Universitat de Girona

THE RELATION BETWEEN PERIPHERAL LEVELS OF CD19+ B CELLS WITH THE INFLAMMATORY ACTIVITY AND THE PROGNOSIS OF MULTIPLE SCLEROSIS

A pilot cohort study in patients with Multiple Sclerosis from Girona

FINAL DEGREE PROJECT

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Aos meus pais, polo seu sempiterno apoio.

INDEX

INDEX OF FIGURES

INDEX OF TABLES

1. LIST OF ABBREVIATIONS

Ab: Antibodies

Ag: Antigens

APC: Antigen presenting cells

BBB: Blood-Brain Barrier

CAM: Cell Adhesion Molecules

CDMS: Clinically Definite Multiple Sclerosis

CIS: Clinically Isolated Syndrome

CNS: Central Nervous System

CSF: Cerebrospinal Fluid

DIS: Dissemination in Space

DIT: Dissemination in Time

DMT: Disease-Modifying Therapy

EBV: Epstein-Barr Virus

EDSS: Extended Disability Status Score

GA: Glatiramer Acetate

Gd: Gadolinium

HLA: Human Leukocyte Antigen

HSCT: Hematopoietic Stem Cell Transplantation

IFN-β: β-Interferon

IgG: Immunoglobulin G

IgM: Immunoglobulin M

MBP: Myelin Basic Protein

MHC: Major Histocompatibility Complex

MOG: Myelin Oligodendrocyte Glycoprotein

MRI: Magnetic Resonance Imaging

MS: Multiple Sclerosis

NFL: Neurofilament

NO: Nitric Oxide

OCB: Oligoclonal Bands

OCGB: IgG Oligoclonal Bands

OCMB: IgM Oligoclonal Bands

PBMC: Peripheral Blood Mononuclear Cells

PLP: Proteolipid Protein

PNS: Peripheral Nervous System

PPMS: Primary Progressive Multiple Sclerosis

RIS: Radiologically Isolated Syndrome

ROS: Reactive oxygen species

RRMS: Relapsing-Remitting Multiple Sclerosis

SPMS: Secondary Progressive Multiple Sclerosis

Th cell: T helper cell

UNIEM: Unitat de Neuroimmunologia i Esclerosi Multiple

2. ABSTRACT

TITLE: The relation between peripheral levels of CD19+ b cells with the inflammatory activity and the prognosis of multiple sclerosis. A pilot cohort study in patients with multiple sclerosis from Girona.

BACKGROUND: Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease of the central nervous system. Globally, it is the first cause of non-traumatic neurological disability in young adults having a great impact in the health system. Although it has been thoroughly investigated, its pathogenesis remains unclear. Recent studies have shown that besides T cells, B cells are also involved in the pathological pathways of the disease. This new finding has led to new fields of investigation as new drugs are being developed and new biomarkers of diagnosis, treatment and prognosis have been proposed.

OBJECTIVE:
The purpose of this study is to determine whether the levels of CD19+ B cells in peripheral blood of patients who have just been diagnosed with Multiple Sclerosis (MS) are related to the inflammatory activity and prognosis of the disease.

DESIGN: The selected study design is a pilot ambispective cohort study.

METHOD: Peripheral blood samples from almost 200 patients involved in the BioEM study carried out in the Unitat de Neuroimmunologia i Esclerosi Multiple (UNIEM), will be analysed to determine the levels of CD19+ B cells at the onset of the disease. Then, a minimum follow-up period of 4 years is required to assess the evolution of the disease which will be evaluated measuring the inflammatory activity and the neurological disability. The first one will be assessed using the information from the basal lumbar puncture and the reports from the annual brain MRI and the latter will be described using the Kurtzke EDSS.
When all the data is compiled, CD19 levels will be compared with the presence or absence of Gadolinium enhancing lesions; presence or absence of new or enlarged T2 lesions; OCGB index and OCMB in CSF; all of them surrogate markers of inflammatory activity. Plus, the levels will be compared to the presence or absence of progression of the neurological disability.

KEYWORDS: Multiple Sclerosis • CD19+ B cells • Prognosis • Biomarkers

3. INTRODUCTION

3.1. Background

Multiple Sclerosis (MS) is a chronic immune-inflammatory disease of the central nervous system (CNS) which is characterised by perivascular infiltrates of mononuclear cells, demyelination, axonal loss and gliosis that lead to the formation of multiple "plaques" located in the brain and spinal cord (1). It represents the main cause of non-traumatic neurological disability in young adults worldwide and approximately half of those affected are in Europe (2,3). The disability is characterised by a large variety of signs and symptoms that are the expression of the presence of these plaques in different locations in the CNS. Characteristically MS does not affect the peripheral nervous system (PNS) (4). During its curse MS typically recurs at unpredictable intervals referred to as "attacks", "relapses" or "flares" of the disease. These inflammatory episodes may last days to months and cause injury not only to the myelin sheaths but to the oligodendrocytes and nerve cells processes, all of it increasing the burden of the disease worsening its clinical presentation.

3.1.1. Epidemiology

Globally it is estimated that more than 2 million people meet the diagnostic criteria for MS. It is notorious that the prevalence and incidence estimates tend to be higher in the Northern regions. The high frequency areas are Europe, Canada, Northern US, South-eastern Australia, New Zealand, Israel and eastern Russia. Medium frequency areas are southern US, rest of Australia, South Africa, Southern Mediterranean basin, inner parts of Russia and Latin America. Countries with low prevalence rates (5 per 100000) are found in the rest of the parts of the world not previously mentioned (2). Are individuals from regions above 40º latitude within the Western hemisphere whom have a higher risk of MS (5). Despite this is a fact that historically has been accepted, recent studies suggest that this latitude gradient is disappearing (6,7).

Fig. 1 Map of the prevalence of MS worldwide. Adapted from (8).

Europe is considered a high prevalence region, defined by a prevalence of ≥ 30 per 100000 (9). As new epidemiological studies are published, we can now compare the past and present incidence and this comparison results in an increased incidence of the disease, especially in Europe and Mediterranean Basin.

The possible reasons for this are the increased incidence of the disease among female population, a better assessment of the MS with a higher availability of Magnetic Resonance Imaging (MRI), and better management of the disease with the use of symptomatic maintenance treatment which lengthens the life expectancy of the patients (2,10). Most of the epidemiological studies show a higher prevalence in women with sex ratios from 1.1 to 3, being the average female-to-male ratio overall 2 (2). This ratio appears to be increasing (3) and this could be explained by the phenomenon that women are more likely to have autoimmune diseases (5).
In Spain, comparing registers from the early 80s with those from the 2000s revealed that both incidence and prevalence are increasing mimicking the global tendency (2,11). The current situation in this country is an incidence of 2.2-5.3 per 100000, a prevalence of 72-77 per 100000 and a female-to-male ratio of 2-3:1 (11).

The age of onset of the disease is most common between de second and third decade of life. Whereas less than 5% of patients has the first symptom under 16 years (juvenile MS) and less than 1% under the age of 10 (childhood MS). Similarly, it is rare to find the onset of the disease at an elderly age (over 50 years old) (12).

3.1.2. Aetiology and risk factors

Our current knowledge about the natural history of MS is that Radiologic Isolated Syndrome (RIS) leads to Clinical Isolated Syndrome (CIS) and then eventually to Clinically Definite MS (CDMS) (13). The associated risk factors seem to have influence in this cascade. The aetiology of MS it is still unclear and the primary target autoantigen has yet to be identified but epidemiology indicates that both genetic and environmental factors are implicated. Factors defined from birth such as sex, place of birth or genetic predisposition, require the involvement of environmental factors (vitamin D deficiency, late Epstein Barr virus (EBV) exposition, smoking) as well as epigenetic factors to finally develop the disease(14). It is still unknown if every factor act in sequence and depend on each other or if they act independently and in an additive way (14).

