk factor for CDMS and MS according to the 2010 McDonald criteria (Polman CH 2011). In total, 155 patients were evaluated. Baseline demographic and clinical characteristics are shown in **Table VI-17**. Median [interquartile range (IQR)] NfL levels were 1238.3 (1782.1) for patients converting to CDMS and 555.8 (825.5) ng/L for those remaining as CIS. Average intra- and interplate CV were 4.9% and 7.0% for the Vall d´Hebron samples (n=93) and 5.1% and 8.8% for the Ramon y Cajal samples (n=62), respectively.

Table VI-16. Correlations between NfL levels and inflammatory and neurodegeneration parameters on MRI at baseline, one year, and five years. Screening phase.

	Baseline			Year One						Year Five					
	T2LN	T2LV	Gd+	T2LN	T2LV	T2New	Gd+	BPFΔ	PBVC	T2LN	T2LV	T2New	Gd+	BPFΔ	PBVC
n	39	39	39	37	37	38	36	29	26	20	20	21	21	17	14
r_s	0.570	0.586	0.495	0.601	0.636	0.553	0.507	-0.558	-0.345	0.544	0.561	0.614	0.206	-0.892	-0.842
p	<0.0001	<0.0001	0.001	<0.0001	<0.0001	<0.0001	0.002	0.001	0.072	0.013	0.010	0.003	0.369	<0.0001	<0.0001

Only MRI studies with volumetric analysis were assessed.
Abbreviations: T2LN: T2 lesion number; T2LV: T2 lesion volume; Gd+: number of gadolinium-enhancing lesions; T2New: number of new T2 lesions; BPFΔ: brain parenchymal fraction percentage change from baseline to one and five years; r_s: Spearman correlation. BPFΔ and PBVC values correspond to partial correlations adjusted for age and Gd+ lesions at baseline.

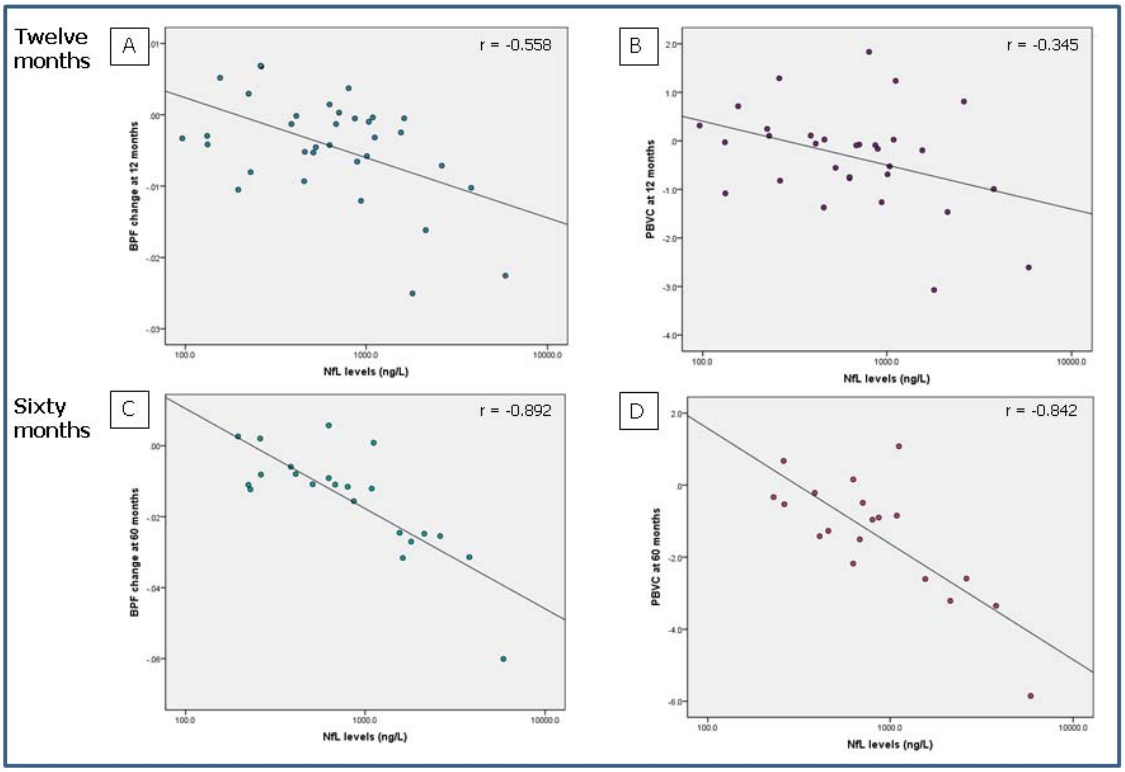


Figure VI-6. Scatter plots showing the correlations between baseline NfL levels and brain volume changes. A: BPF change at 12 months. B: PBVC at 12 months. C: BPF change at 60 months. D: PBVC at 60 months.
Abbreviations: NfL: neurofilament light chain; BPF: brain parenchymal fraction; PBVC: percentage brain volume change.

5.2.1. Conversion to CDMS and fulfilment of the McDonald criteria. Using the cut-off of 900 ng/L as previously established (Villar LM, 2014), 63 (40.6%) patients were NfL positive. Significantly more NfL positive patients converted to CDMS or fulfilled 2010 McDonald MS over time (**Figure VI-8**).

5.2.2. Risk of evolution to MS. When evaluating NfL as a continuous variable, a one point increase in risk for both conversion to CDMS and fulfilment of the 2010 McDonald criteria was observed for every 100 ng/L increment in NfL levels in the univariate analyses. aHR of NfL levels for conversion to CDMS remained significant in the multivariate analysis and showed a trend towards significance (HR 1.004, 95%CI 0.999-1.009, p=0.081) in the case of 2010 McDonald MS that disappeared when adding DMT to the model (**Table VI-18**).

Table VI-17. Baseline demographic and clinical characteristics of the replication phase cohort.

n=155	
Mean age in years (SD)	33.6 (8.8)
Females: n (%)	101 (65.2)
Mean follow-up in years (SD)	3.7 (2.3)
CIS topography: n (%) - Optic nerve - Brainstem - Spinal cord - Other	 59 (38.1) 35 (22.6) 38 (24.5) 23 (14.8)
Positive OCB: n (%)	85 (54.8)
Number of T2 lesions on baseline MR: n (%)* - 0 - 1-3 - ≥4	 41 (27.5) 28 (18.8) 80 (53.7)
Conversion to CDMS: n (%)	48 (31.0)
DMT before CDMS: n (%)	46 (29.7)

*T2 lesions: N=87 VHH, N=62 RyC.
Categorical variables: chi^2 test. Continuous variables: Student´s T test for two groups.
Abbreviations: SD: standard deviation; CIS: clinically isolated syndrome; OCB: oligoclonal bands; MR: magnetic resonance; CDMS: clinically definite multiple sclerosis; DMT: disease modifying treatment.

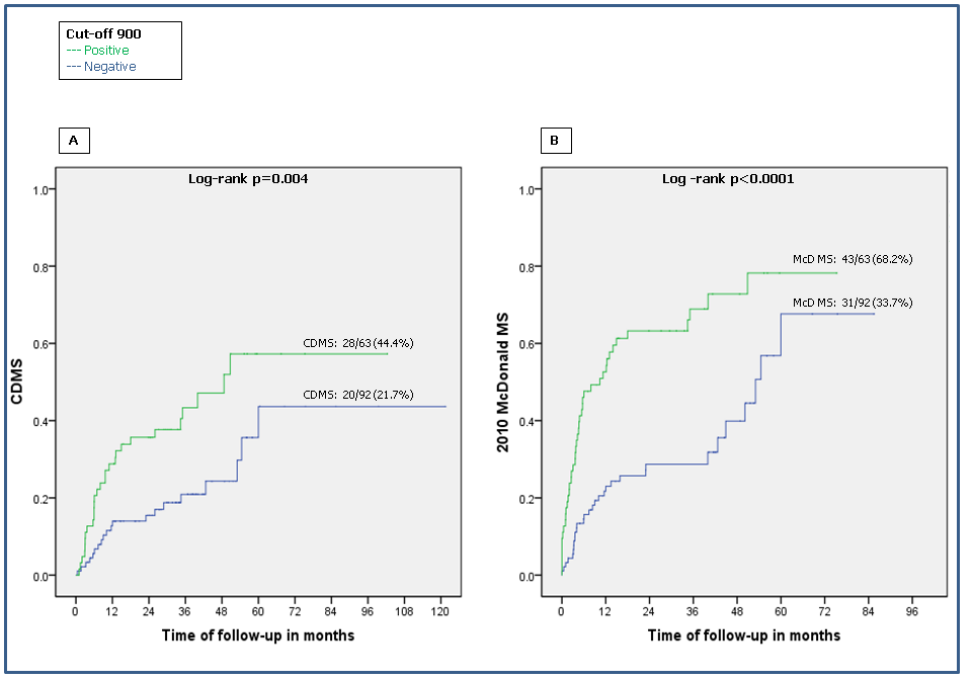


Figure VI-8. Time to CDMS (A) and to 2010 McDonald 2005 (B) for NfL status.
Abbreviations: NfL: neurofilament light subunit, MR: magnetic resonance; IQR: interquartile range.

Table VI-18. Uni- and multivariate analysis for CDMS and 2010 McDonald MS with NfL as a continuous variable.

Conversion to CDMS			
Univariate analysis	**p**	**HR**	**95% CI**
NfL-100*	<0.0001	1.009	1.005-1.014
OCB	<0.0001	6.074	2.580-14.300
T2 lesions 1-3	0.047	8.537	1.026-71.004
T2 lesions ≥4	0.002	21.709	2.982-158.026
Hospital	0.063	1.722	0.971-3.052
DMT**	0.701	1.126	0.615-2.062
Multivariate analysis¤	**p**	**aHR**	**95% CI**
NfL-100	0.040	1.005	1.000-1.011
OCB	0.048	2.597	1.009-6.683
T2 lesions 1-3	0.071	7.225	8.043-61.920
T2 lesions ≥4	0.022	11.469	1.432-91.868
2010 McDonald criteria			
Univariate analysis	**p**	**HR**	**95% CI**
NfL-100	<0.0001	1.009	1.005-1.013
OCB	<0.0001	8.427	4.189-16.951
T2 lesions 1-3	0.020	11.842	1.480-94.741
T2 lesions ≥4	<0.0001	52.103	7.220-375.983
Hospital	0.001	2.232	1.404-3.546
DMT	0.292	0.736	0.416-1.302
Multivariate analysis¥	**p**	**aHR**	**95% CI**
NfL-100	0.155	1.004	0.999-1.008
OCB	0.012	2.669	1.236-5.762
T2 lesions 1-3	0.036	9.593	1.165-79.034
T2 lesions ≥4	0.002	25.676	3.347-196.974

*HR increase for every 100 ng/L.
**DMT: before conversion to CDMS or fulfilment of the 2010 McDonald criteria.
¤Results were not modified before and after adjusting for DMT.
¥Results adjusted for hospital and DMT. When only adjusting for hospital, the NfL aHR remained similar but with a p value of 0.081.
Abbreviations: CDMS: clinically definite multiple sclerosis; HR: hazard ratio; 95% CI: 95% confidence interval; NfL: neurofilament light chain; OCB: oligoclonal bands; DMT: disease modifying treatment; aHR: adjusted hazard ratio.

As for NfL status (positive/negative), having NfL levels above the cut-off value of 900 ng/L was a predictor of evolution to both CDMS and 2010 McDonald MS

in the univariate analysis; however, this significance was lost in the multivariate analyses (**Table VI-19**).

Table VI-19. Uni- and multivariate analysis for CDMS and 2010 McDonald MS with NfL status (cut-off of 900 ng/L).

Conversion to CDMS			
Univariate analysis	**p**	**HR**	**95% CI**
NfL, cut-off of 900 ng/L	0.005	2.279	1.283-4.049
Multivariate analysis¤	**p**	**aHR**	**95% CI**
NfL, cut-off of 900 ng/L	0.530	1.220	0.656-2.269
OCB	0.051	2.615	0.998-6.852
T2 lesions 1-3	0.068	7.631	0.860-62.984
T2 lesions ≥4	0.020	11.874	1.483-95.065
2010 McDonald criteria			
Univariate analysis	**p**	**HR**	**95% CI**
NfL, cut-off of 900 ng/L	<0.0001	2.801	1.761-4.455
Multivariate analysis¥	**p**	**aHR**	**95% CI**
NfL, cut-off of 900 ng/L	0.252	1.347	0.809-2.244
OCB	0.026	2.461	1.112-5.447
T2 lesions 1-3	0.038	9.315	1.132-76.660
T2 lesions ≥4	0.002	24.912	3.234-191.928

*Results were not modified before and after adjusting for DMT.
**Results adjusted for hospital and DMT. When only adjusting for hospital, the NfL aHR did not vary.
Abbreviations: CDMS: clinically definite multiple sclerosis; HR: hazard ratio, 95% CI: 95% confidence interval; NfL: neurofilament light chain; aHR: adjusted hazard ratio; OCB: oligoclonal bands; DMT: disease modifying treatment.

5.2.3. NfL levels at baseline and disability accumulation. Although NfL levels were 2635.6 (1226.4-4044.8) ng/L for the 11 patients with EDSS ≥3.0 and 1560.5 (934.8-2186.2) ng/L for those with lower EDSS scores, this difference was not statistically significant (p=0.172).

5.2.4. NfL levels and inflammatory parameters on MRI. Significant correlations were found between NfL levels and T2 lesion volume and number of Gd-enhancing lesions on baseline and follow-up MRs (**Table VI-20**).

Table VI-20. NfL levels and inflammatory parameters on MRI. Replication phase.

	Baseline brain MRI		Follow-up MRI at 12 months	
	T2LV	Gd+	T2LVNew*	Gd+
N	63	146	36	94
r_s	0.517	0.469	0.420	0.231
p	<0.0001	<0.0001	0.011	0.025

*Since 2009, T2 lesion volume is evaluated only for new T2 lesions.
Spearman correlation.
Abbreviations: NfL: neurofilament light; MRI: magnetic resonance imaging; T2LV: T2 lesion volume; Gd+: gadolinium-enhancing lesions; T2LVNew: T2 lesion volume of new lesions.

Higher NfL levels were associated with a higher number of T2 lesions on MRI scans performed at baseline and at one year (**Figure VI-9**).

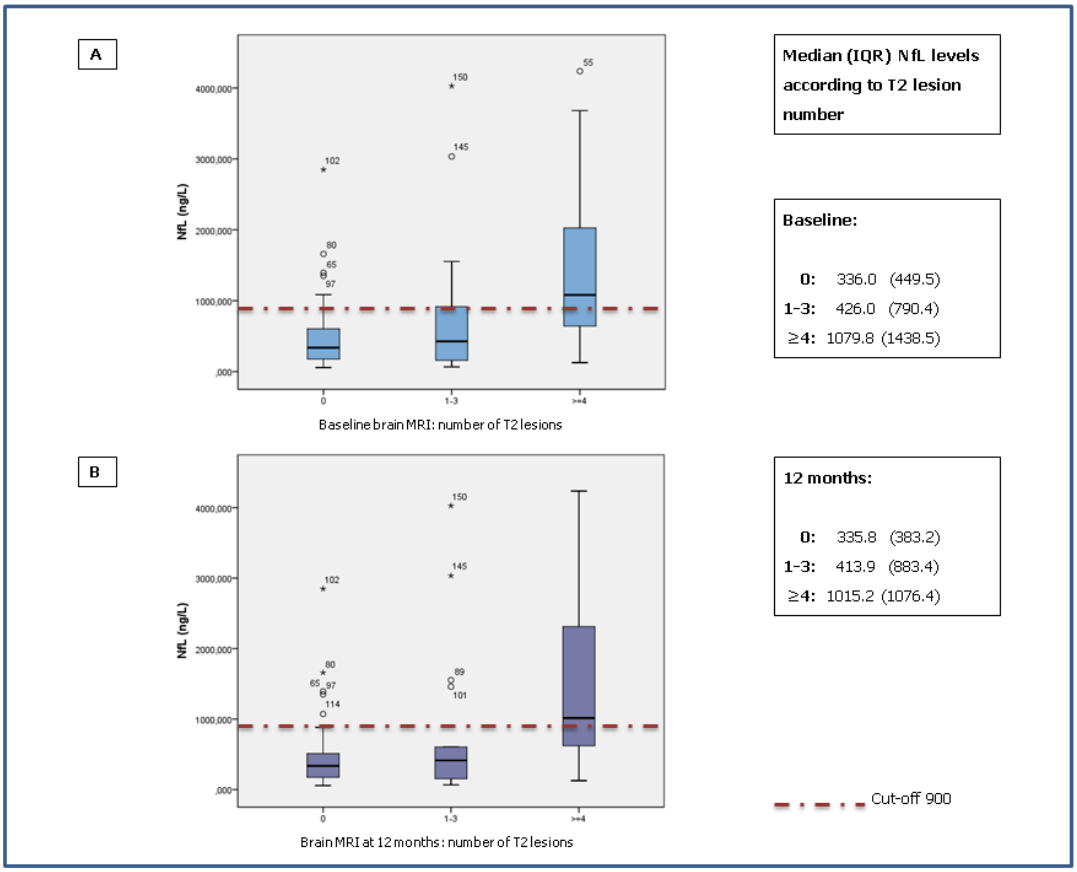
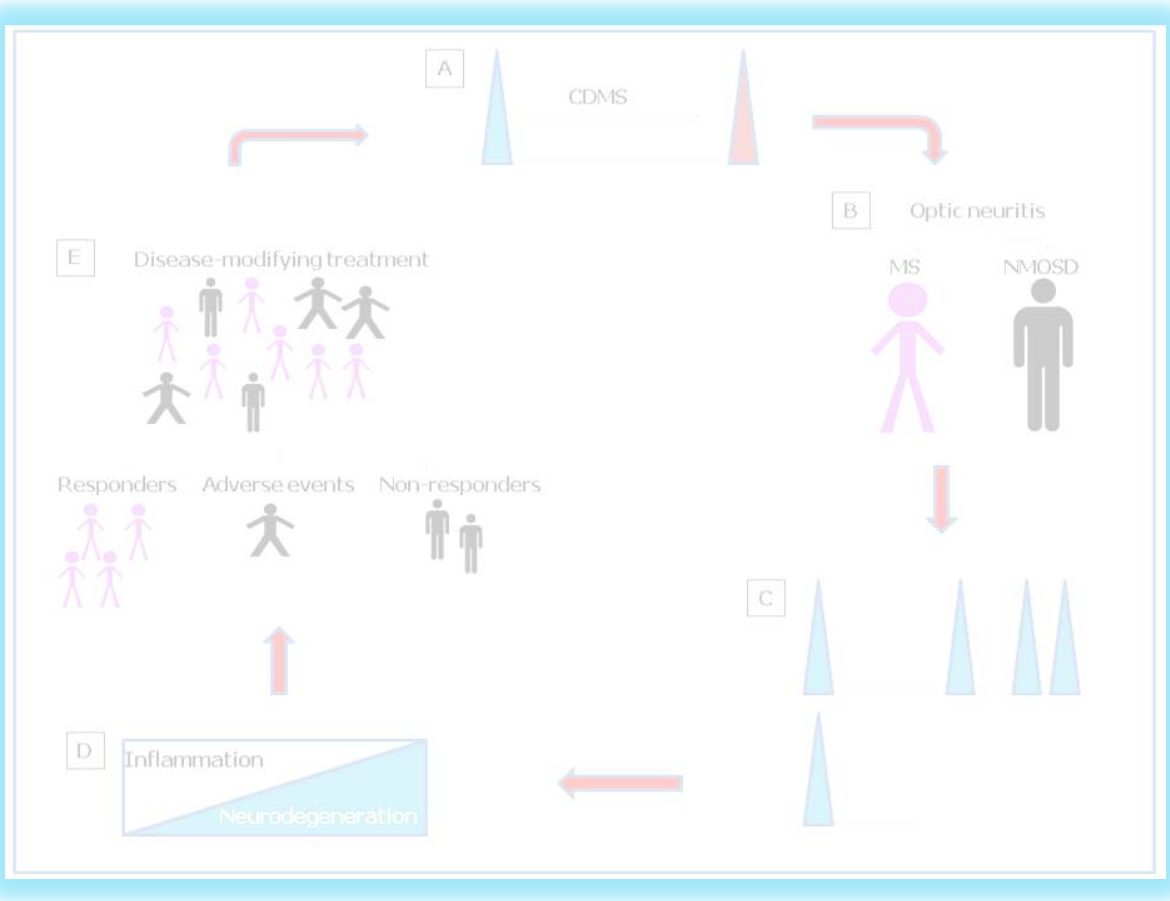