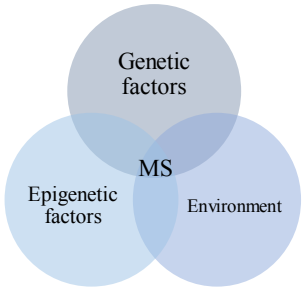


Fig. 2 MS risk factors. Adapted from (58).

The development of MS must start in individuals genetically susceptible. The responsible genes are not mutations but normal polymorphisms and they act independently or through epistasis (10). Twin and family studies have shown that family members have a higher risk of

developing MS than those without this precedent. Monozygotic twins concordance is about 20 to 30% and approximately in dizygotic twins the concordance is about 4% (13,14). There is a particular association with HLA (Human Leucocyte Antigen) haplotypes and the strongest one is found to be the HLA DRB1*15 allele of MHC II (Major Histocompatibility Complex II), which suggests the autoimmune aetiology of MS (13). As new methods of study of the genetic material are available, new genes have been discovered to have an implication in the MS aetiology. On Genomewide Association Studies new genes outside the MHC region have been located and they are the IL2Rα and IL7Rα, CLEC16A, CD58, CD6, TNFRSF1α, IRF8 but these genes can only explain less than 0.2% of the variance in the risk for MS (5,15).

The environmental factors that show the strongest evidence to be associated with the development of the disease include the involvement of EBV, smoking, living at a certain latitude and vitamin D deficiency. Late EBV infection: more than 99% MS diagnosed patients have been infected with EBV and those individuals with higher titters of anti-EBV antibodies (Ab) have a higher risk of developing MS compared to those with low titters (13,16,17).

- Smoking: It exists a dose-dependent relation between smoked, not snuffed, tobacco and MS risk and is also a deleterious factor of the course of the disease (13).
- Latitude and vitamin D: lower sunlight exposure, measured by the actinic damage of the skin, and associated lower vitamin D levels are possible explanations for the association between latitude and MS incidence. Studies have shown that higher consumption and higher levels of 25-OH-colecalciferol were protective against MS (5,13,18).

The timing of exposure is a crucial determinant of risk for MS, particularly for factors that operate early in life as has been reflected on studies of migrant population from low to high prevalence areas and vice-versa (6).

3.1.3. Pathophysiology

The pathologic hallmarks of Multiple Sclerosis lesions are the breakdown of the blood-brain barrier (BBB), multifocal inflammation, demyelination, oligodendrocyte loss, reactive gliosis and axonal degeneration and loss, being the major cause of permanent neurological disability the latter (10,19). This axonal loss is produced early in the disease, but compensatory mechanisms makes it clinically silent until they falter and irreversible neurological disability becomes evident and the disease transitions to the Secondary Progressive MS (SPMS) phase where active inflammation is no longer prominent (19). The partially demyelinated axons conduct impulses at a reduced velocity, which explains the characteristics delays in conduction of evoked potentials. Plus, these demyelinated fibers cannot sustain the fall in membrane capacitance induced by a rise of temperature and conduction fails, this is known as the Uhthoff's phenomenon. Complete demyelinated axons discharge spontaneously and show an increased mechanical activity which

explains the phosphenes and L'Hermitte sign (an electrical sensation running down the spine or limbs when the neck is flexed) (10).

As it was previously mention, MS is a chronic inflammatory disease of autoimmune causes which natural history leads to demyelination and neurodegeneration of the CNS. The traditional view is that myelin-specific autoreactive T lymphocytes, mainly T helpers (Th) cells, were the key factor in the development of the disease and thus most of the current treatment primarily target T cells. These T cells are primed in the periphery into CD4+ cell effectors: Th1, Th2 or Th17. The first and the last produce proinflammatory cytokines, whereas Th2 produce anti-inflammatory cytokines but it has been observed a dysregulation in the activity of these CD4+ T cells and the proinflammatory activity is more prominent (see Figure 4) (1,20). Besides this CD4+ T cells involvement, CD8+ T cells have a role in the pathogenesis of MS as they mediate the suppression of CD4+ cells, kill glial cells, transect axons, promote vascular permeability and activate oligodendrocyte death (21).

Once activated, myelin-specific T cells can cross the BBB (see Figure 3). The transmigration into the CNS is mediated by the interaction of integrins expressed on the surfaces of leucocytes with their ligands, cell adhesion molecules (CAM) expressed on the endothelial cells (20).

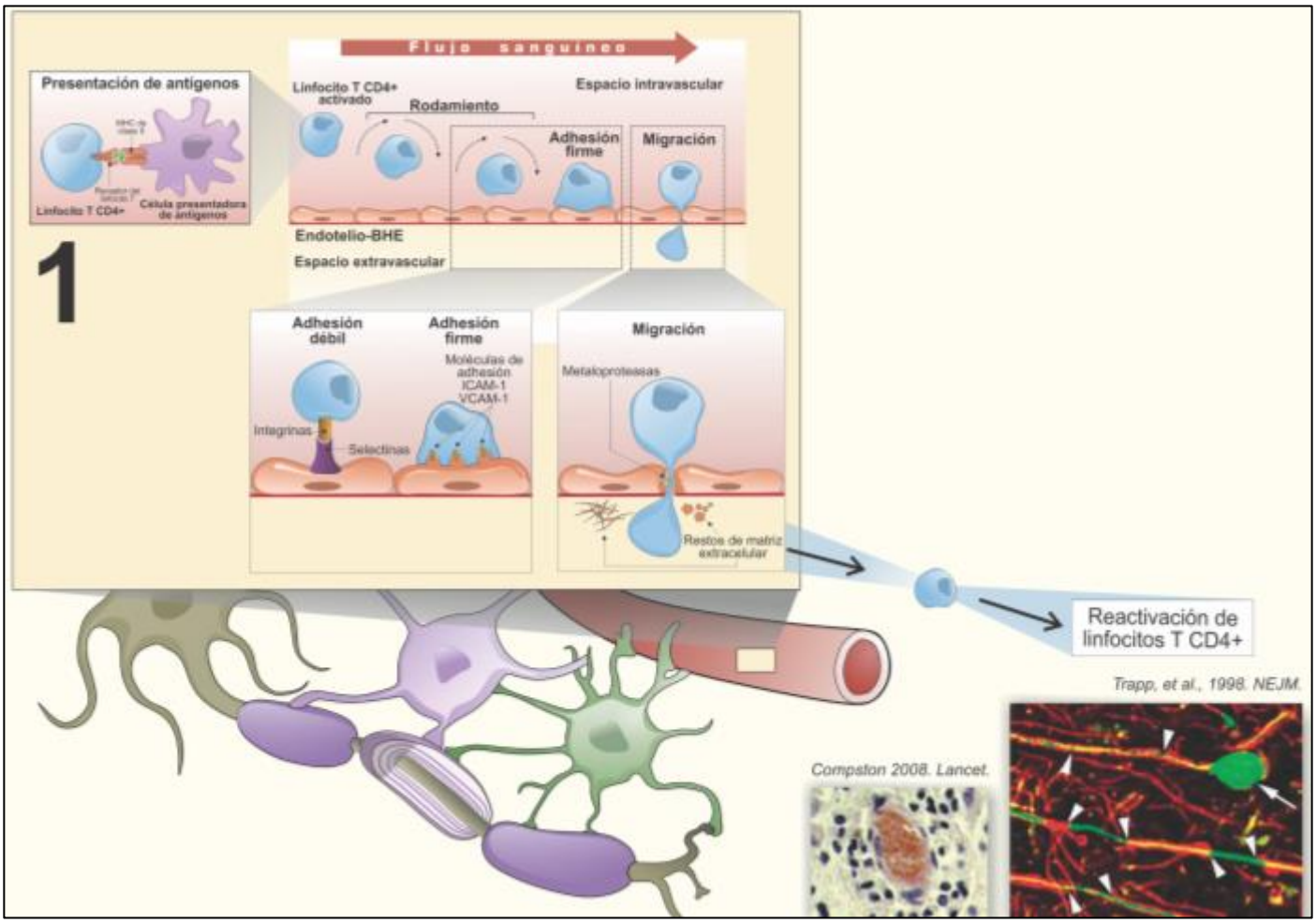


Fig. 3 CD4+ T cell priming process and migration through the BBB (22)

When lymphocytes have already surpassed the BBB they proliferate and secrete proinflammatory (See Figure 4) cytokines which in turn stimulate microglia, macrophages and astrocytes, ultimately resulting in damage to myelin, oligodendrocytes and axons (23). The breakdown of the regulation of the autoimmune responses against myelin components in the CNS is hypothesized to occur through mechanisms such as molecular mimicry, in which is suggested that the presentation of a peptide (the environmental factor) in the groove of MHC II is immunologically indistinguishable from self-antigen and, consequently, an appropriate response to infection generates inappropriate inflammation against of the oligodendrocyte-myelin unit components; bystander activation and epitope spreading (10,23).

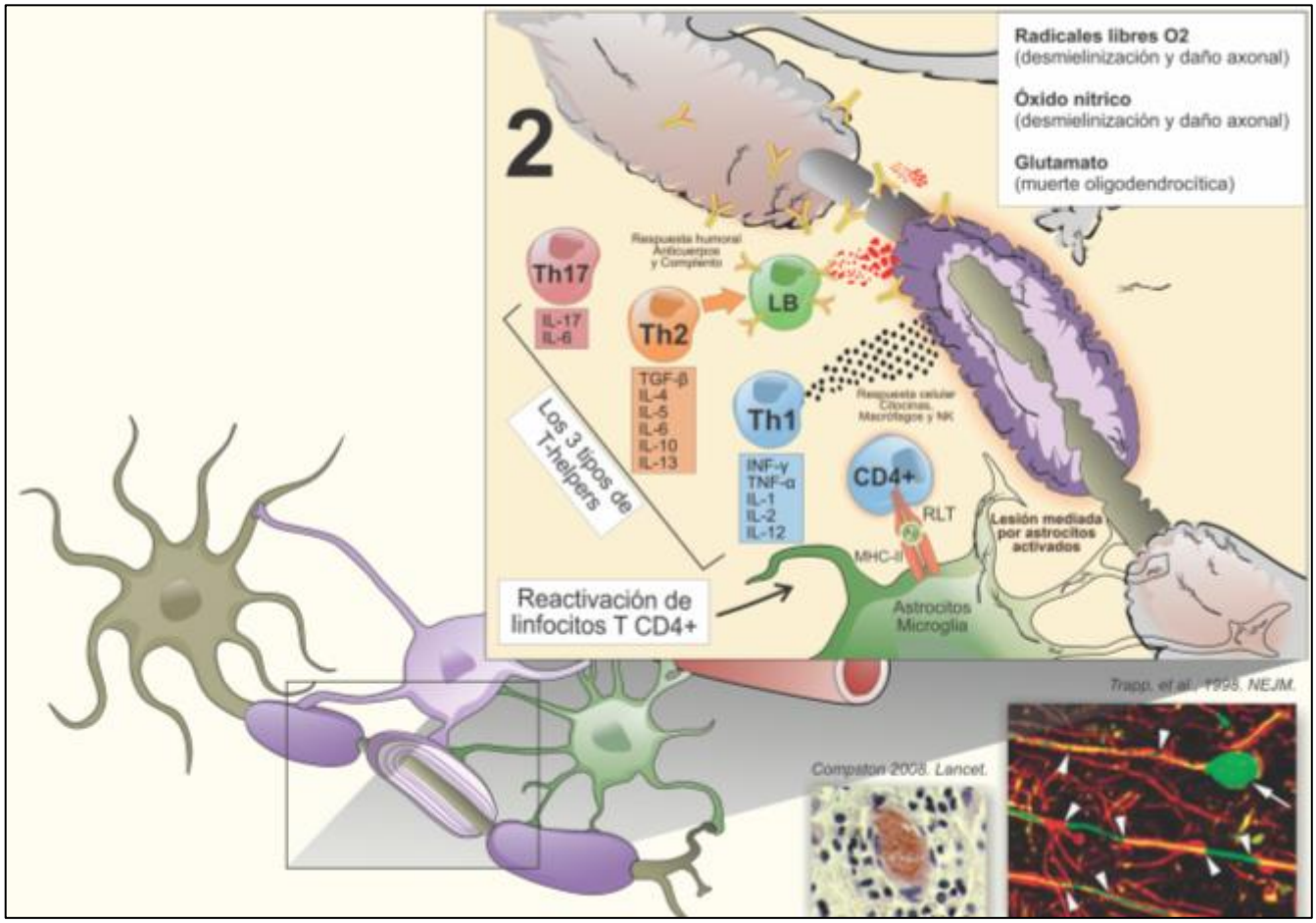


Fig. 4 CD4+ T cell subtypes cytokine release (22)

Microglial cells become activated in response to injury, inflammation and axonal degeneration. They can have a benign protective action or, on the opposite, contribute to neurodegeneration. The exact mechanism that determine one or another remains unknown. Microglia has several harmful mechanisms. One of them, production of reactive-oxygen species (ROS) and nitric oxide (NO), is involved in the mechanism by which inflammation leads to axonal degeneration. ROS and NO induce mitochondrial dysfunction and it contributes to the pathological features that are typical of MS lesions such as demyelination, oligodendrocytes

apoptosis and axonal degeneration (see Figure 5) (20,24). Another one is the released of cytokines and growth-promoting factors that promotes endogenous remyelination.

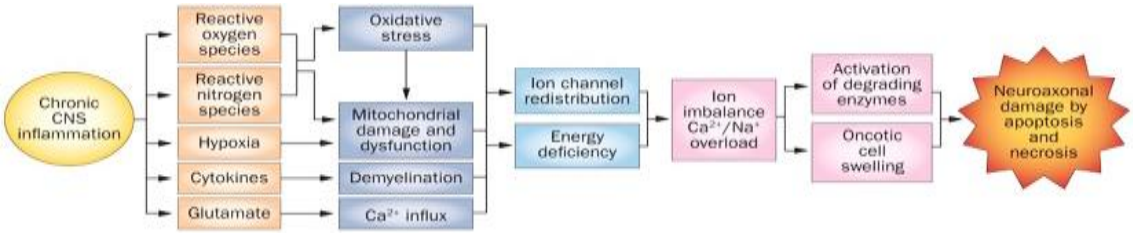


Fig. 5 Cascade leading to inflammation induced neuroaxonal injury. Extracted from (59).

After some time, astrocyte reactivity seals the lesion and the gliosis, name given to the process of hypertrophy and proliferation of astrocytes in demyelinating lesions, causes a physical barrier to further remyelination decreasing the capacity to compensate deficits and marking the transition to the stage of persistent deficit, and as previously mention, to a progressive stage. Lastly, microglia express MHC, re-present antigens to T cells and set up an inflammatory loop (10,20).

In recent years, it has been demonstrated that in addition to T cells, B cells are involved in the pathogenesis of MS too and their role is not limited to only produce autoantibodies. Proof of this is the presence of polyclonal Antibodies (Ab) in CSF, known as oligoclonal bands (OCB), which are produced by plasma cells which in turn derive from B cells, indicating that B cells are activated in the disease. B cells also produce pro and anti-inflammatory cytokines and there seems to be a predominance of the proinflammatory activity over the anti-inflammatory, as it has been seen in the T cell activity. Plus, the discovery of B cell follicle-like aggregates in the meninges, mainly in the progressive forms, supports the central role of B cells in grey matter pathology as a targeted B cell response consequent to antigenic stimulation within the CNS (20,21,25,26). The greatest clue of the B cells involvement in the pathogenesis of MS is the response to treatment with monoclonal antibodies targeting the B cell antigen CD20 (25,26).