Figure VI-9. NfL levels and number of T2 lesions on baseline brain (A) and one year (B) MRI. The discontinuous line represents the cut-off of 900 ng/L.
Abbreviations: NfL: neurofilament light subunit, MRI: magnetic resonance imaging; IQR: interquartile range.



VII. DISCUSSION

VII. Discussion

1. First objective: to assess the value of NMO-IgG status in a cohort of patients regarded as having a CIS at the time of presentation

Recurrent optic neuritis or myelitis may be manifestations of NMOSD, but can also be present in MS and in other metabolic or autoimmune conditions (Miller DH, 2008). Brain and spinal cord MRI, OCB, autoantibody determinations, computer tomography scans or determination of NMO-IgG, among other studies, can aid in the differential diagnosis of individual cases (Sellner J, 2010; Lennon V, 2004). Given the importance of differentiating NMOSD from MS, as both treatment and prognosis differ considerably, we aimed to determine the frequency of NMO-IgG positivity in patients with a first demyelinating event involving the optic nerve or the spinal cord and at least a second attack affecting either topography (classic NMO phenotype) selected from a prospective cohort of patients regarded as having a CIS at the time of presentation. Only one of the NMO-IgG positive patients in group 1 (NMO phenotype) developed clinical NMO. The other two evolved as prototypical MS during a follow-up time of over 9 years and have received the standard treatment for this disease. Of the two positive patients in group 2 (negative controls), one also behaved as the two aforementioned MS cases, and the other has remained clinically and radiologically stable after two demyelinating episodes (**Table VI-2**). It is important to acknowledge that NMO-IgG determination has been useful to detect one patient who was eventually diagnosed with NMO, confirming the previously published data on the diagnostic biomarker's usefulness. Conversely, however, even in patients with an NMO phenotype regarded as CIS at the time of presentation, the frequency of positive cases is low. Therefore, our results suggest that NMO-IgG determination is not routinely necessary in all patients with recurrent optic neuritis or myelitis; however, it could be useful in cases in which the diagnosis is not clear after considering together the available clinical, radiologic, and OCB information, supporting the rational use of medical resources. These findings are in accordance with published data in which even in classic NMO patients,

NMO-IgG determination was needed in only 10.0% of the cases in order to complete the 2006 diagnostic criteria (Collongues N, 2010), as well as with the recommendations included in the 2010 revisions to the McDonald criteria, in which testing for NMO-IgG is advised only in patients suspected of having NMO or NMOSD, especially in Asian and Latin American populations (Polman CH, 2011). Regarding group 3 (NMO positive control group), although we found a lower positivity (44.3%) rate than expected, other studies have reported an NMO-IgG sensitivity ranging from 54.0% to 73.0% when detection was made using indirect immunofluorescence (Lennon VA, 2004; Saiz A, 2007; Collongues N, 2010; Waters P, 2008). We believe the result observed in the NMO-positive control group could be partly explained by the small sample size. Besides, from the time this study was performed, more sensitive cell-based assays have been developed that do not compromise specificity, yielding a mean 76.7% sensitivity in a pooled analysis (Wingerchuk DM, 2015); therefore, it is possible that some of our negative cases may be positive if tested with a cell-based assay.

An important limitation of this study is that the NMO spinal cord MRI criteria could not be used to select the cases as such study has not been routinely performed in the prospective cohort until recently. Our results suggest that the utility of NMO-IgG determination as a routine test in patients presenting with symptoms of the type seen in MS is low, regardless of the affected topography, and should only be performed in selected cases. The 2015 revision of the NMOSD diagnostic criteria by the International Panel for NMO Diagnosis (IPND) further support this conclusion (Wingerchuk DM, 2015): based on evidence collected after the discovery of the NMO-IgG antibodies, the proposed criteria are more complex, yet more specific regarding the clinical and radiological findings that support the diagnosis of NMOSD, and red flags for alternative diagnoses are also described. Therefore, besides considering NMO-IgG testing in cases fulfilling the 2015 clinical and imaging definitions, they could be useful during first attacks indeterminate for MS or NMOSD.

2. Second objective: to assess the added value of presence of ≥2 predictive factors for MS in patients not fulfilling the 2010 DIS criteria

To this day, MRI remains the most important tool for MS diagnosis, hence becoming a key factor in the McDonald criteria (McDonald I, 2001; Polman CH, 2005; Polman CH, 2011). However, a proportion of patients may not fulfil the criteria after a baseline MRI. Therefore, we wanted to evaluate if combinations of other predictive factors could help establish the risk of evolution to MS in those patients. Such predictive factors were age <40 years, presence of OCB, and presence of ≥3 periventricular lesions on baseline MRI (Ruet A, 2011). Nevertheless, first we needed to assess the risk and performance of the 2010 criteria in our cohort to have a better perspective of results concerning the predictive factors. In this study, over 35.0% of patients were diagnosed earlier (after a median of six months) when applying the 2010 criteria (Polman CH, 2011). We observed a 3.8 to 4.2-fold increase in the risk of developing CDMS when evaluating DIS and DIT individually, while the aHR increased to 8.6 in patients who fulfilled both DIS and DIT. This is in accordance with similar findings reported by Swanton and collaborators (Swanton JK, 2007). Regarding performance, DIS and DIT sensitivity is lower than previously reported (Swanton JK, 2007; Nielsen JM, 2010). Such differences could be explained due to inclusion of baseline MRIs exclusively in our case, to our lower proportion of abnormal baseline MRIs (63.0%), and to the timing set for measuring the outcome (CDMS at two years instead of three, as assessed by Swanton JK, 2007). In the case of DIT, an additional reason could have been the MRI timing as the closer to the event, the higher the probabilities of observing Gd-enhancing lesions (Swanton JK, 2007; Rovira A, 2009). Our specificity for DIS and, above all, for DIT, was higher than previously reported (Swanton JK, 2007). Finally, patients with DIS or especially DIT on baseline MRI have a greatly increased risk of fulfilling the 2010 McDonald MS criteria over time. Nonetheless, it is important to note that in this case, DIS and DIT were not adjusted for each other and patients with DIS could also fulfil DIT and vice versa. Furthermore, in comparison to CDMS as the outcome, performance improves as well.

After establishing the risk and performance of fulfilling the 2010 McDonald criteria in our cohort, we evaluated the presence of ≥2 predictive factors in patients who did not fulfil the criteria and found an almost four-fold risk of developing CDMS and a diagnostic accuracy of 70.1%. When evaluating the McDonald MS criteria, the aHR was lower but performance remained similar. We also evaluated combinations of two predictive factors in patients not fulfilling DIS. Despite the fact that presence of one periventricular lesion is already included in the 2010 DIS criteria and that in many settings lumbar puncture is not performed on a routine basis, if such information is available, having ≥2 predictive factors when not fulfilling DIS increases the risk of MS with a high specificity, especially in combinations including three periventricular lesions.

Our study has several limitations. In the inclusion criteria, the upper age limit was <50 years. Thus, the specificity and accuracy of "age ≤40" could have been underestimated. Furthermore, approximately 75.0% of patients underwent a lumbar puncture. When evaluating baseline characteristics, patients with optic neuritis and normal baseline MRIs were more prone to turn this procedure down. This observation was previously reported in our cohort (Tintore M, 2008b) and could bias our results towards a more aggressive disease course. Another limitation is the low number of spinal cord MRIs (23.6% vs 62.5% of the core cohort by Swanton and collaborators) thus possibly limiting the number of cases actually fulfilling DIS or DIT.

In conclusion, our study shows that although sensitivity of the 2010 criteria may differ based on timing and number of MRIs included in the analysis and on differing cut-off times for evaluation, accuracy and specificity remain very high among studies, thus confirming these criteria can be applied with a low risk of misdiagnosis in typical CIS cases. Besides, having ≥2 predictive factors or combinations of predictive factors in patients not fulfilling DIS is highly specific for developing CDMS or McDonald MS, especially if three periventricular lesions are present.

3. Third objective: to analyse the added value of spinal cord MRI in the fulfilment of the 2010 DIS and DIT criteria in non-spinal cord CIS patients, and to evaluate the prognostic value of spinal cord lesions for evolution to MS and disability accumulation

The usefulness of systematically performing a spinal cord MRI at the time of a CIS is still considered controversial, especially in patients presenting with symptoms other than myelitis (Barkhof F, 2014; Rovira A and Tintore M, 2014; Hutchinson M, 2014). Therefore, we aimed to evaluate the added diagnostic value of performing a spinal cord MRI at baseline, as well as the prognostic value of spinal cord lesions.

When evaluating the role of spinal cord MRI on MS diagnosis, we observed that only four additional patients fulfilled radiological DIS and DIT, deeming it necessary to perform 36 scans to diagnose one additional patient. By eliminating normal brain MRIs from the initial analysis, the number needed to scan improved to 23, and to 17 if we assessed those with pathological brain MRI not fulfilling DIS and DIT. However, the relative chance of diagnosing MS is almost 15.0% higher if a spinal cord MRI is performed in addition to a brain scan. Our number needed to scan results differ from the findings published by Sombekke and colleagues, who observed that only seven scans were needed to diagnose one additional patient in a CIS cohort comprising 121 individuals (Sombekke MH, 2013). Conversely, Dalton and colleagues assessed 115 patients with optic neuritis and found that spinal cord MRI allowed the diagnosis in one additional patient at one year and in two at three years after applying the 2001 McDonald criteria (Dalton CM, 2003; McDonald WI, 2001). Such differences can be partly explained by the available MRI resources at the time of the studies, the different diagnostic criteria used in the analyses and, most of all, to the type of patients and their baseline characteristics: in our cohort, approximately two thirds of patients had an abnormal brain MRI, compared to the 95.0% reported by Sombekke and the 70.0% by Dalton. As for spinal cord lesions, we identified them in approximately 45.0% of cases (78.1% spinal cord versus 30.1% non-spinal cord), Sombekke in 67.8% (81.0%

spinal cord versus 53.4% non-spinal cord), and Dalton in 27.0%. Whether a brain MRI is pathological or not appears to be relevant: Perumal and colleagues studied 58 patients with acute transverse myelitis and a normal brain MRI and, after a mean follow-up of five years, observed that 17 (29.3%) patients converted to CDMS or fulfilled the 2005 McDonald diagnostic criteria [7 (41.0%) CDMS, 10 (59.0%) McDonald MS] and, most importantly, none of the patients developed MS after 24 months of disease onset (Perumal J, 2008; Polman CH, 2005). Similarly, Patrucco and colleagues did not observe conversion to CDMS in their cases with normal brain MRI after a mean follow-up of approximately six years (Patrucco L, 2012). All these findings suggest that the added value of spinal cord MRI increases in more pathological cases, but as patients with more brain lesions will most probably fulfil the diagnostic criteria without the need to assess the spinal cord, the role of spinal cord scans appears to be more relevant in pathological cases not fulfilling the criteria with brain MRI alone. From the clinical practice point of view, these findings may implicate that, ideally, an expert should be present at the time the brain MRI scan is being performed to assess whether the spinal cord study is necessary, which is of course unrealistic. Therefore, the decision to perform a spinal cord MRI routinely in CIS may depend not only on work burden, but also on the prognostic value of spinal cord lesions, and part of the aforementioned controversy may originate from the limited evidence on this topic. In our study, we found that the presence of spinal cord lesions is an independent risk factor for evolving to MS regardless of CIS topography and of the symptomatic lesion that was better demonstrated for 2010 McDonald MS, probably due to the higher number of patients reaching this outcome compared to CDMS. The observed trends towards significance could also be due to the sample size. Additionally, when assessing the few patients in the "missing" category for OCB, they had a more homogeneous distribution of spinal cord lesions regardless of outcome fulfilment which may have also influenced the results. To our knowledge, only two other studies have assessed spinal cord lesions prospectively. Patrucco and colleagues studied 75 patients with CIS involving the typical topographies and found an increased risk for CDMS of at least 3.5

times that was independent of brain lesions, presence of OCB, and CIS topography (Patrucco L, 2012). Sombekke and colleagues assessed four different CIS groups in their survival analyses and found that presence of spinal cord lesions conferred the highest risk for CDMS in non-spinal cord cases not fulfilling the MS criteria on brain MRI (Sombekke MH, 2013). As for number of spinal cord lesions, the univariate analyses showed that, the higher the spinal cord lesion number, the higher the risk of evolving to MS. In the multivariate analysis, there is a tendency towards a higher risk with increasing number of spinal cord lesions; however the results are only significant in certain specific subgroups, probably because of a limited sample size in the other subgroups. And once again, the better results when assessing 2010 McDonald MS compared to CDMS may be secondary to the higher proportion of patients who reached the outcome in the former case. However, one of the important advantages in our study is that we were able to assess baseline demographic, clinical, CSF, and brain MRI baseline data all together. All in all, the results point towards the fact that both presence and increasing number of spinal cord lesions are independent factors for predicting MS. These findings also suggest that not only lesion number, but also lesion location, may play a role in defining MS risk, as it has been previously demonstrated with infratentorial lesions (Minneboo A, 2004; Tintore M, 2010). Therefore, spinal cord lesions may further contribute to individualize MS risk (Tintore M, 2015).

As for the prognostic value of spinal cord lesions in disability accumulation, previous evidence is even more limited. Swanton and colleagues evaluated 100 patients with optic neuritis and observed that presence of spinal cord lesions was a predictor for reaching a higher disability outcome (ranked EDSS: 0, 1, 1.5-2.0, ≥2.5) together with Gd-enhancing lesions and new T2 lesions on follow-up MRI three months after the initial scan (OR 3.30, 95% CI 1.26-8.68). When evaluating spinal cord lesion number (0, 1, 2, ≥3), once again, spinal cord lesions were predictors of disability, together with infratentorial and new T2 lesions. And in patients who converted to CDMS, the only predictor was presence or number of spinal cord lesions (Swanton JK, 2009). In our study, we observed a trend when evaluating the 207 patients and found that presence of

spinal cord lesions is an independent risk factor for disability in non-spinal cord CIS. Our study is limited by the few patients who reached the disability outcome during follow-up. That Swanton and colleagues found significant results might be related not only to the longer follow-up in their study, but also to their definition of disability (Swanton JK, 2009), as ours is more restrictive. However, we also consider it a more robust outcome since inter-observer variability is high with lower EDSS scores and, from the clinical point of view, an EDSS of 3.0 better represents the presence of moderate disability. Additionally, we did not test spinal cord lesion number due to the aforementioned limitation. However, the results of the descriptive statistics and performance support that spinal cord lesions might pose a higher risk for disability.