B cells, identified by their expression of CD19 on their surface (see Figure 6), mature in the bone marrow. Then they migrate to secondary lymphoid organs where they differentiate into naïve mature B cells that express only one type of B cell receptor (BCR). Naïve B cells are activated by specific antigen (Ag) binding to its BCR. This Ag is internalized and presented on the surface by MHC II to T cells. After the activation, B cells differentiate into antibodies-secreting plasmatic cells or memory B cells which are distinguished by their expression of CD27. In MS patients, peripheral memory B cells have been found to act as APCs in response to myelin antigens such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) or proteolipid protein (PLP) and memory B cells in CSF have an increased expression of costimulatory molecules that allow them to induce further T cell proliferation (27,28).

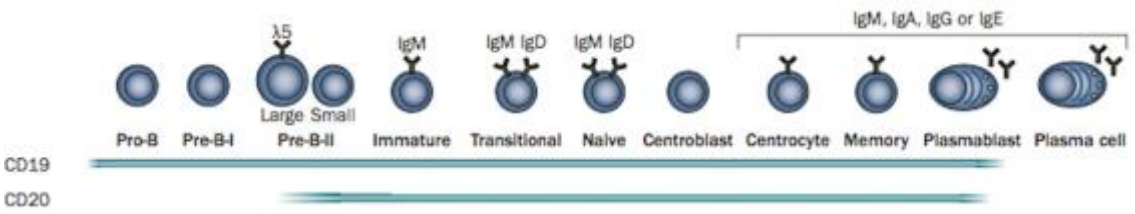


Fig. 6 Expression of surface markers of B cells in different stages of differentiation. Adapted from (29).

B cells can be functionally subdivided according their cytokine profiles. While most B cells stimulate the immune system and contribute to the elimination of antigens by producing proinflammatory cytokines like IFNγ, interleukin 2 (Il-2), TNFα and Il-6, some B cells repress the immune functions by producing immunomodulating mediators such as Il-10 and or TGFβ. The latter are known as regulatory B cells (Breg) (25,28). These functional subpopulations are distributed unequally in MS patients and it has been demonstrated an imbalance between the proinflammatory and anti-inflammatory activity of B cells shown as an increment of the proinflammatory cytokines and a down regulation of anti-inflammatory B cells (25,27,28,30).
In MS patients, B cells are directed into CNS by chemokine signalling during inflammation. CXCL13 and BAAF were found to be up regulated within ectopic lymphoid follicles and lesions from SPMS.

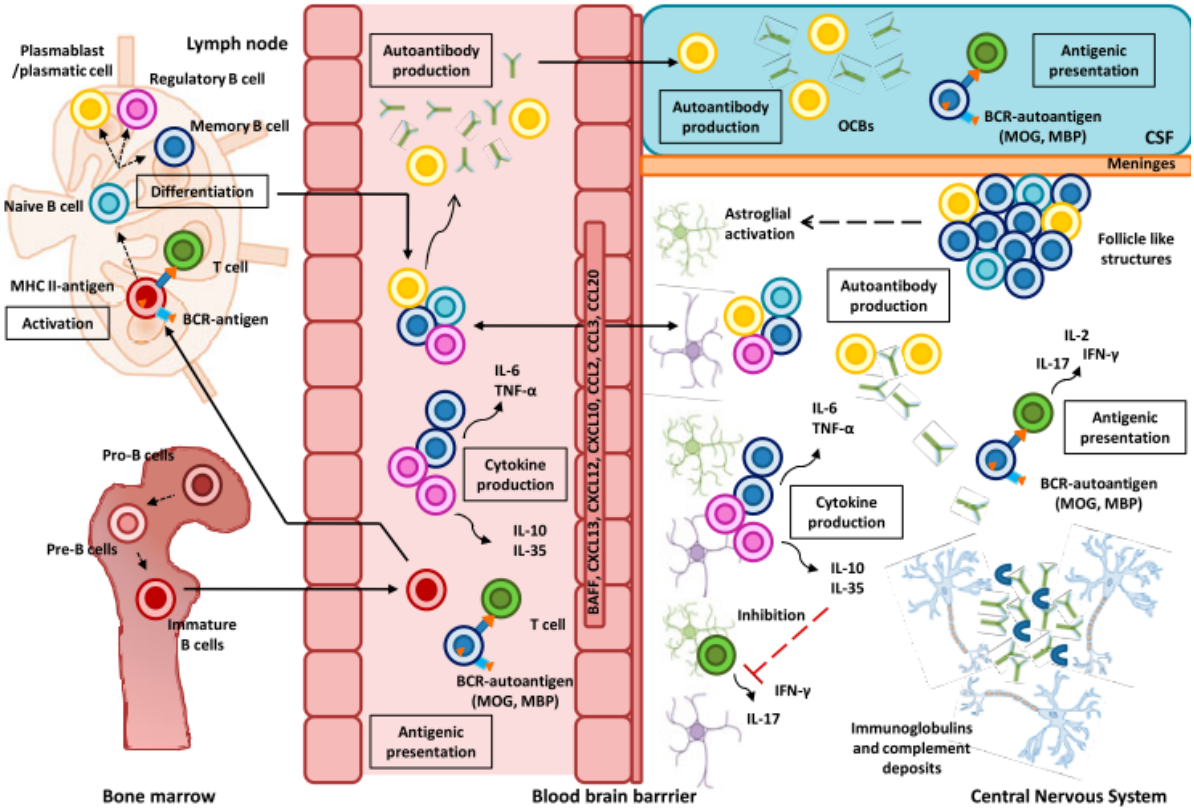


Fig. 7 The B cell involvement in the pathogenesis of MS. Extracted from (27)

Several studies have shown that the production of autoantibodies in the CNS is probably not the initiating cause of MS but after the BBB has been breached ('first hit') antibodies might enhance pathology ('second hit') and this is refuted by the fact that antiCD20 therapies don't target plasma cells and the influence on CSF IgG levels and OCB is minimal or absent, hence, it is unlikely that autoantibodies production mediates the pathogenic role of B cells in MS (26,29).

3.1.4. Clinical course

The course of MS reflects the interplay of the 2 phenomena of inflammation and neurodegeneration. Acute neurological symptoms are considered as relapses that express the focal, disseminated and recurrent inflammation occurring within the CNS (10). Progression, which refers to the steady and irreversible worsening of symptoms and signs over more than 6 months, and accumulation of disability correlate with the early diffuse, chronic and progressive axonal loss, the hallmark of the neurodegenerative process (31).

Clinical forms of MS (See ANNEX I) can be characterised by either episodic acute periods of worsening symptoms, gradual progressive deterioration of the neurological function or a combination of both (32). Classically we differentiated 4 forms of the disease: The Relapsing-Remitting MS (RRMS), the Secondary Progressive MS (SPMS), the Primary Progressive MS (PPMS) and the Progressive-Relapsing MS (PRMS) but after the revision of the Multiple Sclerosis Phenotype Group that included the activity as a modifier of the disease, the latter category disappeared and now we differentiate PPMS as active if there exist acute attacks or non-active if no attacks or MRI activity is demonstrated (33).

The clinical courses definitions are as follow:

- **RRMS**: Clearly defined disease relapses established in hours or days with full recovery or with sequelae and residual deficit upon recovery with periods between disease relapses characterised by a lack of disease progression (32). It is the classic presentation of MS and it accounts the 85% of the total of the patients with MS. The average age of onset in this form is near the 3rd decade of life and the female-to-male ratio is about 3:1 (12).
- **SPMS**: initial RRMS disease course followed by progression with or without occasional relapses, minor remissions, and plateaus (32). This conversion from one form to another can be seen in half of the RRMS patients after 10 years and in the 95% after 25 years (1,11).
- **PPMS**: Gradual progression from onset with occasional plateaus but no distinct relapses and temporary minor improvements allowed (32). It occurs in the 10% of patients and its debut is later in life respect the RRMS form, during the 4th decade and affects more equally to men and women (31).