Regarding performance, our results confirm the specificity that spinal cord lesions provide to both the diagnosis of CDMS and the 2010 McDonald criteria (McDonald WI, 2001; Polman CH, 2005; Polman CH, 2011; O'Riordan JI, 1998; Cordonnier C, 2003; Lycklama a Nijeholt GJ, 2000). The high PPV of spinal cord lesions supports their usefulness as a probability assessment tool of McDonald MS diagnosis in the clinical practice. The differing results in the predictive values between CDMS and McDonald MS have to do with the prevalence of each outcome in our cohort: as the proportion of patients fulfilling McDonald MS is higher, the PPV increases. Whereas applying the McDonald criteria allows for an earlier diagnosis, the prevalence of CDMS is expected to increase with a longer follow-up; therefore, the high predictive value of spinal cord lesions would switch from negative to positive as more patients convert over time. This is influenced by the number of false positives when evaluating CDMS, as it is expected that it will decrease over time if more patients with spinal cord lesions suffer a second attack. Additionally, spinal cord lesions are highly sensitive for disability accumulation, while the high NPV suggests that the probability of reaching an EDSS ≥3.0 in the short term is very low if no spinal cord lesions are present.

Besides some of the previously mentioned limitations, we were not able to assess the spinal cord lesion number as thoroughly as in the brain due to their classification method (0, 1, 2, ≥3) and to the technical limitations compared to

brain scans. Furthermore, presence or number of lesions may not portray the extent of the disease properly and other methods such as lesion load or atrophy measures might be necessary, particularly when assessing disability outcomes (Kearney H, 2015a; Kearney H, 2015b; Lukas C, 2015; Biberacher V, 2015). Many of these techniques will probably aid in our understanding of MS, but it will be important to identify those that will also be useful for prognosis assessment in the clinical practice.

In summary, although the additional diagnostic value of spinal cord MRI is modest, spinal cord lesions are independent predictors of evolution to MS and lesion number appears to be relevant. Moreover, spinal cord lesions are an independent risk factor for disability and the probability of reaching an EDSS ≥3.0 in the short term is very low if no spinal cord lesions are present, especially in non-spinal cord CIS.

4. Fourth objective: to analyse the predictive value of gMS-Classifier2 determination in serum for early conversion to CDMS in a large cohort of CIS patients, and to determine whether gMS-Classifier2 is an independent predictor of conversion to CDMS

Early administration of disease modifying therapies is highly recommended in CIS patients at risk for developing a second attack (Frohman EM, 2006b; Río J, 2011). Therefore, although MRI remains the most important surrogate marker for predicting the risk of a second relapse in CIS patients (Tintore M, 2006; Fisniku LK, 2008), the clinical outcome remains unpredictable due to the high variability of this disease among individuals. Thus, a need remains for auxiliary biomarkers that could provide additional information about the disease course (Fazekas F, 2010; Polman CH, 2011). The presence of OCB at baseline doubles the risk of developing a second attack independent of MRI, but in the revised 2010 diagnostic criteria for MS, CSF was only included in the diagnostic criteria for primary progressive MS. Thus, the testing of CSF may further decline, despite the fact that the International Panel agrees that the inclusion of CSF in

the criteria requires further evaluation (Tintore M, 2008b; Polman CH, 2011). Furthermore, an ideal biomarker should be non-invasive and simple to use, making potential serum prognostic markers a good option, as they would be easy to obtain (Teunissen CE, 2005). Glycans are potential antigens, and indeed, antibodies against various types of glycans have been found in serum. Such antibodies were first described for the human blood group ABO antigens (Pettenkofer HJ, 1960), and later findings have linked antibodies directed against glycans to several autoimmune diseases, either by association only or as etiopathogenic (van Kooyk Y, 2008; Dzhambazov B, 2006; Chiba A, 1992; Sendid B, 1996; Rieder F, 2010). Consequently, diverse assays have been designed to identify anti-glycan antibodies in autoimmune diseases (Schwarz M, 2003; Nimrichter L, 2004) including MS (Menge T, 2005; Lolli F, 2005a; Lolli F, 2005b). Because some of these glycans are found within the type IV collagen matrix of the BBB (Freedman MS, 2009), it has been hypothesized that in MS patients, an inflammatory response could lead to the release of these carbohydrate antigens with the subsequent development of a humoral response (van Horssen J, 2006). Therefore, an array of glycans was screened in RRMS patients and healthy controls, observing that IgM antibodies against various alpha-glucose molecules were elevated in the former group. Among the alpha-glucose, GAGA4 was the most notable and gMS-Classifier Dx was developed as GAGA4 normalized to total IgM. It was further analysed with other neurological disease controls (Schwarz M, 2006; Brettschneider J, 2009), concluding that gMS-Classifier Dx differentiates between MS and non-MS patients. Based on previous experience in Crohn's disease in which broader glycan structures increase performance and address different clinical utilities, gMS-Classifier Dx was extended to include the following anti-alpha glucose antibodies: GAGA2, 3, 4 and 6, thus establishing gMS-Classifier1, which is based on disaccharides covalently bound via a long linker (anti-GAGA2, anti-GAGA3, anti-GAGA4, anti-GAGA6) and was used for the prediction of "early relapse" (within 24 months) (Freedman MS, 2009). Then, in the BENEFIT study, gMS-Classifier1 was analysed on three pre-defined end-points: 1) Time to CDMS, 2) Time to 2005 McDonald, and 3) Time to confirmed EDSS. However, gMS-Classifier1 was only

significant on time to confirmed EDSS (Freedman MS, 2010; Freedman M, 2012). Thus, gMS-Classifier1 seems to be more of a prognostic MS biomarker for progression rather than for diagnosis. As part of the BENEFIT study, several additional alpha-glucose antibodies were analysed due to previously found data that suggested it could be beneficial to further explore them and see their potential value. Among those additional alpha-glucose antibodies were P63, [a polymer based on Glc(a1–3)Glc(a) and Glc(a 1–6)Glc(a)], alpha-ramose, alpha-N-acetyl glucose, and P64 [(a polymer based on Glc(a 1 4)Glc(a) and Glc(a 1–6)Glc(a)]. For each one, time to CDMS with a minimal criterion of 30.0% sensitivity and 90.0% specificity at 24 months was analysed in the BENEFIT placebo sub-cohort and on the entire cohort. Only P63 normalized to age predicted time to CDMS and it was called gMS-Classifier2 (Freedman MS, 2010). A logistic regression model for prediction of early conversion to CDMS was used to develop the classifier; the input data included a number of clinical variables such as age since previous data have shown that IgM levels vary considerably throughout the years (Ritchie RF, 1998a; Ritchie RF, 1998b), plus the raw levels of anti-glycan IgM against eight different glycan antigens. After backward selection only anti-P63 IgM levels and age were found to be independent variables which entered the model. Since this classification rule identified CIS patients at higher risk of converting to CDMS during the first two years of disease evolution (Freedman MS, 2010), the aims of the present study were to confirm those results and to determine whether gMS-Classifier2 is an independent predictor of conversion to CDMS. Our results show that in this hospital cohort, gMS-Classifier2 is an independent predictor for conversion of CIS patients to CDMS that could become a useful prognostic tool when tested within the first two years of disease evolution, and thus could add information to baseline MRI findings, more specifically, in cases in which lumbar puncture or OCB determination cannot be performed. gMS-Classifier2 was positive in 30.0% of CIS patients at baseline, and the median time to CDMS was approximately twice as short for gMS-Classifier2-positive patients than for negative patients. The predictive performance of gMS-Classifier2 was better during the early years of the disease and decreased with long-term follow-up.

When gMS-Classifier2 status was evaluated together with MRI variables in the multivariate analyses at two and five years of follow-up, it remained an independent predictor of conversion to CDMS, but not when evaluated with OCB. With MRI and OCB findings, continuous unit values of gMS-Classifier2 independently predicted the development of an early second relapse, indicating the increased risk of relapse with increased serum levels of the biomarker. When performing the ROC analyses, the model for gMS-Classifier2 units was statistically significant only when OCB were excluded. However, recent publications emphasize that testing for any improvement using discrimination measures such as the change in the area under the ROC curve is extremely conservative (Vickers AJ, 2011; Pepe MS, 2013). Thus, we consider the HRs to be sufficient to support the role of gMS-Classifier2 as an independent predictor of conversion to CDMS. As for the added value of gMS-Classifier2 to OCB findings in predicting early CDMS conversion, the differing results obtained are probably partly due to the higher resolution of a biomarker that is measured in continuous units compared to a dichotomous biomarker (Simel DL, 1993).

We conclude that gMS-Classifier2 is an independent predictor for conversion of CIS patients to CDMS in the first years of the disease course and therefore could be of clinical relevance to determine which patients are at higher risk, particularly in cases in which OCB are not available.

5. Fifth objective: to determine the value of selected biological markers in CSF as prognostic factors for conversion to CDMS and for disability progression in patients with CIS

In the screening phase, we aimed to identify differences in biomarker levels under the rationale that, if none were found between two extreme CIS groups (Comabella M, 2010), they would not be found in consecutive patients either, a scenario that better resembles clinical practice. Additionally, a screening phase allows a more selective use of biological samples.

In this phase, we found no differences in CSF levels of GFAP, fetuin A, and NfH.

Regarding GFAP, we did not find any differences in CSF levels or ratios between CIS groups. Another study compared GFAP levels between CIS-CIS and CIS-CDMS and showed that GFAP levels were good discriminators if combined with other proteins like NfL or MOG, but not if evaluated on their own (Avsar T, 2012). In general, other studies show consistent findings in which GFAP levels are increased in mixed MS phenotypes compared to controls, and when further analysing results between phenotypes, GFAP levels are higher in SPMS, whereas at least two studies showed that levels in RRMS and healthy controls were similar, especially if RRMS patients lacked Gd-enhancing lesions (Avsar T, 2012; Linker RA, 2009; Axelsson M, 2011; Burman J, 2014). We did not find any correlations between GFAP levels and disability. Whilst some previous studies did show correlations, mostly if excluding patients with relapses from the analyses, the studied groups had mixed MS phenotypes including progressive forms (Axelsson M, 2011; Burman J, 2014). More recently, a different study showed GFAP levels to be an independent predictor of disability accumulation (EDSS ≥3.0) in a group including CIS and RRMS patients, but subanalyses on CIS were not described (Martinez MA, 2015). Finally, we did not find any correlations with MRI inflammatory activity or neurodegeneration parameters. To our knowledge, only one study has previously made this analysis and obtained better correlations with atrophy measures than with T2 lesions (Burman J, 2014). However, this study included RRMS and SPMS patients and evaluated measures of atrophy unusually estimated in MS, such as the size of the third and lateral ventricles. All in all, it appears like GFAP may be more useful for assessing disability in later stages of the disease, in contrast to its potential use as biomarker in NMOSD during attacks (Uzawa A, 2013).

When evaluating fetuin A, once again, we did not find significant differences between groups. Whereas a first study showed that CIS-CDMS patients had lower levels in CSF than CIS-CIS (Tumani H, 2009), later studies have found higher levels in SPMS (Ottervald J, 2010; Harris VK, 2010). More recently, significantly higher fetuin A levels have been described in active disease compared to patients in remission and controls with other neurological diseases, but included patients already had CDMS (Harris VK, 2013). We did not

find any correlations between fetuin A levels and EDSS or MRI parameters, but no previous results are available for comparison.

As for neurofilaments, they are type IV intermediate filaments specific for neurons and a main component of the axonal cytoskeleton. They are released into the CSF when axonal damage occurs, a finding observed in neurodegenerative diseases and stroke (Petzold A, 2005a; Nylen K, 2006; Petzold A, 2007; Scherling CS, 2014). Neurofilaments are heteropolymers composed of mainly three subunits: NfL, a medium subunit, and NfH. NfL is the most abundant, smallest, and most soluble, whereas NfH is resistant to CSF proteases in its phosphorylated form (Petzold A, 2005a). Our NfH findings are consistent with a previous study including 41 patients with optic neuritis, in which no differences in CSF levels were observed between patients with white matter lesions on brain MRI + presence of OCB and those with normal findings (Lim ET, 2004). Other studies evaluating CIS-CIS and CIS-CDMS have not demonstrated significant differences in NfH levels (Brettschneider J, 2006; Fialová L, 2013b). NfH levels have indeed proved to be higher in CIS or MS than in controls, but they appear to be higher in clinically relapsing or progressive forms than after one attack, suggesting that levels may accumulate over time (Petzold A, 2005b; Kuhle J, 2011). The time of CSF sampling could also play a role as NfH levels have been found to be increased during relapses compared to remission meaning that, at least in early phases, there may be a temporary increase associated to acute inflammation (Kuhle J, 2011). Similarly, the absence of differences in NfH levels according to the EDSS score could have been due to the early phase of the disease, as correlations between NfH levels and EDSS have been reported in mixed groups of patients with different MS phenotypes (Teunissen CE, 2009; Kuhle J, 2011), and higher levels have been found in patients with progressive forms and have correlated with a higher multiple sclerosis severity score (MSSS) (Petzold A, 2005b; Petzold A, 2006; Teunissen CE, 2009). That very few of our patients reached an EDSS ≥3.0 could also have influenced our results. As for MRI, we did not find any correlations with MRI inflammatory activity and neurodegeneration parameters. A small study of CIS patients compared to controls demonstrated a correlation

of NfH levels with brain volume change at one year ($r=0.518$, $p<0.01$), but not with change in T2 lesion load (Khalil M, 2013). Therefore, although acute attacks may temporarily increase NfH levels, this protein is not a very sensitive biomarker in early stages of the disease, may rather reflect chronic irreversible damage, and has a better prognostic value for disease progression or disability as MS evolves (Teunissen CE, 2012). Furthermore, some studies suggest that NfL levels could be a more sensitive tool, particularly at earlier stages of MS (Kuhle J, 2013a; Kuhle J, 2013b).

In the screening phase, only NfL levels were significantly higher in patients who converted to MS compared to non-converters. Significantly higher NfL levels in CIS-CDMS compared to CIS-CIS were reported in a study with 38 patients (Teunissen CE, 2009) but this was not confirmed in three other investigations including 9-36 patients per group, two of them using the same ELISA as herein described (Avsar T, 2012; Fialová L, 2013a; Khalil M, 2013). Our study was properly powered with a well-balanced distribution between groups. Additionally, NfL levels were marginally higher in CIS-CDMS compared to other inflammatory neurological diseases but not to other non-inflammatory neurological diseases, probably due to the inclusion of patients with Parkinson's disease, Alzheimer's disease, and stroke in the latter group (Petzold A, 2005a; Nylen K, 2006; Petzold A, 2007; Kuhle J, 2010; Scherling CS, 2014). Other studies found significantly higher NfL levels in CIS or MS patients than in controls, one of them also differentiating between CIS groups, but controls were selected under differing definitions (Malmestrom C, 2003; Norgren N, 2004; Teunissen CE, 2009; Fialová L, 2013a; Kuhle J, 2013a).

No associations between NfL levels and disability as measured by EDSS were observed. Except for one study, most have shown significant correlations, but the analyses have included CIS and other MS phenotypes, with the strongest correlations observed in progressive forms (Semra YK, 2002; Malmestrom C, 2003; Norgren N, 2004; Teunissen CE, 2009; Kuhle J, 2013a; Trentini A, 2014). Khalil and collaborators (Khalil M, 2013) evaluated CIS exclusively and found a marginal correlation coefficient of $r_s=0.324$, $p<0.05$; however, no separate

results for CIS-CDMS and CIS-CIS were reported. When assessing NfL levels according to EDSS ≥3.0, they were higher in patients with more severe disability but the difference was not significant, a finding observed again in the replication phase that could be partly influenced by the few patients (n=11-15) who reached such EDSS score. In this sense, Salzer and collaborators (Salzer J, 2010) evaluated RRMS patients assessing the MSSS composite instead, observing that evolution to SPMS was more likely with higher NfL levels.

Our results showed significant associations between NfL levels and MRI inflammatory parameters. Correlations with T2 lesion number and Gd-enhancing lesions on baseline brain MRI have been reported in CIS and/or MS groups (Teunissen CE, 2009; Burman J, 2014; Villar LM, 2015). However, other studies using baseline or follow-up MRIs did not show correlations with Gd-enhancing lesions in acute optic neuritis or with T2 lesion volume in CIS (Khalil M, 2013; Modvig S, 2013). We observed correlations with number and volume of T2 lesions on baseline MRI and, interestingly, on follow-up at one and five years, with similar findings during the replication phase, even though T2 lesion volume data were available only at Vall d'Hebron Hospital. As for Gd-enhancing lesions, in the screening we found correlations with NfL levels at baseline and one year, but not at five years. In the replication phase, we found correlations again at baseline and one year. The most striking findings concern MRI neurodegenerative parameters in the screening phase: our results suggest that baseline NfL levels are not only increased in association with lesion load, accrual and activity, but also independently of these inflammatory parameters (Perez-Miralles F, 2013; Bielekova B and McDermott MP, 2015). On the contrary, a previous study did not find any correlations between NfL levels and brain volume at baseline or brain volume change in CIS (Khalil M, 2013). Although the authors did adjust for age and used the SIENA software, results were not controlled by Gd-enhancing lesions and the follow-up was only assessed at one year. Our study is the first to evaluate the association between NfL levels at the time of a CIS and changes in brain volume at five years. Our findings are partly supported by those of Burman and colleagues, in which NfL levels were elevated irrespective of Gd-enhancing lesions in progressive MS (Burman J,

2014). Unfortunately, our appraisal of neurodegenerative markers is limited: our sample size was small in the screening and we were not able to repeat this evaluation in the replication phase due to the few patients with calculated BPF at the time of the analysis. However, by estimating both BPFΔ and PBVC, the latter a robust method for evaluating atrophy, we believe our results are reliable and should be assessed in future studies.