Besides the main clinical courses, it should be mentioned the Radiologically-Isolated Syndrome (RIS) and Clinically-Isolated Syndrome (CIS) as they are part of the natural history of the disease (1). RIS is defined as incidentally identified white matter anomalies in brain MRI associated with demyelination of the CNS suggestive of MS in non-symptomatic people and non-neurological dysfunction after excluding other possible processes (34). The presentation that occurs in the 85% of patients that will later develop MS is characterised by an acute or subacute episode of neurological dysfunction due to a single white matter lesion and this is known as CIS (35).

The semiology of the disease is related to the location of the damaged area of the CNS. The most common locations of this damage are the visual tracts (92%), spine (74%), brainstem/cerebellum (55%) and brain cortex (40%) (11).

Common symptoms at the onset include (in a high to low order): weakness in one or more limbs, optic neuritis, paraesthesiae, diplopia, vertigo and disturbance of micturition. Late symptoms are the alteration of the pyramidal tract, paroxysmal symptoms (L'Hermitte sign and trigeminal neuralgia), internuclear ophthalmoplegia and cognitive disorders (4,11).

Table 1. Signs and symptoms of Multiple Sclerosis. Adapted from (11).

<table>
<tr><th>Visual symptoms</th><th>Muscular tone and power</th><th>Reflexes</th><th>Sensation</th></tr>
<tr><td>▪ Decreased visual acuity
▪ Altered colour perception
▪ Scotomas
▪ Disc pallor</td><td>▪ Weakness
▪ Spasticity
▪ Tetra/para/hemi/monoparesis</td><td>▪ Tendon:
-Hyperreflexia
-Clonus
-Hyporreflexia

▪ Cutaneous:
-Absent abdominal
-Unilateral absence
-Babinski
-Equivocal plantar</td><td>▪ Alteration of the vibratory, thermalgesic, arthrocynetic sensitivity and two-point discrimination</td></tr>
<tr><th>Sphincters</th><th>Brainstem</th><th>Cerebellum</th><th>Superior functions</th></tr>
<tr><td>▪ Urinary urgency, hesitancy, incontinence
▪ Increased urinary frequency
▪ Bowel dysfunction</td><td>▪ Nystagmus
▪ Ocular movement disorders
▪ Dysarthria
▪ Facial paresis
▪ Dysphagia
▪ Dysarthria</td><td>▪ Ataxia of the upper limb, lower limb or combined.

Other symptoms

▪ Fatigue (75%), pain, paroxysms, tremor, sexual dysfunction</td><td>▪ Mood alteration: depression
▪ Cognitive impairment: loss of memory, especially visual memory, slow processing speed and impaired executive functions</td></tr>
</table>

3.1.5. Diagnosis

There is no single clinical or paraclinical feature or diagnostic test that is sufficient to diagnose MS. Therefore, the diagnosis relies on the demonstration of signs and symptoms attributable to white matter lesions which also have to prove dissemination in time and space with the exclusion of other conditions with similar characteristics (1,36). The 2010 Revised McDonald Criteria (see ANNEX II) allow a more rapid diagnosis with higher sensitivity and specificity. They should be applied only when a patient experiences a typical CIS or progressive paraparesis/cerebellar/cognitive syndrome in the case of suspected PPMS (36).

The diagnosis of **RRMS** requires the demonstration of dissemination of lesions in time (DIT) and space (DIS) defined as (36):

- DIT: any new T2 or Gadolinium (Gd) enhancing lesions on follow-up scan at any time after the baseline scan or the simultaneous presence of asymptomatic enhancing and non-enhancing lesions on the same scan regardless the timing.
- DIS: at least one T2 lesion in at least 2 of 4 key locations: juxtacortical, periventricular, infratentorial and spine.

Otherwise, **PPMS** may be diagnosed in patients with (36):

1) One year of disease progression (retrospectively or prospectively determined)
2) Plus 2 of the 3 following criteria:
   i) Evidence for DIS in the brain based on ≥1 T2 lesions in at least 1 area characteristic for MS (periventricular, juxtacortical, or infratentorial)
   ii) Evidence for DIS in the spinal cord based on ≥2 T2 lesions of the same.
   iii) Positive cerebrospinal fluid (CSF): isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index.

The paraclinical information is nowadays obtained by performing a lumbar puncture to obtain CSF, a MRI, evoked potentials and blood tests.

- CSF analysis: When analysing the CSF, we should assess the macroscopic aspect, the cell and protein count, serologies and the presence of OCB. In MS, it is characteristic a transparent colour with less than 5 cells/mm^3, the type often encountered are lymphocytes T CD4+ and activated lymphocytes and plasmatic cells. The protein count is normal or subtly elevated, less than 50mg/dL. Serologies are performed to rule out mainly VIH, Borrelia Burgdorferi and syphilis (12,37).

  The analysis of CSF in MS has gained prominence in the MS study in recent years as it has helped to figure out new data about the physiopathology as it reflects the increased intrathecal synthesis of immunoglobulins (Ig). This gain can be calculated quantitatively or qualitatively with the IgG index and the presence of OCB (37). The first one is the

easiest way to demonstrate quantitatively the increase of intrathecal synthesis and values bigger than 0.7 in the quotient (IgG CSF/IgG serum) / (albumin CSF/albumin Serum) indicate activity of the disease conferring and increasing the risk of developing a secondary progressive form. The OCB patron most frequently seen in MS patients is the positive patron, this is, the presence of ≥2 OCB of IgG in CSF without OCB in serum (38).

Depending on the type of OCB we study, we obtain different information:

- IgG OCB (OCGB): have a diagnostic role as it allows a differential diagnosis with other inflammatory diseases of the CNS. Their absence make the diagnosis of MS unlikely (39,40). Plus, they have a prognosis role as they predict the development of MS in CIS patients with a high positive predictive value (26).
- IgM OCB (OCMB): its role is mainly prognostic. Only 30 to 40% of MS patients present OCMB and it is considered that they have a higher risk of attacks and disability and worse response to β interferon and glatiramer acetate treatment (40–42).

- MRI: A brain MRI must be performed in every patient with CIS and in patients with suspected MS with the objective of ruling out other condition that may explain the symptomatology and to identify the possible existence of demyelinating lesions in the CNS (43). The MRI sequences used in the study are weighted in T1, T2, FLAIR and T1 with Gadolinium contrast.

  The radiologic characteristics of the plaque of demyelination are:

  - Hyperintense T2 sequences, which translates the increased free water, not the plaque composition, its oedema contribution, demyelination or remyelination, inflammation, axonal damage or gliosis. Typically, the lesions are small (<25mm) and multiple with a nodular morphology and are most commonly located in the juxtaventricular white matter, juxtacortical white matter, infratentorial parenchyma and corpus callosum (44).
  - Despite the nodular shape is a common finding, in most patients is present a plaque with ovoid morphology known as Dawson's fingers (12).
  - If these T2 lesions are present it is recommended the use of T1 sequences after Gd contrast administration. The Gd enhanced lesions allow the selective identification of lesions with acute inflammatory activity and to determine the temporal and spatial dissemination of demyelinating lesions for the initial diagnosis (43). The spine MRI should be performed when: there's a normal brain study but high clinical suspicion; nonspecific brain MRI findings; a clinically isolated syndrome with only spinal cord symptoms; atypical new spinal cord symptoms and after the diagnosis of PPMS to exclude other conditions and confirm demyelinating lesions (45). It is important to

take into account that no symptomatic correlations can be made for the great majority of brain abnormalities seen on MRI and even established chronic demyelination in highly eloquent areas may be asymptomatic (11).

- Black holes can be seen too in the brain MRI as T1 hypointense images compared to the normal grey. Their significance is different depending on the stage of the disease. At first they indicate oedema and demyelination and it can resolve. But in a chronic phase, this hyposignal reflects the irreversibility of the process (12).

- Evoked potentials: the findings that show demyelination in MS are delayed latencies of the visual (mainly), somatosensory and auditory potentials on electrophysiological studies (1,10,11).
- Blood tests: it should be determined ANA, antiRo and antiLa levels, syphilis, VIH, VHB, VHZ, Rubella, Measles, Borrelia Burgdoferi serologies and vitamin D levels. The main objective of performing a blood test is to rule out other diseases that can mimic MS (1).

There are more than 70 entities that can be included in the differential diagnosis of MS but they can be reduced to 3 categories: CNS tumours, CNS infections and systemic autoimmune diseases (11,46).

Once the diagnosis of MS is done, the severity of disability must be assessed. The currently most extended scale is the Kurtzke Extended Disability Status Scale (EDSS) (see ANNEX III). It is based on the results of the neurological examination and the patient ability to walk and it measures the disability in 8 functional systems: 1. Pyramidal 2. Cerebellar 3. Brainstem 4. Sensory 5. Bowel and bladder 6. Visual 7. Cerebral or mental 8. Other or miscellaneous functions (47).

Table 2. Correspondence between the EDSS score and the clinical repercussion (Extracted from All About Multiple Sclerosis, available at www.mult-sclerosis.org)

0: Normal neurological exam	**6.5**: Bilateral support needed
1-4.5: Fully ambulatory without aid	**7-9.5**: Restricted to wheelchair
5-5.5: Disability impairs daily activity	**10**: Death due to MS
6: Unilateral support needed	

The recovery from the first neurological episode is considered as complete when the irreversible score after the episode was ≤2 on the EDSS, and incomplete when the score was ≥3. The disability is irreversible when a given score persists at least 6 months, excluding transient worsening of disability related to relapses (31).

3.1.6. Treatment

MS is a disease that still does not have a definitive treatment. For this, it is required a multidisciplinary team that works together to ameliorate the patients' symptoms and stop the disease progression and to try finding the best therapeutic option for each patient, what is commonly called "personalized medicine". The treatment of the MS is different depending on whether we want to control the attacks and relapses or if we want to modify the natural history of the disease. The main objective of the treatment is to control the inflammatory activity to prevent new relapses and burden of disease and for this reason early treatment is crucial (48).

- Treatment of the relapses: Relapses are defined as a worsening of neurological impairment or an appearance of a new symptom or abnormality attributable to MS lasting at least 24 hours and preceded by stability of at least 1 month (49). Its treatment consists in the administration of 1gr/day of methylprednisolone during 3-5 days dissolved in 250cc of saline 1-3h together with the evaluation of the necessity of oral prednisone. If after the withdrawal of the corticoids symptoms return, a new dose of intravenous methylprednisolone can be administered. If even after these methylprednisolone pulses the attacks are still not controlled, plasmapheresis is a strategy to test (12).

- Treatment to modify the natural history of the disease: Disease Modifying Therapy (DMT): In Spain are approved as immunomodulators: **β-interferon** (IFN) **1a, 1b**; **Glatiramer Acetate** (GA); and as immunodepressors: **Azathioprine**; **Mitoxantrone**; **Natalizumab**; **Fingolimod**.

  The therapy election depends on the characteristics of clinical course (48):

Table 3. Therapeutic election according the clinical course

CIS	After the first demyelinating episode, it is indicated the treatment with either of the following: β-IFN 1a s.c. or i.m; β-IFN 1b s.c.; GA.
RRMS	First line of treatment can be: β-IFN 1a or 1b or GA. This option is indicated if the patient is older than 16 years old, has had less than 2 relapses in the last 3 years, has an EDSS <5.5 and has no contraindications.
	As second line of treatment it is used natalizumab or fingolimod. They are second line therapies because of their serious secondary effects: progressive multifocal leukoencephalopathy (LMP) and cardiotoxicity (12).
	Natalizumab and fingolimod can also be used as first line treatments but their use is restricted to those cases of an aggressive onset, fast functional impairment and evidence of inflammatory activity.
SPMS	The treatment depends on the presence or absence of attacks. If the SPMS has no attacks no treatment has demonstrated to be effective. In case of being a SPMS form with attacks, it is indicated the use of β-IFN s.c.
PPMS	Unfortunately, nowadays there is still no recommendation for the use of DMT in this form as it has no effect on its course.

- Symptomatic treatment: pharmacologically each symptom can be treated as follow (11,12):
    - Spasticity: baclofen, cannabinoids
    - Neuralgic pain: carbamazepine, phenytoin, gabapentin
    - Paroxysmal symptoms: carbamazepine, acetazolamide
    - Urinary retention: anticholinergics
    - Sexual dysfunction: papaverine or fentolamine
    - Gastrointestinal symptoms: laxative or enemas
    - Mood swing: fluoxetine, amitriptyline
    - Ambulation disorders: fampridine

There are also many in study treatments that still do not have the indication for the disease but account with long clinical experience and promising results, between them are: cyclophosphamide, alemtuzumab, daclizumab, monoclonal antibodies antiCD20 (rituximab, ocrelizumab, ofatumumab) and autologous hematopoietic stem cell transplantation (HSCT) (12).

The development of monoclonal antibodies has revolutionized the treatment of MS. The only cell specific Ab which has proved in clinical trials to be highly efficient in RRMS is rituximab, which acts depleting B cells which are positive for the CD20 antigen. This protein is expressed in different stages of B cells differentiation, from pre-B cells to naïve and memory B, but absent in early stages (pro B cells) and plasma cells cells (see Figure 6). The decrease of CD20+ B cells after the treatment with rituximab have been followed by the diminution of the number of Gd enhancing lesions in MRI and the proportion of patients experiencing relapses and similar results have been obtained with second generation antiCD20 molecules (ocrelizumab and ofatumumab) (26,27).

3.1.7. Prognosis

Clinical and demographic features at disease onset along with the findings in MRI, CSF and evoked potentials can be used as tools to predict the risk of relapses, relapse severity and recovery or disability (50). And these predictors can help clinicians determine when to initiate the first line of DMT. Clinical factors of **good** prognosis are: female sex, young age of onset, relapsing-remitting course, optic neuritis and sensitivity alterations as initial symptoms, no sequelae after the first attack and low number of relapses within the first 2 years. On the other hand, clinical factors of **bad** prognosis are: male sex, late age of onset, primary progressive form, pyramidal and cerebellar symptoms in the onset, existence of sequels after the first attack and elevated number of relapses in the first 2 years (51). What evidence has shown is a limited value when these factors are applied at an individual level, consequently finding paraclinical predictors is needed (52).