Of note, there was a significant correlation between NfL and NfH levels. Similar results have been found in CIS and RRMS before (Teunissen CE, 2009; Khalil M, 2013; Kuhle J, 2013a). However, in comparison to our negative findings for NfH, some of these and other studies did show correlations between NfH and EDSS (Brettschneider J, 2006; Teunissen CE, 2009; Kuhle J, 2011), T2 lesion number (Brettschneider J, 2006) or accelerated global brain volume decrease (Khalil M, 2013). Thus, the origin of this correlation in our study is a matter of debate and deserves further study.

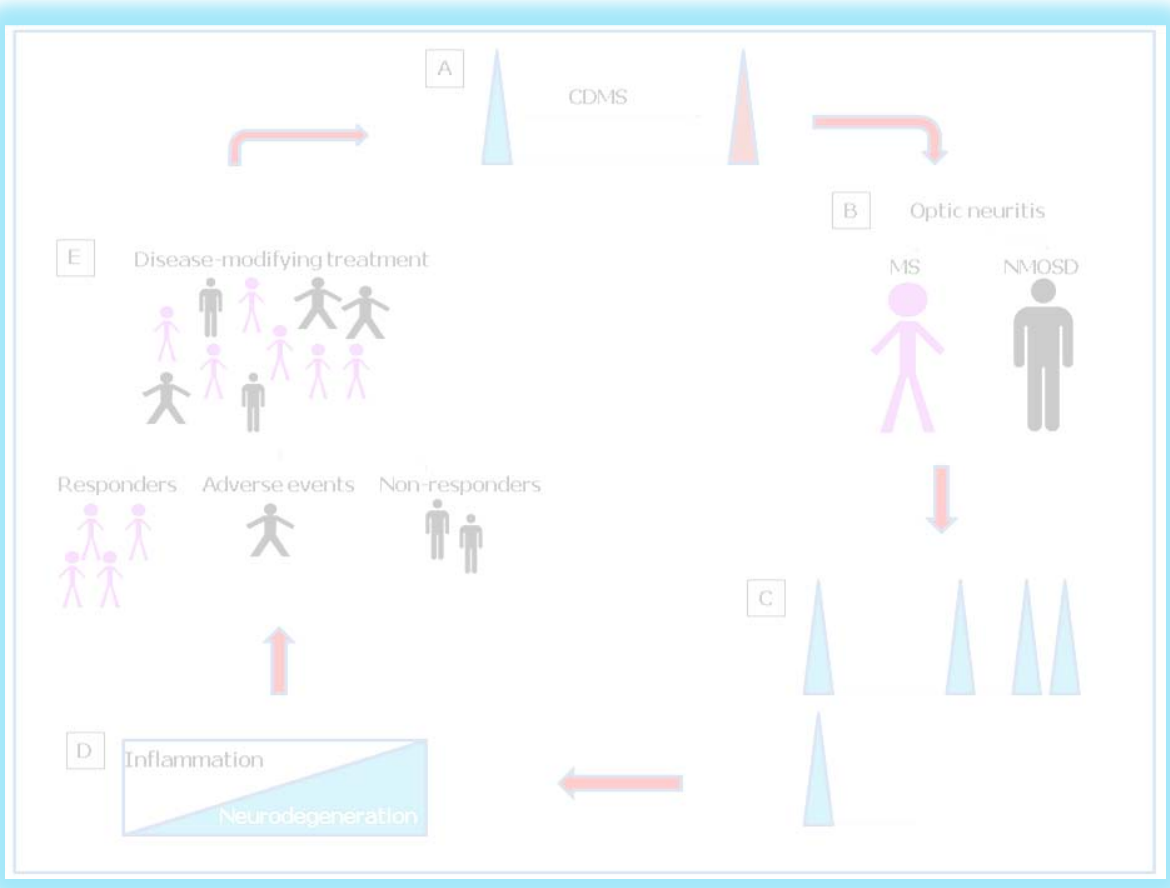
In the replication phase, we aimed to explore NfL as a risk factor for MS. Under the rationale that a dichotomic biomarker (positive/negative) could be more useful in the clinical practice, we explored a 900 ng/L cut-off obtained from a control group with non-inflammatory or neurodegenerative diseases by calculating the mean + 3 standard deviations of the control values (Villar LM, 2014), observing that more NfL-positives than negatives evolved to CDMS and 2010 McDonald MS. To our knowledge, only Malmeström and collaborators and Salzer and collaborators have evaluated cut-off values, the former exploring 674 ng/L for identifying relapses with good sensitivity and specificity, and the latter showing that RRMS patients with NfL levels >386 ng/L more likely evolved to SPMS (Malmestrom C, 2003; Salzer J, 2010). One study explored NfL levels and fulfilment of the 2010 DIS criteria in optic neuritis (Modvig S, 2013). However, no cut-off values or survival analyses for fulfilment of the 2010 McDonald criteria have previously been explored in CIS. Another original contribution is the assessment of NfL as an independent risk factor for MS over time. In the univariate analyses, we found a risk for CDMS and 2010 McDonald MS of 1.009 times for every 100 ng/L increase in NfL levels that was maintained

for CDMS in the multivariate analysis, observing a trend towards significance for 2010 McDonald MS. When assessing NfL status (positive/negative), the increased risk for both CDMS and 2010 McDonald MS in the univariate analyses was lost in the multivariate models. Conversely, the aHR of OCB was maintained, confirming its usefulness as an independent predictor of MS risk (Tintore M, 2008b). Thus, although NfL status could be more practical in the clinical setting, only NfL as a continuous variable is an independent risk factor for CDMS. The non-significant results of NfL as a continuous variable in predicting 2010 McDonald MS could be conditioned by the distribution of patients over the different categories included in the multivariate analysis, the correlations between NfL levels and T2 lesions, and the strong predictive value of T2 lesions in DIS fulfilment as part of the 2010 McDonald criteria. Even if NfL levels are independent risk factors, the higher aHRs for T2 lesion number, particularly in 2010 McDonald MS, underscore the major diagnostic role of MRI. Furthermore, a shortcoming of NfL levels is their determination in CSF as lumbar puncture is not routinely carried out in some centres, especially since the publication of the 2010 McDonald criteria (Polman CH, 2011). With the available ELISA, it has not been possible to quantify NfL levels in serum (Avsar T, 2012; Fialová L, 2013a); however, a new electrochemiluminescence array has shown promising results in detecting serum NfL levels in neurodegenerative diseases and spinal cord injury (Gaiottino J, 2013; Kuhle J, 2014), and in detecting higher levels in CIS compared to healthy controls (Disanto G, 2015). In any respect, NfL determination could add useful information for MS prognosis in cases in which a lumbar puncture is performed, although it would be considered a low impact risk factor for MS (Tintore M, 2015).

Besides the aforementioned limitations, other issue pertains to the wide confidence intervals in the Cox regression models. This was due to the very low number of patients with either negative OCB or zero T2 lesions who had converted to CDMS (n=6 and n=1, respectively) or who fulfilled 2010 McDonald MS (n=9 and n=1). This, in turn, could be related to the mean follow-up of 3.7 (2.3) years, as patients with negative baseline studies may require more time to evolve to MS.

Our biomarker list was probably not thoroughly comprehensive. We did, however, search for updates throughout the duration of the study. Besides, the properties of the selected assays could have influenced our findings. As for NfH levels, we did not compare the electrochemiluminescence assay to other available methods. Finally, control groups including other neurological diseases were only assessed during the first biomarker determination (fetuin A and NfL), but as the main objective of the screening was to evaluate whether biomarkers can differentiate between CIS-CDMS and CIS-CIS, we decided not to assess controls in the following determinations if there were no differences between CIS groups.

In summary, NfL levels are markers of axonal damage in patients who will develop MS and higher levels indicate a higher risk of evolving to MS. Besides, higher NfL levels are associated with MRI inflammatory parameters on baseline and follow-up scans and are, above all, strongly associated with brain volume loss at medium-term follow-up.



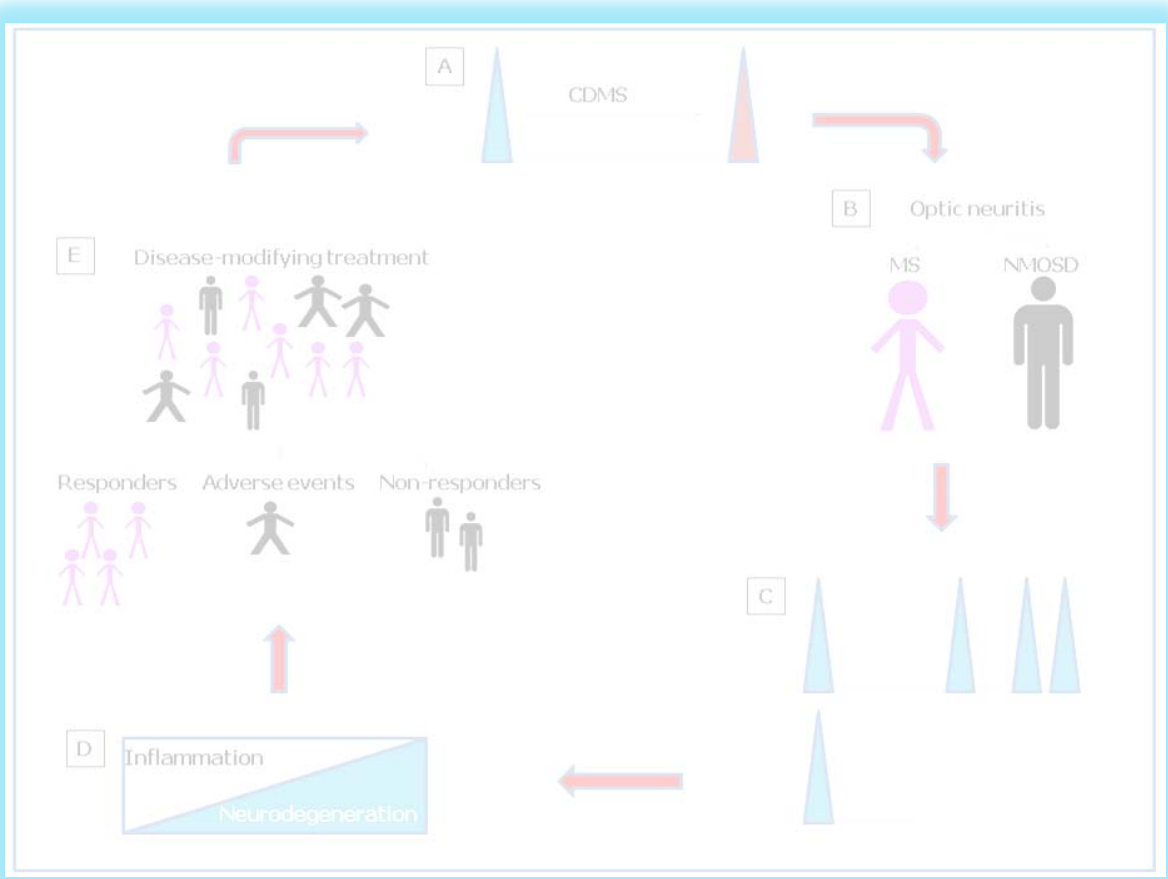
VIII. CONCLUSIONS

VIII. Conclusions

At the time of a CIS, a number of markers have been identified with varying degrees of impact upon diagnosis and prognosis.

1. NMO-IgG antibodies are highly specific for NMOSD.
2. NMO-IgG antibodies should only be determined in suggestive cases according to the 2015 revised criteria for NMOSD or in cases with indeterminate characteristics for MS or NMOSD.
3. The 2010 McDonald criteria are highly specific despite their simplification compared to the 2005 revisions.
4. In cases not fulfilling the 2010 criteria, other combinations of two or more low to high impact prognostic factors (age <40 years, presence of OCB, and ≥3 periventricular lesions) also provide high specificity for MS diagnosis and patients with these characteristics should be monitored closely.
5. Spinal cord MRI contributes modestly to MS diagnosis as most patients with cord lesions will already fulfil the diagnostic criteria with brain MRI alone.
6. Spinal cord lesions are independent predictors of evolution to MS and lesion number appears to be relevant.
7. Spinal cord lesions are an independent risk factor for disability.
8. The probability of reaching an EDSS ≥3.0 in the short term is very low if no spinal cord lesions are present, especially in non-spinal cord CIS.
9. gMS-Classifier2 determined in serum is an independent predictor for conversion to CDMS in the first years of the disease course.
10. gMS-Classifier2 determined in serum could be of clinical relevance in cases in which OCB are not available.
11. NfL levels are markers of axonal damage in patients who will evolve to MS.
12. Baseline NfL levels in CSF are a low impact independent risk factor for MS.

13. Higher NfL levels are associated with MRI inflammatory parameters on baseline and follow-up scans.
14. Higher NfL levels are strongly associated with brain volume loss at medium-term follow-up.
15. MRI findings pose the highest risk for MS, followed by OCB, when evaluating gMS-Classifier2 and NfL levels.



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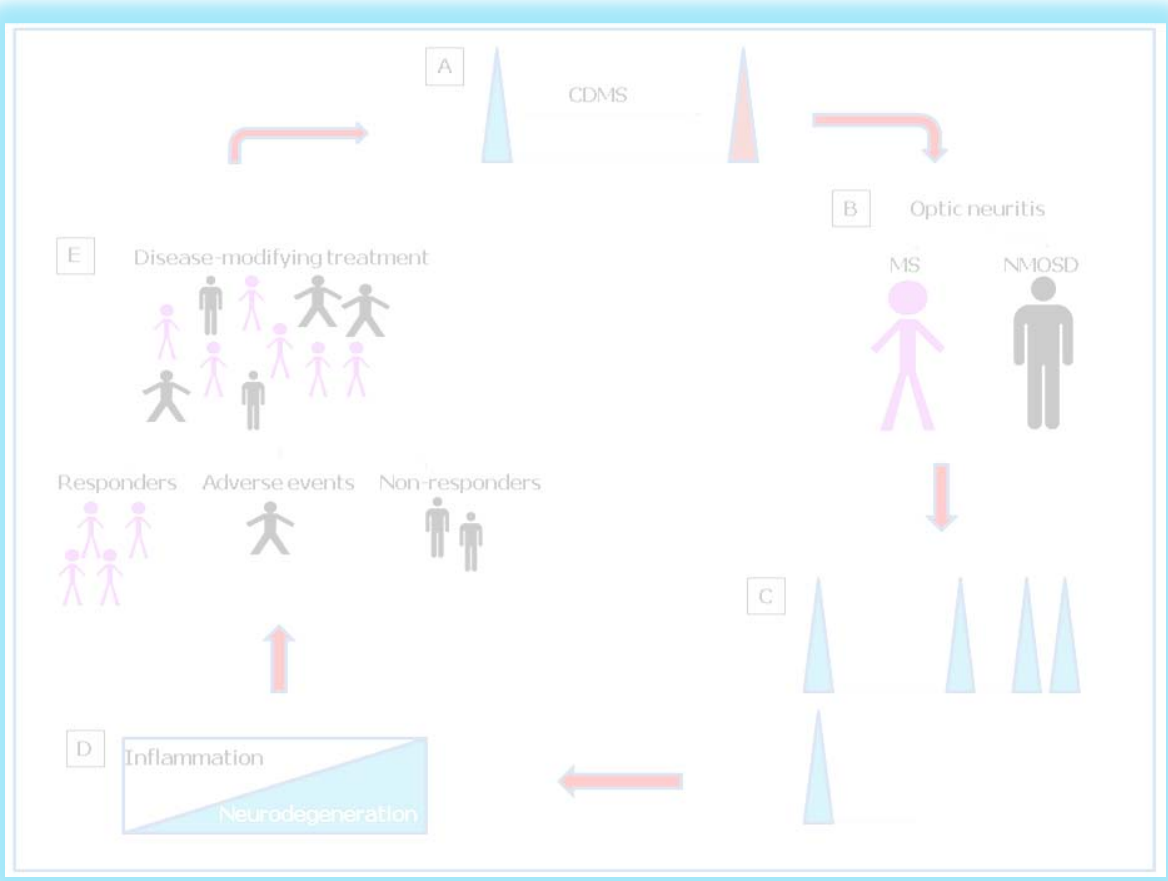
A
CDMS
B
Optic neuritis
MS
NMOSD
C
D
Inflammation
Neurodegeneration
E
Disease-modifying treatment
Responders
Adverse events
Non-responders

X. ANNEXES

X. Annexes

Annex 1.

Value of NMO-IgG determination at the time of presentation as CIS

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ABSTRACT

Background: Despite the availability of diagnostic criteria, an overlap between neuromyelitis optica (NMO) and multiple sclerosis (MS) exists, particularly in the early stage of the disease.

Objective: To study the value of NMO-immunoglobulin G (IgG) determination in Caucasian patients with a first demyelinating episode who develop a relapsing form of optic neuritis or myelitis.

Methods: This study was based on a prospectively acquired cohort of patients regarded as having a clinically isolated syndrome (CIS) at the time of presentation. From this cohort, 2 different groups were selected: group 1 (NMO phenotype), consisting of a first attack involving the optic nerve or the spinal cord, and at least a second event affecting either topography, and group 2 (negative control group), consisting of a first attack involving the brainstem or the cerebral hemispheres and at least 1 relapse in any topography. Group 3 was composed of patients with NMO according to the 2006 revised diagnostic criteria. Serum NMO-IgG was determined by indirect immunofluorescence.