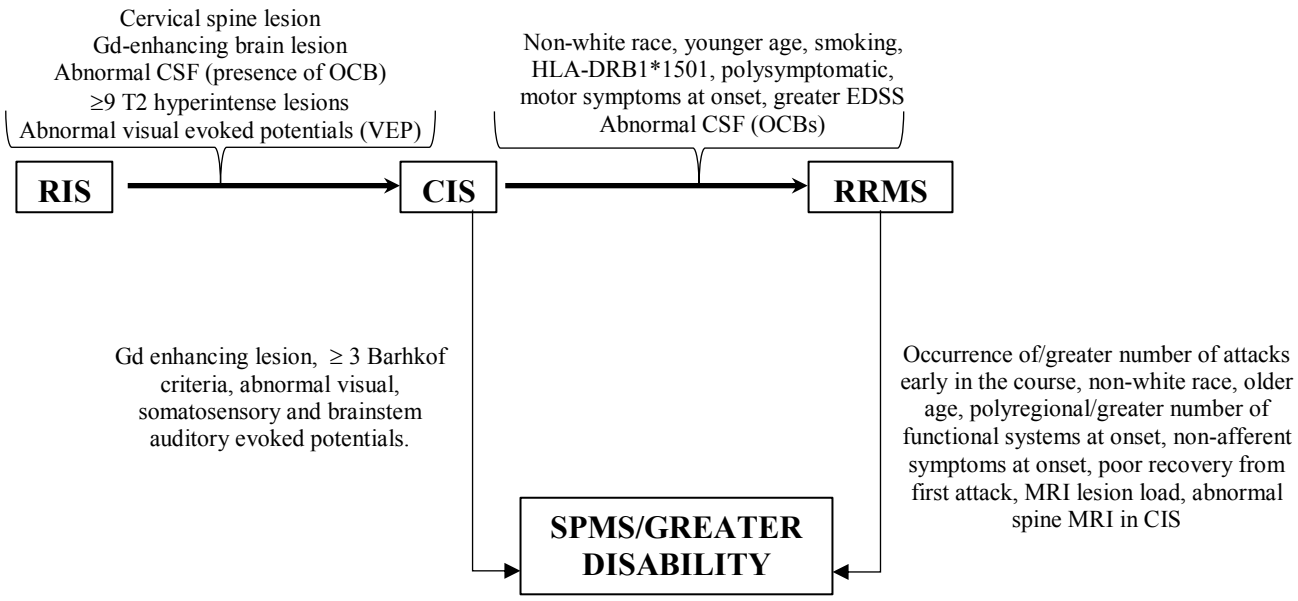


Fig. 8: Predictors of progression in early and intermediate course of MS. Adapted from (50).

The natural history of the disease leads to chronicity and accumulation of disease burden due to the neurodegenerative process that occurs in the CNS. Although the life expectancy can be similar to that of the general population, mortality is increased in MS patients with a reduced life-spam of 5 to 10 years (53). The mortality is related to the neurological disability and the potential complications of the disease mainly pulmonary and urinary infections (53).

3.1.8. Biomarkers

One of the major fields of investigation on MS is the validation of biomarkers in biological fluids that would allow a more accurate patient management. Identifying these biomarkers could improve the disease diagnosis, the prediction of disease progression and improve clinical outcomes resulting on the prevention of long-term neurological disability. There are 3 main areas of research into biomarkers for MS: biomarkers that can predict individuals at risk of developing MS, biomarkers of progression that can predict individuals that are at risk of developing severe attacks or progressive disease and biomarkers that can differentiate individuals that may be responsive to specific treatments (54). To this day, there are only 3 biomarkers used in the diagnosis and treatment of MS: OCB in the CSF, lesions found on MRI and JC viral antibody titers in patients treated with natalizumab. As mentioned above in the diagnosis section, higher levels of OCGB predicts the evolution from a clinically isolated syndrome to clinically definite multiple sclerosis (39). For their part, the presence of OCMB gives a higher risk of new attacks and disability. Besides its diagnostic role, the use of MRI also gives information about the state

of the disease as Gd enhancing images show active inflammation and lesion burden. Also, number and size of enhancing MRI lesions are predictive of the onset and severity of relapses. On the contrary to white matter damage, grey matter atrophy is correlated with cognitive dysfunction and it can be used as a biomarker for prediction of clinical severity. Lastly, JC viral antibodies titers in CSF and serum are determined prior to the treatment with natalizumab because it may reactivate the virus which can lead to an encephalopathy known as progressive multifocal leukoencephalopathy (54). Potential biomarkers that will be soon used in the clinical practice as they have been long studied are the CSF levels of light and heavy chains of CNS Neurofilaments (NFL), which are proteins that form part of the axonal cytoskeleton liberated when there is axonal damage and this could be a prognostic marker for an aggressive disease course and higher risk for SPMS (39,54).

To summarize the evidence, we can only obtain prognosis information from CSF, which requires the performance of a lumbar puncture, and from MRI studies. Because of the difficulties in obtaining CSF samples and the necessity to carry out a lumbar puncture to make a diagnosis has been reduced, as it is no longer needed for the diagnose of RRMS forms, the research of blood-based biomarkers may provide useful tools in clinical practice for the diagnosis and prognosis of the disease as well as a tool to better understand the physiopathology of MS.

3.2. Justification

Multiple Sclerosis (MS) is a potentially progressive disease with a yet unknown complex pathophysiology. This complexity is reflected in the different clinical courses it can take. Thus, the disease can debut as a clinical isolated syndrome (CIS) which in the 85% of cases will later develop a Clinical Definite Multiple Sclerosis (CDMS). Some (15%) of MS diagnosed patients have a progressive course from the beginning, it is called Primary Progressive MS (PPMS). But the utmost number of patients, the 85% of them, will have the so-called Relapsing-Remitting MS (RRMS) with attacks and remissions of the disease in an unpredictable basis. Characteristically this last form will eventually become more progressive, with less relapses and it is known as Secondary Progressive MS (SPMS) which reflects the change from a prominent inflammatory activity to a neurodegenerative state.

To this day it cannot be given an accurate prognosis to a patient just diagnosed with MS. There are still difficulties predicting the behaviour of the disease as it can take an aggressive or a more stable course, or its clinical course or when the patient will have a relapse. Hence many biomarkers have been sought and proposed in an attempt to clarify the prognosis. These biomarkers can be a determinant of the risk of an individual of developing CDMS from a CIS stage; of the risk of new attacks or progression and of the response to therapy. After carrying out a considerable amount of studies, only 3 biomarkers have been validated for having clinical relevance. These are: the determination of oligoclonal bands (OCB) in cerebrospinal fluid (CSF), the determination of JC virus titers in patients treated with natalizumab and serial MRI. Briefly, the first one positively correlates the presence of IgM OCB (OCMB) with a more aggressive course. The second one serves to monitor the secondary effects of the treatment with natalizumab as it predisposes the JC virus infection. And the latter gives information about active inflammation and lesion burden according the presence of Gadolinium (Gd) and new or enlarged T2 enhancing lesions, being the location and number of the lesions predictive of the onset of the disease and its severity. Also, there is a positive correlation between grey matter atrophy and cognitive dysfunction making it a better biomarker for the prediction of clinical severity. Summarizing, nowadays we have two prognostic biomarkers and one that monitors the secondary effects of the treatment. These two prognostic biomarkers are restricted to 2 out of the three compartments where MS has proved to provoke alterations according its pathogenesis, this is, they are limited to the CSF and CNS, leaving peripheral blood without any biomarker where to obtain information for the prognosis.

Additionally, recent investigations showed that B cells have a more prominent role in the pathogenesis of MS than previously thought as they have shown a proinflammatory activity and in patients treated with antiCD20, such as rituximab, ocrelizumab or ofatumumab, the decreased number of these cells ameliorate the symptoms and signs.

Taking all the above into account, in this study we want to assess if the levels of B cells expressing CD19 in peripheral blood once the diagnosis of MS is established would be a good biomarker of the inflammatory activity of the disease. If so, this would be a method to give MS patients a more accurate prognosis. Plus, it would be a less invasive method compared to the lumbar puncture, more affordable compared to the MRI, generally available and reproducible. A consequence of finally having a new prognostic biomarker could be a better management of the patients adjusting even more the treatment to the needs of the patients.

4. HYPOTHESIS

The levels of CD19+ B cells in peripheral blood of patients who have just been diagnosed with Multiple Sclerosis (MS) are related to the inflammatory activity and prognosis of the disease.

5. OBJECTIVES

5.1. *Main Objective*

- Determine if the CD19+ B cells in peripheral blood of MS patients at the onset of the disease can be a biomarker for the inflammatory activity.

5.2. *Secondary Objectives*

- Analyse if there is a difference in the CD19 levels at the onset of the disease depending the main clinical forms of MS (PPMS and RRMS).