Results: A total of 3.1% of the group 1 patients were positive for NMO-IgG in comparison to 3.9% of group 2 and 44.5% of group 3, NMO. One of the positive patients in group 1 evolved to NMO.

Conclusions: NMO-IgG determination is crucial in detecting patients who will develop NMO; however, its value as a routine test in cases presenting with symptoms of the type seen in MS is low, and should only be performed in those patients in which the initial diagnosis is not clear. *Neurology*® 2012;78:1608-1611

GLOSSARY

AQP4 = aquaporin-4; **CIS** = clinically isolated syndrome; **IgG** = immunoglobulin G; **MS** = multiple sclerosis; **NMO** = neuromyelitis optica; **OCB** = oligoclonal bands.

Despite having a different pathophysiology and prognosis, neuromyelitis optica (NMO) and multiple sclerosis (MS) can have phenotypical similarities at disease onset and can present a relapsing clinical course.[1,2] In recent years, the presence of autoantibodies against aquaporin-4 (AQP4), or NMO-IgG, in both serum and CSF of patients with NMO has been widely demonstrated and it has become a biomarker of the disease.[3,4] For this reason, NMO-IgG detection may play an important role in differentiating NMO and MS at onset.

The objective of the present study is to asses the value of NMO-IgG determination in a cohort of Caucasian patients regarded as having a clinically isolated syndrome (CIS) at the time of presentation, who develop an NMO phenotype consisting of sequential or relapsing optic neuritis and myelitis.

METHODS The study is based on longitudinal clinical, CSF, and MRI data prospectively collected from a cohort of patients presenting for the first time with monophasic neurologic symptoms consistent with demyelination, recruited since 1995. Inclusion

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Study funding: Carme Costa is supported by the "Sara Borrell" program (CD09/00363; CD08/00187) from the FIS, Ministry of Science and Innovation, Spain. Georgina Arrambide's research is made possible by the McDonald Fellowship, awarded by the Multiple Sclerosis International Federation. Alejandro Horga was supported by a postgraduate training fellowship from the Carlos III Health Institute, Ministry of Science and Innovation, Spain.

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criteria are as follows: a first demyelinating event of the CNS involving the optic nerve, brainstem, spinal cord, or other topography; age <50 years; and onset of symptoms within 3 months of both clinical and MRI examinations. Patients are seen every 3 to 6 months; CSF and serum samples are collected within 3 months of disease onset and always before a second attack; IgG oligoclonal bands (OCB) are examined at this point. Brain MRI is performed after the first demyelinating event and repeated at specific time points.[1] Spinal cord MRIs were previously performed only in cases of myelitis; from 2006 onwards, a baseline spinal cord MRI is performed in all presentations.

For this study, 3 different Caucasian patient groups were selected:

1. Group 1: patients selected from the aforementioned prospective cohort with a first demyelinating event involving the optic nerve or the spinal cord, and at least a second demyelinating event affecting either topography (NMO phenotype).
2. Group 2: patients selected from the prospective cohort with a first demyelinating event involving either the brainstem or the brain hemispheres, and at least 1 relapse in any topography (negative control group).
3. Group 3: patients were selected from a retrospective NMO cohort. Diagnosis was made according to the 2006 revised diagnostic criteria.[5]

In all groups, the analysis was performed if a minimum of one 200 μL serum aliquot was available. Serum NMO-IgG was determined by indirect immunofluorescence.[3] Briefly, cover slides containing mouse cerebellum, neocortex, hippocampus, kidney, and liver were incubated overnight with serum samples, and FITC goat antihuman IgG (Southern Biotechnology) was used as secondary antibody. This technique was previously validated in our laboratory with a sensibility of 50%–60% and a specificity of >90%, consistent with published data.[3,6] The result was considered positive when staining was observed in the pia mater, capillaries, or in the kidney's distal collecting tubes. The rabbit antibody anti-AQP4 (Chemicon AB3594) was used as a positive control.

For the statistical analysis, descriptive statistics and Fisher exact test for group comparisons were performed.

Standard protocol approvals, registrations, and patient consents. This study has received approval from the local ethical committee and all patients have signed written informed consent.

Table 1 Demographic characteristics and results of NMO-IgG determination of each studied group[a]

	Group 1 (NMO phenotype)	Group 2 (negative control group)	Group 3 (NMO)
No.	101	59	14
No. of available samples	97	51	9
Female (%)	67 (69.1)	31 (61.0)	8 (89.0)
Median age, y (range)	28 (13–48)	25 (17–47)	36 (22–49)
Mean ± SD follow-up, y	7.1 ± 3.1	7.4 ± 3.1	4.5 ± 2.2
NMO IgG+ patients (%)	3 (3.1)	2 (3.9)	4 (44.5)

Abbreviations: IgG = immunoglobulin G; NMO = neuromyelitis optica.
[a] Fisher exact test for group comparisons was used and no significant difference was found between group 1 and 2 NMO IgG positivity percentages ($p = 0.64$).

RESULTS Between 1995 and 2008, 822 patients were included in the prospective cohort. Of them, 101 fulfilled the group 1 criteria and 59 the group 2 criteria. Sample availability was of 97 for the former and 51 samples for the latter group. Fourteen patients were identified for group 3 (retrospective NMO positive control group), and 9 serum samples were available. The results of NMO-IgG detection and the group characteristics are summarized in table 1.

Mean follow-up was 7.1 ± 3.1 years for group 1 (NMO phenotype), 7.4 ± 3.1 for group 2 (negative control group), and 4.5 ± 2.2 for group 3 (positive control group).

Only 3 (3.1%) of the group 1 patients (NMO phenotype) were positive for NMO-IgG in comparison to 4 (44.5%) of the NMO-positive control cases. Two (3.9%) group 2 patients (negative control group) were positive for NMO-IgG. After obtaining the abovementioned percentages of NMO-IgG positivity in groups 1 and 2, no significant difference was found between these results ($p = 0.64$).

The characteristics of all CIS patients who were positive for NMO-IgG are shown in table 2; only one of them developed NMO during follow-up.

DISCUSSION Recurrent optic neuritis or myelitis may be manifestations of NMO, but can also be present in MS and in other metabolic or autoimmune conditions.[7] Brain and spinal cord MRI, OCB, autoantibody determinations, CT scans, or determination of NMO-IgG, among other studies, can aid in the differential diagnosis of individual cases.[2,3] Given the importance of differentiating NMO from MS, as both prognosis and treatment differ considerably, we aimed to determine the frequency of NMO-IgG positivity in patients with a first demyelinating event involving the optic nerve or the spinal cord and at least a second attack affecting either topography (NMO phenotype) selected from a prospective cohort of patients regarded as having a CIS at the time of presentation.

Only one of the NMO-IgG positive patients in group 1 developed clinical NMO. The other 2 evolved as prototypical MS during a follow-up time of over 9 years and have received the standard treatment for this disease. Of the 2 positive patients in group 2, one also behaved as the 2 aforementioned cases, and the other has remained clinically and radiologically stable after 2 demyelinating episodes (table 2).

It is important to acknowledge that NMO-IgG determination has been useful to detect one patient who was eventually diagnosed with NMO, confirming the previously published data on the diagnostic

Table 2 Clinical and paraclinical features of group 1 and group 2 patients who were positive for NMO-IgG

	Group 1 (NMO phenotype)			Group 2 (negative control group)	
Patient	1	2	3	4	5
Gender	Female	Female	Female	Male	Female
Topography of first demyelinating episode	Optic nerve	Spinal cord	Optic nerve	Brainstem	Brainstem
OCB	Positive	Positive	Positive	Positive	Positive
Baseline MRI: Barkhof criteria	3	3	0	1	1
Baseline spinal cord MRI	1 cervical lesion extending <1 vertebral segment	NA	Not done	Normal	NA
Second attack topography	Spinal cord	Spinal cord	Optic nerve and spinal cord	Spinal cord	Polyregional
Total years of follow-up	11.51	12.12	5.5	3.80	13.33
Follow-up brain MRIs	DIS and DIT with typical MS lesions	DIS and DIT with typical MS lesions	Normal	DIT, no DIS	DIS and DIT with typical MS lesions
Follow-up spinal cord MRIs	Not done	Not done	First MRI: 1 cervical lesion extending <1 vertebral segment; second MRI: 1 cervical lesion from bulbo-medullary junction to C7 and 1 thoracic lesion extending <1 vertebral segment	Not done	Normal
Treatment	IFN-β1b; switch to natalizumab due to clinical and radiologic activity	IFN-β1a IM; switch to glatiramer acetate due to application side effects	IFN-β1b; switch to mitoxantrone. Plasmapheresis due to clinical and radiological activity despite immunosuppression	No DMD	Glatiramer acetate
Diagnosis and evolution	Prototypical MS	Prototypical MS	NMO	Prototypical MS	Prototypical MS
	Clinically stable	Clinically stable	Severe relapsing myelitis and ON despite treatment	Clinically stable	Clinically stable
Last visit EDSS[b]	5.5	2.5	9.5	1.0	2.5

Abbreviations: DIS = dissemination in space; DIT = dissemination in time; DMD = disease-modifying drug; EDSS = Expanded Disability Status Scale; IFN = interferon; IgG = immunoglobulin G; MS = multiple sclerosis; NA = not available (spinal cord MRI performed in another center that was not retrieved); NMO = neuromyelitis optica; OCB = oligoclonal band; ON = optic neuritis.

[a] Mean follow-up time for these 5 cases was 9.3 ± 4.3 years.

[b] As of 2011, none of these patients had been lost to follow-up.

biomarker's usefulness. Conversely, however, even in patients with an NMO phenotype regarded as CIS at the time of presentation, the frequency of positive cases is low. Therefore, our results suggest that NMO-IgG determination is not routinely necessary in all patients with recurrent optic neuritis or myelitis; however, it could be useful in cases in which the diagnosis is not clear after considering together the available clinical, radiologic, and OCB information, supporting the rational use of medical resources. These findings are in accordance with published data in which even in NMO patients, NMO-IgG determination was needed in only 10% of the cases in order to complete the 2006 diagnostic criteria,[8] as well as with the recommendations included in the 2010 revisions to the McDonald criteria, in which testing for NMO-IgG is advised only in patients suspected of having NMO or NMO spectrum disorders, especially in Asian and Latin American populations.[9]

Regarding group 3 (NMO positive control group), although we found a lower positivity (44.3%) rate than expected, other studies have reported an NMO-IgG sensitivity ranging from 54% to 73% when detection was made using indirect immunofluorescence.[3,6,8,10] We believe the result observed in the NMO-positive control group could be partly explained by the small sample size.

An important limitation of this study is that the NMO spinal cord MRI criteria could not be used to select the cases as such study has not been routinely performed in the prospective cohort until recently.

Our results suggest that the utility of NMO-IgG determination as a routine test in patients presenting with symptoms of the type seen in MS is low,

regardless of the affected topography, and should only be performed in selected cases. This observation deserves further investigations in different ethnic populations.

AUTHOR CONTRIBUTIONS

Carme Costa contributed in selecting the patient and serum samples, testing and interpretation of NMO-IgG test, discussing the results, and writing the paper. Georgina Arrambide contributed in selecting the patients, analyzing the clinical data, developing the statistical studies, discussing the results, and writing the paper. Mar Tintore has contributed in selecting the patients, analyzing the clinical data, developing the statistical studies, and discussing the results and writing the paper. Joaquín Castilló has contributed in visiting and selecting the patients and in revising the manuscript. Jaume Sastre-Garriga has contributed in acquisition of clinical data and drafting/revising the manuscript for content. Carmen Tur has contributed in acquisition of clinical data and drafting/revising the manuscript for content. Jordi Río has contributed in visiting and selecting the patient and in revising the manuscript. Albert Saiz has contributed in the optimization and validation of NMO-IgG detection test in our center and drafting/revising the manuscript for content. Anka Vidal-Jordana has contributed in visiting and selecting the patient and in revising the manuscript. Cristina Auger has contributed in analyzing the MRI data and discussing the results and writing the manuscript. Carlos Nos has contributed in visiting and selecting the patient and in revising the manuscript. Alex Rovira has contributed in analyzing the MRI data and discussing the results and writing the manuscript. Manuel Comabella has contributed in visiting and selecting the patient and in revising the manuscript. Alejandro Horga has contributed in acquisition of clinical data and drafting/revising the manuscript for content. Xavier Montalban has contributed in directing the study, selecting the patients, analyzing the clinical data, developing the statistical studies, discussing the results, and writing the paper.

ACKNOWLEDGMENT

The authors thank the FEM (Fundació Esclerosi Multiple), María José Vicente and Rosalía Horno (MS nurses at the Unit of Clinical Neuroimmunology), Mireia Castillo, Zoraida Castro, Nuria Vilarrasa, and Alba Garcia (laboratory technicians, for technical support in processing serum samples and testing NMO-IgG); and REEM (Red Española de Esclerosis Múltiple) for supporting in part the MS Unit at their center.

DISCLOSURE

The authors report no disclosures relevant to the manuscript. **Go to Neurology.org for full disclosures.**

Received June 15, 2011. Accepted in final form January 17, 2012.

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Annex 2.

MULTIPLE SCLEROSIS JOURNAL | MSJ

Research Paper

Early predictors of multiple sclerosis after a typical clinically isolated syndrome

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Multiple Sclerosis Journal

1–6

DOI: 10.1177/1352458514533397

Abstract

Background: The 2010 McDonald criteria allow diagnosing multiple sclerosis (MS) with one magnetic resonance imaging (MRI) scan. Nevertheless, not all patients at risk fulfil criteria at baseline. Other predictive factors (PFs) are: age ≤40 years, positive oligoclonal bands (OBs), and ≥3 periventricular lesions.
Objective: The purpose of this study was to evaluate the 2010 McDonald criteria performance and to assess other PFs in patients without dissemination in space (DIS).
Methods: Patients with clinically isolated syndrome (CIS) underwent baseline MRI and OB determination with clinical and radiological follow-up. Adjusted hazard ratios (aHRs) for clinically definite MS were estimated for DIS, dissemination in time (DIT), and DIS+DIT. Diagnostic properties at two years were calculated. In cases without DIS, combinations of ≥2 PFs were assessed.
Results: A total of 652 patients were recruited; aHRs were 3.8 (2.5–5.8) for DIS, 4.2 (1.9–9.2) for DIT, and 8.6 (5.4–13.8) for DIS+DIT. Sensitivities were 69.6%, 42.3%, and 36.4%, and specificities were 67.3%, 87.9%, and 90.2%, respectively. In patients without DIS, aHRs varied between 2.7–5.5 and specificities ranged from 73.5–89.7% for PF combinations.
Conclusion: The high specificity of the 2010 McDonald criteria is confirmed. In patients without DIS, PF combinations could be helpful in identifying those at risk for MS.

Keywords: Multiple sclerosis, clinically isolated syndrome, diagnostic, magnetic resonance imaging, cerebrospinal fluid

Date received: 14 December 2013; revised: 16 March 2014; accepted: 6 April 2014

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Introduction

Multiple sclerosis (MS) is diagnosed based on demonstration of central nervous system demyelination with dissemination in space (DIS) and time (DIT) after excluding alternative diagnoses.[1–3] In 2010, the International Panel on the Diagnosis of Multiple Sclerosis adopted the definitions for DIS and DIT proposed by the european Magnetic Resonance Imaging in MS (MAGNIMS) group,[4–6] producing a revised version of the McDonald criteria[3] with a higher sensitivity and similar specificity to the 2005 version.[2] Nevertheless, a significant proportion of patients do not fulfil DIS and DIT at baseline and they are still at risk of developing MS. In this sense, Ruet et al. analysed spinal cord (SC) clinically isolated syndrome (CIS) patients and observed that age of onset ≤40 years, inflammatory cerebrospinal fluid (CSF), and ≥3 periventricular (PV) lesions were independent predictive factors (PFs) for MS.[7] Presence of ≥2 factors predicted conversion to MS with better accuracy than the 2005 DIS criteria. Study limitations included its retrospective nature and focus on SC CIS.

Thus, the aims of this study were to evaluate the performance of the 2010 DIS and DIT criteria in a large prospective cohort of CIS patients, and to assess the added value of the PFs in predicting MS in patients not fulfilling the 2010 DIS criteria.

Materials and methods

Patients

The study was based on longitudinal data acquired from a cohort of CIS patients recruited consecutively at Vall d'Hebron University Hospital. Inclusion criteria were: a typical CIS not attributable to other diseases, <50 years of age, and symptom onset within

3–5 months of clinical, CSF, and magnetic resonance imaging (MRI) examinations. Due to technical changes in MRI protocols in 2001, we selected patients seen for the first time between 2001–2011.

This study received approval from the local ethics committee and patients signed an informed consent.

Baseline assessments

Clinical data. Gender, age, and presenting symptoms were recorded. Follow-up was performed every 3–6 months or in case of relapses.

Serum and CSF analyses. Immunoglobulin G (IgG) oligoclonal bands (OBs) were examined using agarose isoelectric focusing combined with immunoblotting.

MRI acquisition and analysis. Baseline brain and SC MRI were performed within five months of disease onset and brain scans were repeated at 1, 5 and 10 years. SC MRI was systematically performed at baseline since November 2007. MRIs were obtained on a 1.5-Tesla magnet between 2001–2010 and on a 3-Tesla since 2010. The following brain sequences were performed: transverse proton density/T2-weighted fast spin-echo, sagittal and transverse T2-weighted fast-fluid-attenuated-inversion recovery, and transverse T1-weighted spin-echo. For all sequences, 44–46 contiguous axial sections were acquired with a 3 mm section thickness. The transverse T1-weighted sequence was repeated in patients with focal white matter lesions on T2-weighted sequences after gadolinium (Gd) injection (0.2 mmol/kg; scan delay, 5–10 min). SC sequences included sagittal proton density/T2-weighted fast spin-echo, sagittal short-tau inversion-recovery (STIR) and, in patients in whom brain Gd-enhanced sequences were obtained or who had symptomatic SC lesions, a Gd-enhanced sagittal T1-weighted sequence was obtained. Additional transverse T2-weighted fast spin-echo sequences were obtained to confirm signal abnormalities detected on sagittal views. All sequences were acquired with a contiguous 3 mm section thickness.

MRI scans were assessed by two neuroradiologists blinded to clinical follow-up. Lesions were defined as areas of increased signal intensity larger than 3 mm on both proton-density and T2-weighted images. MRI scans were considered abnormal if at least one lesion was observed.

Demographic, clinical, and biological variables. These were age, gender, CIS topography, OBs, and disease-modifying treatment (DMT). DMT was proposed to patients who had a second attack within three years of disease onset or to CIS patients fulfilling 3–4 Barkhof-Tintoré criteria,[8,9] according to the Catalan Institute of Health guidelines.

MRI variables. These were on brain, number and location of T2 lesions, number of Gd-enhancing lesions, and number of new T2 lesions, and on SC, number of T2 lesions and presence of Gd-enhancing lesions. We applied the 2010 McDonald criteria: presence of ≥1 T2 lesion in ≥2 of the four MS-characteristic areas provided evidence of DIS in any MRI, excluding the symptomatic lesion in brainstem or SC CIS.[3,4] DIT was fulfilled by simultaneous presence of asymptomatic Gd-enhancing and non-enhancing lesions on any MRI, or when new T2 lesions appeared on follow-up scans.[5]

Predictive factors[7]. Patients were classified according to age (≤40 or >40), OBs (present or absent), and PV lesions (≥3 or <3). A cut-off of ≥2 PFs was applied after observing similar outcomes for two or three PFs (data not shown).

Outcomes

Clinically definite multiple sclerosis (CDMS) was diagnosed after a second attack with a new neurological abnormality confirmed by neurological examination.[10]

The 2010 McDonald criteria were met by demonstration of DIS and DIT on baseline or follow-up MRIs. Patients with a second clinical attack also fulfilled the criteria.[3]

Statistical analysis

Nonparametric descriptive statistics were performed. Results are shown as median and interquartile percentiles for continuous variables and as percentages for categorical variables.

Uni- and multivariate Cox proportional hazard regressions were performed to assess the risk of CDMS by estimating DIS only, DIT only, and DIS+DIT compared to No DIS+No DIT on baseline MRI. The risk of developing CDMS in patients not fulfilling DIS at baseline was evaluated for ≥2 PFs and in the following combinations: age≤40 and ≥3PV, age≤40 and OBs, and ≥3PV and OBs. A similar analysis for 2010 McDonald MS was done evaluating DIS and DIT unadjusted for each other and the combinations of PFs. CIS topography, gender, and DMT before MS diagnosis were considered potential covariates. As treatment was the only

Table 1. Demographic and clinical findings in the clinically isolated syndrome cohort.

	652 patients	401 patients followed for at least two years
Age at onset (years)	31 (26–38)	31 (26–38)
Sex		
Male	220 (33.7)	125 (31.2)
Female	432 (66.3)	276 (68.8)
CIS topography		
Optic neuritis	232 (35.6)	127 (31.7)
Brainstem syndrome	180 (27.6)	115 (28.7)
Spinal cord syndrome	165 (25.3)	110 (27.4)
Other	75 (11.5)	49 (12.2)
Follow up (months)	44.6 (13.7–76.0)	66.7 (51.1–92.7)
CDMS	201 (30.8)	176 (43.9)
2010 McDonald MS	237 (36.3)	202 (50.4)

CIS: clinically isolated syndrome CDMS: clinically definite multiple sclerosis; MS: multiple sclerosis.
Data are the median (interquartile percentiles) for age and follow up or number (percentage) of patients otherwise.

term with a significant effect, it was included in the Cox model to obtain an adjusted hazard ratio (aHR) for prediction of CDMS or McDonald MS. Since treatment is not a baseline static characteristic, DMT was defined as a time-dependent variable.

Finally, the performance of the 2010 DIS and DIT criteria on baseline MRI and of the PFs in the previously described combinations was assessed with CDMS as the outcome during the first two years in patients followed for ≥2 years. Sensitivity, specificity, and accuracy, all with exact binomial 95% confidence intervals (CIs), were calculated.

Statistical analyses were performed at the 0.05 level of significance using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, Illinois, USA) version 19.0.

Results

Study population

A total of 652 patients with a median follow-up of 44.6 (13.7–76.0) months were included. Median age was 31 (26–38) years, and the female: male ratio was 2:1 (Table 1).

CSF

Of 492 patients (75.5%) with lumbar puncture (LP), 272 (55.3%) had positive OBs (Table 1). Among those, 125 (46.0%) developed CDMS and 146 (53.7%) McDonald MS compared to 49 (22.3%) and 54 (24.5%) with negative OBs, respectively. Patients who did not undergo the procedure were more likely to have optic neuritis and a normal MRI (data not shown).

Baseline MRI

At the time of the analysis, brain MRI was available in 505 patients (77.5%) and it was abnormal in 318 (63.0%). Among these, 156 (49.1%) developed CDMS and 190 (59.7%) fulfilled the McDonald criteria. Of the 187 (37.0%) with normal brain MRI, 22 (11.8%) developed CDMS and 24 (12.8%) fulfilled the McDonald criteria.

SC MRI was performed in 154 (23.6%) patients and was abnormal in 79 (51.3%). Among these, 30 (38.0%) had CDMS and 37 (46.8%) McDonald MS. Of the 75 (48.7%) patients with a normal SC MRI, three (4.0%) developed CDMS and eight (10.7%) fulfilled McDonald MS. However, 60 (75.9%) patients with SC lesions also had an abnormal brain MRI, of which 36 (60.0%) had a SC syndrome.

Treatment

Altogether 236 patients (36.2%) were on DMT: 136 (57.6%) before CDMS and 87 (36.9%) before McDonald MS.

Predicting MS after CIS

DIS and DIT. Baseline DIS only and DIT only had similar aHR for conversion to CDMS, whereas the estimated aHR for DIS+DIT was higher (Table 2). The risk of McDonald MS was also higher in cases fulfilling DIS or DIT on baseline MRI (Table 3).

Table 2. Risk assessment and performance of the 2010 McDonald criteria and the predictive factors (PFs) by Ruet et al. for predicting conversion to clinically definite multiple sclerosis (CDMS) in clinically isolated syndrome (CIS) patients.

CDMS	Cox regression models in the entire cohort n=652		Performance in patients followed for at least two years n=401			
	n (%)	aHR (95% CI)	n (%)	Sensitivity (95% CI)	Specificity	Accuracy
2010 DIS and DIT criteria according to baseline MRI						
2010 DIS only	459 (70.4)	3.8 (2.5–5.8)[a]	360 (89.8)	69.6 (61.8–76.7)	67.3 (60.4–73.7)	68.3 (63.3–73.1)
2010 DIT only	459 (70.4)	4.2 (1.9–9.2)[a]	339 (84.5)	42.3 (34.2–50.6)	87.9 (82.4–92.2)	67.8 (62.6–72.8)
DIS+DIT	459 (70.4)	8.6 (5.4–13.8)[a]	344 (85.8)	36.4 (28.8–44.6)	90.2 (85.1–94.0)	66.6 (61.3–71.5)
Predictive factors in cases not fulfilling DIS						
≥2 PFs	266 (40.8)	3.7 (2.1–6.7)[a]	184 (45.9)	60.4 (45.3–74.2)	73.5 (65.3–80.7)	70.1 (62.9–77.0)
Age≤40 and ≥3PV	266 (40.8)	5.5 (1.6–19.2)[b]	184 (45.9)	31.3 (18.7–46.3)	88.2 (81.6–93.1)	73.4 (66.4–79.6)
Age≤40 and +OBs	212 (32.5)	2.7 (0.8–9.0)	184 (45.9)	54.2 (39.2–68.6)	77.9 (70.0–84.6)	71.7 (64.6–78.1)
≥3 PV and +OBs	212 (32.5)	3.6 (1.7–7.9)[b]	184 (45.9)	25.0 (13.7–39.6)	89.7 (83.3–94.3)	72.8 (65.8–79.1)

aHR: adjusted hazard ratio; CI: confidence interval; DIS: dissemination in space; DIT: dissemination in time; MRI: magnetic resonance imaging; PV: periventricular; OBs: oligoclonal bands.
The value of n represents the size of the sample for each variable of interest.
[a]p value <0.001; [b]p value <0.01.

PFs in patients not fulfilling DIS. Having ≥2 PFs or the PV and OB combination resulted in similar aHRs for CDMS as DIS only or DIT only (Table 2). The risk of McDonald MS when having ≥2 PFs was higher than for CDMS (Table 3).

Predicting MS in the first two years after a CIS

Of 652 patients, 401 (61.5%) were followed for ≥2 years. Sensitivity, specificity, and accuracy of DIS, DIT, DIS+DIT, and PFs for CDMS are shown in Table 2. Performance of DIS, DIT, and PFs for McDonald MS is shown in Table 3.

Discussion

To this day, MRI remains the most important tool for MS diagnosis, hence becoming a key factor in the McDonald criteria.[1–3] In this large prospective CIS cohort, over 35.0% of patients were diagnosed earlier (after a median of six months) when applying the 2010 criteria.[3] Furthermore, we observed a 3.8–4.2 increase in the risk of developing CDMS when evaluating DIS and DIT individually, while the aHR increased to 8.6 in patients who fulfilled both DIS and DIT. This is in accordance with findings by Swanton et al. for DIS only and DIT only, but not for DIS+DIT, in which case they report a HR of 12.33.[4] Regarding performance, DIS sensitivity is lower than previously reported.[4,11] Such differences could be explained due to inclusion of both baseline and follow-up MRIs in the analysis by Swanton et al. compared to only baseline MRIs in our case, and to the proportion of abnormal baseline MRIs: 78.4% in their publication and 63.0% in our study. Besides the aforementioned differences on MRI characteristics, one more possibility for our lower DIS performance is that in the study by Swanton et al., the outcome was CDMS after three years[4] and in ours it was two years, whereas in the study by Nielsen et al., sensitivity and specificity were based on MRI scans performed in one group of patients with a diagnosis of MS and another with other neurological diseases.[11] Our specificity was higher than in the former study and lower than the latter, thus obtaining a similar accuracy to that reported by Swanton et al.[4] As for DIT, our sensitivity was certainly lower and specificity higher than in the aforementioned study. Once again, their use of both baseline and follow-up MRIs may account for this difference. Indeed, when evaluating only new Gd lesions, their results and ours are more similar.[4] Interestingly, Rovira et al. used an identical definition of DIS+DIT as ours, obtaining 53.1% sensitivity and 86.6% specificity. Furthermore, when evaluating patients with MRI

done 61–90 days after the CIS, sensitivity was 37.5% and specificity 85.7%,[5] these being the most similar results to ours, probably due to similar time windows for performing baseline MRIs. Finally, patients with DIS or specially DIT on baseline MRI have a greatly increased risk of fulfilling McDonald MS criteria over time. Nonetheless, it is important to note that in this case, DIS and DIT were not adjusted for each other and patients with DIS could also fulfil DIT and vice versa. Furthermore, in comparison to CDMS as the outcome, performance improves as well.

However, since a proportion of patients do not fulfil DIS criteria at baseline, we evaluated ≥2 PFs in those cases and found an almost four-fold risk of developing CDMS and a diagnostic accuracy of 70.1%. When evaluating McDonald MS criteria, the aHR was lower but performance remained similar. We also evaluated combinations of two PFs in patients not fulfilling DIS. Despite the fact that presence of one PV lesion is already included in the 2010 DIS criteria and that in many settings LP is not performed on a routine basis, if such information is available, having ≥2 PFs when not fulfilling DIS increases the risk of MS with a high specificity, especially in those combinations including three PV lesions.

Our study has several limitations. In the inclusion criteria, the maximum age was <50 years. Thus, specificity and accuracy of 'age ≤40' could have been underestimated. Furthermore, approximately 75% of patients underwent a LP. When evaluating baseline characteristics, patients with optic neuritis and normal baseline MRIs were more prone to turn this procedure down. This observation was previously reported in our cohort[12] and could bias our results towards a more aggressive disease course. Another limitation is the low number of SC MRIs (23.6% vs 62.5% of the core cohort by Swanton et al.) thus possibly limiting the number of cases actually fulfilling DIS or DIT.

In conclusion, our study shows that although sensitivity of the 2010 criteria may differ based on timing and number of MRIs included in the analysis (baseline and/or follow-up MRIs) and on differing cut-off times for evaluation (e.g. two or three years), accuracy and, above all, specificity remain very high among studies, thus confirming these criteria can be applied with a low risk of misdiagnosis in typical CIS cases. Besides, having ≥2 PFs or combinations of PFs in patients not fulfilling DIS is highly specific for developing CDMS or McDonald MS, especially in those with three PV lesions.

Table 3. Risk assessment and performance of the 2010 McDonald criteria and the predictive factors (PFs) by Ruet et al. for predicting fulfilment of 2010 McDonald multiple sclerosis (MS) (second relapse or magnetic resonance imaging (MRI) criteria for dissemination in space (DIS) and dissemination in time (DIT)) in clinically isolated syndrome (CIS) patients.

2010 McDonald MS	Cox regression models in the entire cohort *n*=652		Performance in patients followed for at least two years *n*=401			
	n (%)	aHR (95% CI)	*n* (%)	Sensitivity (95% CI)	Specificity	Accuracy
2010 DIS and DIT criteria according to baseline MRI						
2010 DIS only	495 (75.9)	5.1 (3.7–7.0)[a]	360 (89.8)	72.3 (65.2–78.6)	75.6 (68.5–81.7)	73.9 (69.0–78.4)
2010 DIT only	459 (70.4)	10.7 (7.9–14.5)[a]	339 (84.5)	47.1 (39.5–54.8)	97.6 (93.9–99.3)	71.7 (66.6–76.4)
Predictive factors in cases not fulfilling DIS						
≥2 PFs	266 (40.8)	3.3 (1.9–5.8)[a]	184 (45.9)	60.8 (46.1–74.2)	74.4 (66.2–81.6)	70.7 (63.5–77.1)
Age≤40 and ≥3 PV	266 (40.8)	4.6 (1.3–16.2)[c]	184 (45.9)	29.4 (17.5–43.8)	88.0 (81.2–93.0)	71.8 (64.6–78.1)
Age≤40 and +OBs	212 (32.5)	2.7 (0.8–9.1)	184 (45.9)	54.9 (40.3–68.9)	79.0 (71.0–85.5)	72.3 (65.2–78.6)
≥3 PV and +OBs	212 (32.5)	2.9 (1.3–6.2)[b]	184 (45.9)	23.5 (12.8–37.5)	89.5 (83.0–94.1)	71.2 (64.1–77.6)

aHR: adjusted hazard ratio; CDMS: clinically definite multiple sclerosis; CI: confidence interval; PV: periventricular; OBs: oligoclonal bands.
The value of *n* represents the size of the sample for each variable of interest.
[a]*p* value<0.001 ; [b]*p* value<0.01 ; [c]*p* value<0.05.

Acknowledgement
The authors thank Xavier Vidal for statistical analyses (Department of Pharmacology, Hospital Vall d'Hebron, Spain). M Tintore had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflicts of interest
None declared.

Funding
This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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Annex 3.

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Serum Biomarker gMS-Classifier2: Predicting Conversion to Clinically Definite Multiple Sclerosis

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Abstract

Background: Anti-glycan antibodies can be found in autoimmune diseases. IgM against glycan P63 was identified in clinically isolated syndromes (CIS) and included in gMS-Classifier2, an algorithm designed with the aim of identifying patients at risk of a second demyelinating attack.

Objective: To determine the value of gMS-Classifier2 as an early and independent predictor of conversion to clinically definite multiple sclerosis (CDMS).

Methods: Data were prospectively acquired from a CIS cohort. gMS-Classifier2 was determined in patients first seen between 1995 and 2007 with ≥ two 200 μL serum aliquots (N = 249). The primary endpoint was time to conversion to CDMS at two years, the factor tested was gMS-Classifier2 status (positive/negative) or units; other exploratory time points were 5 years and total time of follow-up.

Results: Seventy-five patients (30.1%) were gMS-Classifier2 positive. Conversion to CDMS occurred in 31/75 (41.3%) of positive and 45/174 (25.9%) of negative patients (p = 0.017) at two years. Median time to CDMS was 37.8 months (95% CI 10.4–65.3) for positive and 83.9 months (95% CI 57.5–110.5) for negative patients. gMS-Classifier2 status predicted conversion to CDMS within two years of follow-up (HR = 1.8, 95% CI 1.1–2.8; p = 0.014). gMS-Classifier2 units were also independent predictors when tested with either Barkhof criteria and OCB (HR = 1.2, CI 1.0–1.5, p = 0.020) or with T2 lesions and OCB (HR = 1.3, CI 1.1–1.5, p = 0.008). Similar results were obtained at 5 years of follow-up. Discrimination measures showed a significant change in the area under the curve (ΔAUC) when adding gMS-Classifier2 to a model with either Barkhof criteria (ΔAUC 0.0415, p = 0.012) or number of T2 lesions (ΔAUC 0.0467, p = 0.009), but not when OCB were added to these models.

Conclusions: gMS-Classifier2 is an independent predictor of early conversion to CDMS and could be of clinical relevance, particularly in cases in which OCB are not available.

Citation: Arrambide G, Espejo C, Yarden J, Fire E, Spector L, et al. (2013) Serum Biomarker gMS-Classifier2: Predicting Conversion to Clinically Definite Multiple Sclerosis. PLoS ONE 8(3): e59953. doi:10.1371/journal.pone.0059953

Editor: Markus Reindl, Innsbruck Medical University, Austria

Received October 8, 2012; **Accepted** February 20, 2013; **Published** March 28, 2013

Funding: The "Red Española de Esclerosis Múltiple (REEM, www.reem.es)" (RD07/0060) sponsored by the Fondo de Investigación Sanitaria (FIS), Ministry of Economy and Competitively, Spain; the "Ajuts per donar Suport als Grups de Recerca de Catalunya" (2009 SGR 0793, http://www10.gencat.cat/agaur_web/AppJava/catala/a_beca.jsp?categoria=recerca&id_beca=13601), sponsored by the "Agència de Gestió d'Ajuts Universitaris i de Recerca" (AGAUR, www.gencat.cat/agaur/), Generalitat de Catalunya, Spain. The biomarker study was funded by the Fondo de Investigación Sanitaria (FIS, www.isciii.es); grant number PI08/0788; Ministry of Science and Innovation, Spain. Georgina Arrambide's research is made possible by the McDonald Fellowship, awarded by the Multiple Sclerosis International Federation (www.msif.org). Carmen Espejo is partially supported by the "Miguel Servet" program (CP07/00146) from the FIS, Ministry of Economy and Competitively, Spain (www.isciii.es). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials

Competing Interests: Georgina Arrambide has received travel expenses for the 2011 CMSC (Consortium of Multiple Sclerosis Centers) meeting from Glycominds. Carmen Espejo has received speaking honoraria from Merck Serono and Almirall. Jennifer Yarden, Ella Fire, Larissa Spector, and Nir Dotan are employees and stock holders of Glycominds. Avinoam Dukler is an employee and share and stock holder of Glycominds. Alex Rovira serves on scientific advisory boards for NeuroTEC and on the editorial board of the American Journal of Neuroradiology and Neuroradiology, has received speaker honoraria from Bayer Schering Pharma, Sanofi-Aventis, Bracco, Merck Serono, Teva Pharmaceutical Industries Ltd. and Biogen Idec, receives research support from Bayer Schering Pharma, and serves as a consultant for Novartis. Xavier Montalban has received speaking honoraria and travel expenses for scientific meetings, has been a steering committee member of clinical trials or participated in advisory boards of clinical trials in the past with Bayer Schering Pharma, Biogen Idec, EMD Merck Serono, Genentech, Genzyme, Novartis, Sanofi-Aventis, Teva Pharmaceuticals and Almirall. Mar Tintoré has received compensation for consulting services and speaking honoraria from Bayer-Schering, Merck-Serono, Biogen-Idec, Teva, Sanofi-Aventis, and Novartis. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Evidence exists that both the number of lesions observed using baseline magnetic resonance imaging (MRI) [1,2] and the presence of IgG oligoclonal bands (OCB) in the cerebrospinal fluid (CSF) [3–5] of patients with clinically isolated syndromes (CIS) are independent predictors of conversion to clinically definite multiple sclerosis (CDMS). However, MS is a highly heterogeneous disease, and the search for other biomarkers that could improve the prediction of conversion to CDMS may still be necessary for early and appropriate therapeutic decision making [6,7].

A complex array of covalently attached glycans densely covers the surface of all cells and many proteins, and these molecules are a major component of the extracellular matrix. Thus, glycans are a prime antigen source and play a vital role in immunity. Indeed, antibodies against these molecules have been implicated in a number of autoimmune diseases [8], for example, those directed against galactose in collagen type II in rheumatoid arthritis [9], ganglioside GQ1b in Miller-Fisher syndrome [10] and oligomannose, mannobioside, laminaribioside, chitobioside, laminarin and chitin epitopes in Crohn's disease [11,12]. IgM antibodies directed against glycans composed of alpha-glucose disaccharides have been found in MS patients and demonstrated to distinguish relapsing-remitting MS patients from those with other neurological diseases [13–15]. One of the identified antibodies was directed against P63, a polymer of alpha-glucose molecules comprising two different carbohydrate structures [Glc(α1,6)Glc(α) and Glc(α1,3)Glc(α)]. Thus, a classification rule named gMS-Classifier2, which is based on the combination of polyclonal serum IgM antibody levels against P63 and age, was developed after an exploration analysis of clinical data and anti-glycan antibody levels in samples collected in the "Betaferon® in Newly Emerging multiple sclerosis For Initial Treatment" (BENEFIT) trial. In this study, this classification rule identified CIS patients at higher risk of converting to CDMS during the first two years of disease evolution [16]. To validate these preliminary results, herein we aimed to analyze the gMS-Classifier2 predictive value for early conversion of CIS patients to CDMS and to determine whether gMS-Classifier2 is an independent predictor of conversion to CDMS.

Patients and Methods

Ethics Statement

This study received approval from the Clinical Research Ethics Committee (CREC) of Vall d'Hebron University Hospital and Research Institute (Comitè Ètic d'Investigació Clínica –CEIC- de l'Hospital Universitari Vall d'Hebron-Institut de Recerca). Participants provided their written informed consent to participate in this study.

The present study is based on longitudinal clinical, CSF, serum and MRI data prospectively acquired from a cohort of CIS patients which started in 1995. Patients presenting for the first time with monophasic neurologic symptoms of the type seen in MS were recruited at the Vall d'Hebron University Hospital in Barcelona, Spain. Inclusion criteria were as follows: a CIS suggestive of central nervous system (CNS) demyelination involving the optic nerve, brainstem, spinal cord or other topography that were not attributable to other diseases; age <50 years; and onset of symptoms within three months of both clinical and MRI examinations. Patients were seen every three to six months and if relapses occurred. IgG OCB were examined using agarose isoelectric focusing combined with immunoblotting. The remaining biological samples were stored at −80°C until testing. Brain MRIs were performed after the first demyelinating event and repeated after twelve months and five years of follow-up. From 2001 onwards, baseline cranial MRIs were performed at three months after the first demyelinating event. Further clinical, CSF and MRI assessments have been detailed elsewhere [1].

Cases were selected from the CIS cohort based on the following eligibility criteria: consecutive patients older than 18 years of age seen between 1995 and 2007, with a minimum of two available 200 µL stored serum aliquots that had not undergone previous thawing.

A diagnosis of conversion to CDMS was made when new symptoms occurred after an interval of at least one month and only when other diagnoses had been excluded [17]. Time of follow-up was calculated based on the difference between the date of the baseline visit and the date of the last visit. De-identified and coded serum samples obtained at the time of enrolment in the department-wide sample repository were shipped to Glycominds, Ltd. (Modi'in, Israel) for analysis. Clinical data were not shared with collaborators at Glycominds until after the results of the serological analysis had been returned.

Serum samples were thawed according to the following protocol to prevent IgM precipitation: i) Samples were allowed to reach room temperature; ii) Samples were incubated at 37°C for 2 hours; iii) Samples were vortexed to homogeneity. IgM antibody measurement is stable if these conditions are met for no more than two freeze-thaw cycles.

Levels of anti-P63 IgM antibodies were measured in IgG-depleted serum samples by enzyme immunoassay (EIA) in duplicate. Briefly, microtiter 96-well plates with immobilized P63 were prepared as described elsewhere [18]. IgG was depleted from the samples using rheumatoid factor removal reagent (Chemicon, Australia, Cat. RFRR) according to the manufacturer's instructions. Following IgG removal, serum samples (using a dilution of 1:600 instead of the 1:1200 originally described) were dispensed into microtiter wells in duplicate, incubated for 180 minutes at 4°C, and washed with wash buffer. Bound antibodies were labelled with horseradish peroxidase (HRP)-conjugated goat anti-human IgM antibody, washed, and 3, 3′, 5, 5′-tetramethylbenzidine was added for detection. After 30 minutes, the enzymatic reaction was stopped by adding 1% sulphuric acid solution to the wells, and the optical density (OD) was read at 450 nm using a Victor 1420 plate reader (Wallac, Turku, Finland). Each plate included a 5-point calibration curve. Anti-P63 serum levels were reported in arbitrary EIA units (EU). gMS-Classifier2 units were calculated according to the following algorithm: [1.171 − 0.082×age in years at the time of blood collection]+[0.015 × anti-P63 (EU)]. The gMS-Classifier2 was considered positive when the number of units was equal to or greater than 0.289 [16,19].

Statistical Analysis

Parametric and nonparametric comparative statistics were performed depending on the normality of the distributions of the continuous variables. Fisher's exact test was performed to compare categorical variables. Kaplan-Meier analysis was used to estimate cumulative survival probabilities and to construct survival plots. To assess whether gMS-Classifier2 can independently predict time to CDMS, a multivariate analysis using Cox proportional hazard regression was performed for both gMS-Classifier2 status (positive or negative) and continuous values. Baseline MRI parameters such as number of Barkhof Criteria (BC), the number of T2 lesions (0, 1–9, >9 lesions) and OCB were considered as potentially relevant covariates. Age was already included in the gMS-Classifier2 algorithm as a covariate; the role of gender and CIS topography as

possible covariates was also evaluated. Time to event analysis was performed primarily at two years; it was additionally assessed at five years and total time of follow-up to evaluate the length of time during which the biomarker could be useful. To assess the clinical utility of gMS-Classifier2, a Hosmer and Lemeshow goodness-of-fit test was performed as a calibration measure for two models: one with number of Barkhof criteria, OCB and gMS-Classifier2; and one for number of T2 lesions, OCB and gMS-Classifier2. As discrimination measures, two ROC curve analyses were made: one model using number of Barkhof criteria, OCB and gMS-Classifier2 continuous units and compared with a model without OCB. The second ROC curve analysis compared one model using number of T2 lesions, OCB and gMS-Classifier2 continuous units versus another in which OCB were excluded. Statistical tests were performed at the 0.05 level of significance using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) version 17.0.

Results

Between 1995 and 2007, 723 patients were included in the CIS cohort. gMS-Classifier2 units were determined in a subgroup of 249 (34%) patients that met the present study's selection criteria. The screened cohort was similar to the non-screened cohort in age, follow-up time, and proportion of both positive OCB and baseline number of Barkhof criteria. There were differences in the proportion of females and the topography of disease presentation (Table 1). When comparing the demographic variables between patients with positive and negative gMS-Classifier2 status, including median time of follow-up, there was a difference in the proportion of females and in the distribution of CIS topography, but since the gMS-Classifier2 hazard ratio (HR) estimate was not substantially modified when gender or topography were included in the model, it was not considered necessary to adjust the results for these clinical variables (data not shown). There was also a difference in the median time of follow-up; however, as it was of approximately 5 months between groups, it was not considered relevant when the total follow-up time was of up to 14 years.

gMS-Classifier2 Status at Specified Time Points and Conversion to CDMS

The median value of gMS-Classifier2 was −0.24 units (range −2.3 to 5.5 units) and seventy-five patients (30.1%) were positive for gMS-Classifier2.

The median time to CDMS was 37.8 months for gMS-Classifier2-positive patients (95%CI 10.4–65.3 months) and 83.9 months (95%CI 57.5–110.5) for gMS-Classifier2-negative patients. gMS-Classifier2 predicted conversion to CDMS within two years (HR = 1.8, 95%CI 1.1–2.9; p = 0.013) and within five years of follow-up (HR = 1.5, 95%CI 1.0–2.4; p = 0.033) but not for total follow-up time, although a trend was observed (HR = 1.4, 95%CI 1.0–2.2; p = 0.060) (Figure 1). Table 2 shows the proportion of CDMS patients that were positive and negative for gMS-Classifier2 at 2 years, 5 years and total time of follow-up.

Predictive Value of gMS-Classifier2 Status for Conversion to CDMS

In a univariate analysis, gMS-Classifier2 status (positive/negative), Barkhof criteria, number of T2 lesions and presence of OCB were predictors for early conversion to CDMS at two years of follow-up (Table 3).

In the multivariate analyses at two years of follow-up, gMS-Classifier2 status remained significant when tested with number of Barkhof criteria (HR = 1.8, 95%CI 1.1–2.8, p = 0.014) or number of T2 lesions (HR = 1.7, 95%CI 1.1–2.7, p = 0.020). When combining gMS-Classifier2 status and OCB, the formers significance was lost (HR = 1.5, 95%CI 0.9–2.4, p = 0.095). When combining gMS-Classifier2 status with OCB and either Barkhof criteria or number of T2 lesions the HR were non-significant (HR 1.5, 95%CI 0.9–2.5, p = 0.081 and HR 1.5, 95%CI 0.9–2.4, p = 0.100, respectively) (Table 4). When adding treatment to these models, there were no statistically significant changes in the HR of gMS-Classifier2 (data not shown).

Table 1. Demographic, clinical and MRI characteristics of screened and non-screened patients: gMS-Classifier2 serum assay.

Group characteristics (1995–2007)	Screened CIS cohort (N = 249)	Non-screened CIS cohort (N = 474)	p-value
Mean age in years ± SD	31.6±7.9	31.6±7.9	0.455
Females (%)	187 (75.1)	315 (66.5)	0.017
Median follow-up in months (range)	68.7 (0.53–177.0)	63.2 (0.30–171.2)	0.002
Topography N (%):			
ON	106 (42.6)	154 (32.5)	
Brainstem	51 (20.5)	144 (30.4)	
Spinal cord	65 (26.1)	122 (25.7)	
Other	27 (10.8)	54 (11.4)	0.014
Positive OCB N (%)*	152 (64.4)	181 (60.1)	0.311
Barkhof criteria on baseline MRI N (%):**			
0	88 (35.5)	173 (38.6)	
1–2	56 (22.6)	104 (23.2)	
3–4	104 (41.9)	171 (38.2)	0.601

Abbreviations: CIS = clinically isolated syndrome; SD = standard deviation; ON = optic neuritis; OCB = oligoclonal bands; MRI = magnetic resonance imaging. *The total number of patients with available cerebrospinal fluid for OCB determination was 236 for the screened CIS cohort and 301 for the non-screened CIS cohort. Percentages in the table correspond to these figures. **The total number of patients with available baseline MRI for Barkhof criteria determination was 248 for the screened CIS cohort and 448 for the total CIS cohort. Percentages in the table correspond to these figures.

doi:10.1371/journal.pone.0059953.t001

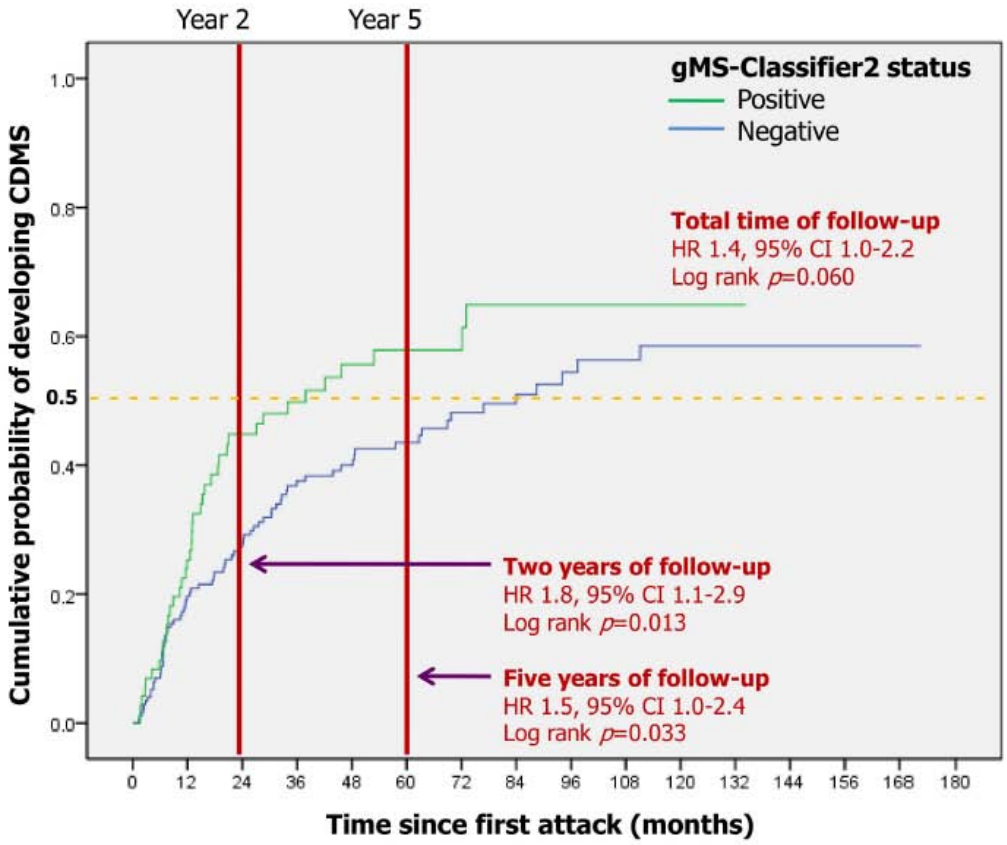

Figure 1. Time to reach CDMS based on gMS-Classifier2 status. Dotted line: median time of follow-up. HR = hazard ratio; CI = confidence interval.
doi:10.1371/journal.pone.0059953.g001

Predictive Value of gMS-Classifier2 Continuous Unit Values for Conversion to CDMS

In the univariate analysis, gMS-Classifier2 continuous units, Barkhof criteria, number of T2 lesions and presence of OCB were predictors for early conversion to CDMS at two and five years of follow-up (Table 3).

In the multivariate analyses at two years of follow-up, gMS-Classifier2 continuous units remained significant when tested with number of Barkhof criteria (HR = 1.3, 95%CI 1.1–1.5, p = 0.003) or number of T2 lesions (HR = 1.3, 95%CI 1.1–1.6, p = 0.001). When combining gMS-Classifier2 continuous units and OCB, the formers significance was lost (HR = 1.1, 95%CI 0.9–1.4, p = 0.088), but when combining gMS-Classifier2 continuous units with OCB and either Barkhof criteria or number of T2 lesions the HR were once again statistically significant (HR 1.2, 95%CI 1.0–1.5, p = 0.020 and HR 1.3, 95%CI 1.1–1.5, p = 0.008, respectively) (Table 5).When adding treatment to these models, there were no statistically significant changes in the HR of gMS-Classifier2 (data not shown).

Table 2. gMS-Classifier2 status and number of patients converting to CDMS at specified time points.

Specified time points	CDMS in positive patients (75)	CDMS in negative patients (174)	p-value
	N (%)	N (%)	
Two years	31 (41.3)	45 (25.9)	0.017
Five years	38 (50.7)	67 (38.5)	0.093
Total time of follow-up (up to 14 years)	40 (53.3)	77 (44.3)	0.214

Abbreviations: CDMS = clinically definite multiple sclerosis.
doi:10.1371/journal.pone.0059953.t002

Table 3. Univariate Cox proportional hazard regression for conversion to CDMS.

Univariate model	N	Two years of follow-up			Five years of follow-up			Total time of follow-up		
		HR	95%CI	p	HR	95%CI	p	HR	95%CI	p
gMS-Classifier2 status (positive or negative)	249	1.8	1.1–2.8	0.017	1.5	1.0–2.3	0.034	1.4	1.0–2.1	0.061
gMS-Classifier2 continuous units*	249	1.2	1.0–1.4	0.027	1.1	1.0–1.3	0.038	1.1	1.0–1.3	0.117
1–2 Barkhof criteria‡	56	3.9	1.6–9.4	0.002	4.3	2.1–8.6	<0.0001	5.3	2.7–10.6	<0.0001
3–4 Barkhof criteria	104	7.5	3.4–16.5	<0.0001	6.7	3.6–12.8	<0.0001	8.1	4.3–15.4	<0.0001
1–9 T2 lesions	76	5.6	1.7–18.9	0.005	10.0	3.1–32.6	<0.0001	11.5	3.6–37.4	<0.0001
≥10 T2 lesions	107	12.5	3.9–39.9	<0.0001	17.4	5.5–55.2	<0.0001	21.4	6.7–68.1	<0.0001
Positive OCB	152	3.7	1.9–7.3	<0.0001	3.1	1.8–5.3	<0.0001	2.9	1.8–4.8	<0.0001

Abbreviations: HR = hazard ratio, CI = confidence interval, OCB = oligoclonal bands.
*For continuous values, HR indicates how much the hazard (for CDMS) increases per unit increase in gMS-Classifier2.
‡Barkhof criteria and number of T2 lesions on baseline MRI.
doi:10.1371/journal.pone.0059953.t003

Predictive Value of gMS-Classifier2 Status and Continuous Unit Values for Conversion to CDMS at Five Years and Total Time of Follow-up

Similar results were obtained at five years of follow-up in the uni- and multivariate analyses for gMS-Classifier2 status and continuous units (Tables 3, 4 and 5). At total time of follow-up, gMS-Classifier2 status and continuous units were not predictive of conversion to CDMS in the univariate analysis (Table 3), but when included in the multivariate models, gMS-Classifier2 continuous units remained independent predictors except when combined with OCB, whereas gMS-Classifier2 status yielded mostly negative results (Tables 4 and 5).

Discrimination Measures: ROC Curve Analyses

To assess the clinical utility of gMS-Classifier2, the calibration measures for number of Barkhof criteria, OCB and gMS-Classifier2 continuous units yielded a p value of 0.303; and the one performed for number of T2 lesions, OCB and gMS-Classifier2 continuous units showed a p value of 0.664. When performing the ROC analyses as discrimination measures, in the model using number of Barkhof criteria, the ROC association

Table 4. Multivariate Cox proportional hazard regression for conversion to CDMS according to gMS-Classifier2 status (positive or negative).

Multivariate models	Two years of follow-up			Five years of follow-up			Total time of follow-up		
	HR	95%CI	p	HR	95%CI	p	HR	95%CI	P
gMS-Classifier2 status	1.8	1.1–2.8	0.014	1.6	1.1–2.4	0.019	1.5	1.0–2.2	0.031
Number of Barkhof criteria, N = 1–2 vs. 0‡	4.1	1.7–9.8	0.002	4.4	2.2–8.9	<0.0001	5.5	2.8–10.9	<0.0001
Number of Barkhof criteria, N = 3–4 vs. 0	7.5	3.4–16.5	<0.0001	6.9	3.6–13.1	<0.0001	8.3	4.4–15.7	<0.0001
gMS-Classifier2 status	1.7	1.1–2.7	0.020	1.5	1.0–2.3	0.044	1.4	1.0–2.1	0.074
Number of T2 lesions, N = 1–9 vs. 0	5.4	1.6–18.0	0.007	9.6	3.0–31.3	<0.0001	11.2	3.4–36.4	<0.0001
Number of T2 lesions, N≥10 vs. 0	12.2	3.8–39.1	<0.0001	17.2	5.4–54.8	<0.0001	21.4	6.7–67.9	<0.0001
gMS-Classifier2 status	1.5	0.9–2.4	0.095	1.3	0.9–2.0	0.217	1.2	0.8–1.8	0.307
Positive OCB	3.6	1.8–7.0	<0.0001	3.1	1.8–5.2	<0.0001	2.9	1.7–4.6	<0.0001
gMS-Classifier2 status	1.5	0.9–2.5	0.081	1.4	0.9–2.1	0.126	1.3	0.9–2.0	0.151
1–2 Barkhof criteria	3.1	1.2–7.6	0.015	3.5	1.7–7.3	0.001	4.4	2.2–9.0	<0.0001
3–4 Barkhof criteria	5.2	2.3–11.9	<0.0001	5.3	2.7–10.2	<0.0001	6.5	3.3–12.5	<0.0001
Positive OCB	2.2	1.1–4.5	0.022	1.9	1.1–3.4	0.014	1.8	1.1–2.9	0.023
gMS-Classifier2 status	1.5	0.9–2.4	0.100	1.3	0.9–2.0	0.204	1.2	0.8–1.9	0.267
Number of T2 lesions, n = 1–9 vs. 0	3.9	1.1–13.7	0.030	7.6	2.3–25.2	0.001	9.1	2.7–30.0	<0.0001
Number of T2 lesions, n≥10 vs. 0	8.2	2.5–27.1	0.001	13.1	4.0–42.7	<0.0001	16.5	5.1–53.3	<0.0001
Positive OCB	2.2	1.1–4.4	0.024	1.9	1.1–3.2	0.021	1.7	1.0–2.8	0.030

Abbreviations: HR = hazard ratio, CI = confidence interval, OCB = oligoclonal bands.
‡Barkhof criteria and number of T2 lesions on baseline MRI.
doi:10.1371/journal.pone.0059953.t004

Table 5. Multivariate Cox proportional hazard regression models for CDMS conversion according to gMS-Classifier2 continuous units.

Multivariate models	Two years of follow-up			Five years of follow-up			Total time of follow-up		
	HR[#]	95%CI	p	HR	95%CI	p	HR	95%CI	p
gMS-Classifier2 units[‡]	1.3	1.1–1.5	0.003	1.3	1.1–1.5	0.001	1.2	1.1–1.4	0.004
1–2 Barkhof criteria	4.5	1.8–10.9	0.001	4.9	2.4–10.0	<0.0001	6.0	3.0–12.0	<0.0001
3–4 Barkhof criteria	8.3	3.7–18.4	<0.0001	7.6	4.0–14.5	<0.0001	9.1	4.8–17.4	<0.0001
gMS-Classifier2 units	1.3	1.1–1.6	0.001	1.3	1.1–1.5	0.001	1.3	1.1–1.5	0.001
Number of T2 lesions, n = 1–9 vs. 0	6.0	1.8–20.5	0.004	10.7	3.3–34.8	<0.0001	12.4	3.8–40.3	<0.0001
Number of T2 lesions, n≥10 vs. 0	15.0	4.6–48.8	<0.0001	20.9	6.5–67.3	<0.0001	25.8	8.0–82.9	<0.0001
gMS-Classifier2 units	1.1	0.9–1.4	0.088	1.1	0.9–1.3	0.157	1.1	0.9–1.2	0.283
Positive OCB	3.4	1.9–7.2	<0.0001	3.1	1.8–5.2	<0.0001	2.9	1.8–4.7	<0.0001
gMS-Classifier2 units	1.2	1.0–1.5	0.020	1.2	1.0–1.4	0.015	1.2	1.0–1.4	0.020
1–2 Barkhof criteria	3.4	1.3–8.4	0.010	3.8	1.8–7.9	<0.0001	4.8	2.3–9.8	<0.0001
3–4 Barkhof criteria	5.7	2.5–13.1	<0.0001	5.8	2.9–11.3	<0.0001	7.1	3.6–13.8	<0.0001
Positive OCB	2.2	1.1–4.4	0.025	1.9	1.1–3.3	0.019	1.7	1.1–2.9	0.031
gMS-Classifier2 units	1.3	1.1–1.5	0.008	1.2	1.1–1.5	0.007	1.2	1.0–1.4	0.009
Number of T2 lesions, n = 1–9 vs. 0	4.4	1.3–15.2	0.020	8.3	2.5–27.6	0.001	9.9	3.0–32.9	<0.0001
Number of T2 lesions, n≥10 vs. 0	9.9	2.9–33.1	<0.0001	15.6	4.8–51.3	<0.0001	19.6	6.0–64.2	<0.0001
Positive OCB	2.2	1.1–4.3	0.029	1.8	1.1–3.1	0.031	1.7	1.0–2.7	0.044

Abbreviations: HR = hazard ratio, CI = confidence interval, OCB = oligoclonal bands.
[‡]Barkhof criteria and number of T2 lesions on baseline MRI.
[#]For continuous values, HR indicates how much the hazard (for CDMS) increases per unit increase in gMS-Classifier2.
doi:10.1371/journal.pone.0059953.t005

statistics showed that when number of Barkhof criteria, OCB and gMS-Classifier2 were put together, the area under the curve (AUC) was 0.7786 (95%CI 0.7169–0.8403), in comparison with an AUC ROC of 0.7552 (95%CI 0.6945–0.8160) when not including gMS-Classifier2. Thus, the AUC change (ΔAUC) ROC was 0.0233 (p = 0.0788). But when the model excluded OCB, the results were the following: AUC ROC 0.7651 (95%CI 0.6990–0.8312) with gMS-Classifier2 versus AUC ROC 0.7236 (95%CI 0.6616–0.7856) without it, with a ΔAUC ROC of 0.0415, p = 0.012. Similar findings were observed when using number of T2 lesions instead of number of Barkhof criteria: when adding OCB to the model, the AUCs were 0.7855 (95%CI 0.7255–0.8455) with gMS-Classifier2 and 0.7528 (95%CI 0.6927–0.8128) without it, leading to a ΔAUC of 0.0328, p = 0.0515. When OCB were excluded from the model, the AUCs were 0.7733 (95%CI 0.7102–0.8364) and 0.7266 (95%CI 0.6669–0.7864), respectively, with a ΔAUC of 0.0467, p = 0.009.

Discussion

Early administration of disease modifying therapies is highly recommended in CIS patients at risk for developing a second relapse [20,21]. Therefore, although MRI remains the most important surrogate marker for predicting the risk of a second relapse in CIS patients [1,2], the clinical outcome remains unpredictable due to the high variability of this disease among individuals. Thus, a need remains for auxiliary biomarkers that could provide additional information about the disease course [22,23]. The presence of IgG OCB at baseline doubles the risk of developing a second attack independent of MRI, but in the revised 2010 diagnostic criteria for MS, CSF was only included in the diagnostic criteria for primary progressive MS. Thus, the testing of CSF may further decline, despite the fact that the International Panel agrees that the inclusion of CSF in the criteria requires further evaluation [5,23]. Furthermore, an ideal biomarker should be non-invasive and simple to use, making potential serum prognostic markers a good option, as they would be easy to obtain [24].

Glycans are potential antigens, and indeed, antibodies against various types of glycans have been found in serum. Such antibodies were first described for the human blood group ABO antigens [25], and later findings have linked antibodies directed against glycans to several autoimmune diseases, either by association only or as etiopathogenic [8–12]. Consequently, diverse assays have been designed to identify anti-glycan antibodies in autoimmune diseases [18,26] including MS [27–29]. Because some of these glycans are found within the type IV collagen matrix of the blood-brain barrier [14], it has been hypothesized that in MS patients, an inflammatory response could lead to the release of these carbohydrate antigens with the subsequent development of a humoral response [30]. Therefore, an array of glycans was screened in RRMS patients and healthy controls, observing that IgM antibodies to various alpha-glucose are elevated in the former group. Among the alpha-glucose, GAGA4 was the most notable and gMS-Classifier Dx was developed as GAGA4 normalized to total IgM. It was further analysed with other neurological diseases controls (13, 31), concluding that gMS-Classifier Dx differentiates between MS and non-MS patients. Based on previous experience in Crohn's disease in which broader glycan structures increase performance and address different clinical utilities, gMS-Classifier Dx was extended to include the following anti-alpha glucose antibodies: GAGA2, 3, 4 and 6, thus establishing gMS-Classifier1, which is based on disaccharides covalently bound via a long linker (anti-GAGA2, anti-GAGA3, anti-GAGA4, anti-GAGA6) and was established for the prediction of "early relapse" (within 24 months)

(14). Then, in the BENEFIT study, gMS-Classifier1 was analysed on three pre-defined end-points: 1) Time to CDMS, 2) Time to McDonald, and 3) Time to confirmed EDSS. However, Classifier1 was only significant on Time to confirmed EDSS (16, 32). Thus, Classifier1 seems to be more of a prognostic MS biomarker for progression rather than for diagnosis. As part of the BENEFIT study, several additional alpha-glucose antibodies were analysed due to previously found data that suggested it could be beneficial to further explore them and see their potential value. Among those additional alpha-glucose antibodies were P63, [a polymer based on Glc(α1–3)Glc(α) and Glc(α 1–6)Glc(α)], alpha-ramose, alpha-N-acetyl glucose and P64 [(a polymer based on Glc(α 1–4)Glc(α) and Glc(α 1–6)Glc(α)]. For each one, time to CDMS with a minimal criterion of 30% sensitivity and 90% specificity at 24 months was analysed in the BENEFIT placebo sub-cohort and on the entire cohort. Only P63 normalized to age predicted time to CDMS and it was called gMS-Classifier2 (16).

A logistic regression model for prediction of early conversion to CDMS was used to develop the classifier; the input data included a number of clinical variables such as age since previous data have shown that IgM levels vary considerably throughout the years [33,34], plus the raw levels of anti-glycan IgM against 8 different glycan antigens. After backward selection only anti-P63 IgM levels and age were found to be independent variables which entered the model. Since this classification rule identified CIS patients at higher risk of converting to CDMS during the first two years of disease evolution [16], the aims of the present study were to confirm those results and to determine whether gMS-Classifier2 is an independent predictor of conversion to CDMS.

Our results show that in this hospital cohort, gMS-Classifier2 is an independent predictor for conversion of CIS patients to CDMS that could become a useful prognostic tool when tested within the first two years of disease evolution, and thus could add information to baseline MRI findings, more specifically, in cases in which lumbar puncture or OCB determination cannot be performed. gMS-Classifier2 was positive in 30% of CIS patients at baseline, and the median time to CDMS was approximately twice as short for gMS-Classifier2-positive patients than for negative patients. The predictive performance of gMS-Classifier2 was better during the early years of the disease and decreased with long-term follow-up. When gMS-Classifier2 status was evaluated together with MRI variables in the multivariate analyses at two and five years of follow-up, it remained an independent predictor of conversion to CDMS, but not when evaluated with OCB. With MRI and OCB findings, continuous unit values of gMS-Classifier2 independently predicted the development of an early second relapse, indicating the increased risk of relapse with increased serum levels of the biomarker. When performing the ROC analyses, the model for gMS-Classifier units was statistically significant only when OCB were excluded. However, recent publications emphasize that testing for any improvement using discrimination measures such as the change in the area under the ROC curve is extremely conservative [36,37]. Thus, we consider the HRs to be sufficient to support the role of gMS-Classifier2 as an independent predictor of conversion to CDMS.

As for the added value of gMS-Classifier2 to OCB findings in predicting early CDMS conversion, the differing results obtained are probably partly due to the higher resolution of a biomarker that is measured in continuous units compared to a dichotomous biomarker [35].

We conclude that gMS-Classifier2 is an independent predictor for conversion of CIS patients to CDMS in the first years of the disease course and therefore could be of clinical relevance to determine which patients are at higher risk, particularly in cases in which OCB are not available.

Acknowledgments

The authors wish to thank the people at the FEM (Fundació Esclerosi Multiple); Maria José Vicente, Rosalia Horno and Isabel Martín (MS nurses at the MS Unit); Mireia Castillo, Zoraida Castro and NuriaVilarrasa (laboratory technicians) for their technical support in processing serum; and Xavier Vidal for statistical advice.

Author Contributions

Contributed in analyzing the MRI data: AR. Conceived and designed the experiments: GA CE JY ND XM MT. Performed the experiments: EF LS. Analyzed the data: GA CE JY ND AD AR XM MT. Contributed reagents/materials/analysis tools: GA CE JY EF LS ND AD XM MT. Wrote the paper: GA CE JY AR XM MT.

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