- Assess the relation between the levels of peripheral CD19 and the neurological disability of MS patients.

6. MATERIALS AND METHODS

6.1. *Study design*

The gist of the study is as follows: it will be determined the peripheral blood percentage (%) of CD19+ B cells at the onset of the disease along with a follow up period of 4 years using a brain MRI and a neurological disability assessment using the Kurtzke EDSS score. CD19+ levels will be then compared with the presence or absence of Gd enhancing lesions; new or enlarged T2 lesions; OCGB index and OCMB in CSF of the basal lumbar puncture, as they give information about the inflammatory activity of the disease. Plus, these CD19+ levels will be also compared to the score in the EDSS to assess their clinical repercussion.

The study design that is more suitable for the consecution of the objectives is a pilot ambispective cohort study in which will participate patients from the Unitat de Neuroimmunologia i Esclerosi Múltiple (UNIEM) from Hospital Santa Caterina and Doctor Josep Trueta from Girona.

6.2. *Population of study*

6.2.1. Definition of the population

The population of the study will be patients diagnosed with MS using the revised McDonald 2010 criteria (See ANNEX II) from Girona who have been diagnosed in the UNIEM of Doctor Josep Trueta and Santa Caterina hospitals from Girona and who are already involved in the BioEM study that is being carried out in the Unit.

6.2.2. Inclusion and exclusion criteria

INCLUSION CRITERIA
✓ Patients diagnosed with MS following the revised 2010 McDonald criteria
✓ Individuals between the ages of 18 and 55
✓ Individuals capable to cooperate and agree with their participation in the study by signing the consent form
EXCLUSION CRITERIA
× Patients who had received any DMT treatment in the previous year or were receiving any treatment with DMT in the moment of the diagnosis.
× Patients who had received immunosuppressive treatment in the previous year or were then receiving immunosuppressive treatment in the moment of the blood collection.
× Patients who were immunocompromised.
× Patients with a selective CD19+ B cells immunodeficiency.
× Patients with a selective CD20+ B cells immunodeficiency.

- Patients with haematological disorders.
- Patients who were undergoing an infectious process.
- Pregnant and breastfeeding patients.
- Patients who had suffered a relapse or had received corticoids in the previous 28 days.
- Patients who cannot be performed a MRI.

6.3. *Sample and sampling method*

For this study, it cannot be calculated the sample size as there is a gap of knowledge in this subject which make it not possible to establish reliable parameters. Therefore, this protocol has been proposed as a pilot study.

It will be used a cohort of approximately 200 patients that is already participating in the BioEM study carried out in the UNIEM. These patients have their samples already available in the Biobanc for their analysis, provided that they had signed the consent form that allows the use of their samples for other research programs (See ANNEX V).

6.4. *Variables & Measurement methods:*

6.4.1. Independent variable:

- Variable A: **Levels of CD19+ B cells in peripheral blood**

In this study CD19 levels will be expressed as a quantitative continue variable expressed in percentages (%) to assess whether there is a relation between the levels of CD19 and the dependent variables or not.

To obtain the number of CD19 in peripheral blood it will be performed an immunophenotyping of the same. The processing of the samples will follow the protocols of the responsible laboratory, in this case are from the IDIBGI. The protocol describes the processes for the obtainment of CD19+ in 2 steps. The first one is a PBMC (peripheral blood mononuclear cells) purification:

1) 3 CPT tubes (sodium citrate 8mL) will be centrifuged at 1800g during a period of 30 minutes at 19ºC. Then they are inverted 3 times gently.
2) The supernatant (plasma and PBMC) is collected with a Pasteur pipette and transferred to a sterile Falcon of 50mL.
3) A new centrifugation is done, this time at 800g during 15 minutes at the same temperature.
4) Afterwards it will be removed the supernatant (plasma) leaving approximately 5-10mL and the pellet is resuspended.
5) Enriched saline solution is added to complete 20-25 mL

6) Again, it is centrifuged at 950g for 10 minutes and 19ºC.
7) Then it is eliminated all the supernatant and the pellet is resuspended again, this time in 1mL of complete RPMI.
8) In the next step, it takes place the recount with a haemocytometer (Neubauer's camera): to 10µL of cell solution is added 90 µL of regular saline and then 10µL of triptan blue.
9) Later, aliquots of $5x10^6$ cells in a maximum volume of 500µL (if is lower to 500µL it will be balanced with RPMI until the 500µL) mixed with 500µL of freezing medium and finally are transferred to cryovials.
10) These samples are then frozen at -80ºC for at least 24h.

After all this process, it will be performed the flow cytometric immunophenotyping using a cytometer to differentiate the lymphocyte subpopulations. But before, the samples must be prepared:

1) 200.000 to 500.000 cells are cleaned with 2mL of FACS buffer.
2) Then it is centrifuged at 800g for 5 minutes.
3) Then it is decanted the supernatant.
4) It is blocked with final 10% of serum from the animal from where it is being produced the Ab for 20 minutes at 4ºC. The correspondent quantity of Ab is added.
5) Later, in a dark environment, it is incubated for 30 minutes at 4ºC.
6) Then it is clean with 2mL of FACS buffer.
7) It is centrifuged again at 800g for 5 minutes.
8) Afterwards, the pellet is decanted and resuspended in a final volume of 150µL, it is marked with FITC.
9) Lastly is transferred to an Accuri C6 of BD Bioscience cytometer for the determination of the subpopulation of the selected B cells.

6.4.2. Dependent variables. Variables B, C and D.

- Variable B: **Inflammatory activity**:

  Inflammatory activity is determined in two different ways:

  - **Brain MRI findings**: it will be determined if there are new lesions or enlarged previous lesions in T2 sequences and/or Gd-enhancing lesions per T1-weighted MRI scan, surrogate markers of inflammatory activity. It will be presented as a dichotomous variable according the presence or absence of new or enhancing lesions (if determine with Gd) or if there are or not new or enlarged lesions in T2 sequences.

- OCB IN CSF:

IgG index: As has been said throughout the paper, the presence of OCGB in CSF is a validated biomarker for the diagnosis and prognosis of MS as their absence makes unlikely the MS diagnosis as well as their presence is related with a higher risk of developing CDMS in CIS patients. The presence of OCGB is a sign of inflammation of the SNC so it will be used to compared the peripheral compartment to the CSF compartment.
It will be considered an index higher than 0,7 an indicator of inflammation. This variable will be considered a dichotomous variable according the presence of an index higher than 0,7 or not.

IgM OCB: As well as the OCGB, OCMB can be used to predict the aggressiveness of the disease and the presence of these bands in the CSF is related to clinical forms of worse prognosis. As well as the IgG index, this variable will be expressed as dichotomous according its presence or absence in the CSF.

- Variable C: **Neurological disability** (See ANNEX III):

  The neurological disability progression will be assessed using the Kurtzke **EDSS score** (See ANNEX III). It ranges from scores between 0 to 10, where 0 refers to a normal neurologic exam and 10 refers to death due to MS. In this study, will be analysed in both ways, using the numeric score (1 to 10) as a quantitative discrete variable and the presence or absence of progression as a dichotomous variable. EDSS progression is defined as increase in ≥1.0 on EDSS from a baseline score of ≥1.0 or an increase in ≥1.5 from baseline score of 0 for more than one year (55).

- Variable D: **Clinical forms of MS**: There will be presented the two main presentations of the disease, these are the PPMS and the RRMS. They are considered categorical variables.

6.4.3. Covariates

These variables must be considered as they have proven to have a part in the pathogenesis of MS as they may vary the course of the disease and, also, are prognostic factors. So, they can alter the outcome of the study acting as a confounder variable. Besides, these other variables must be contemplated as they can better define the population of the study and would make possible a more detailed analysis. The covariates have been selected according literature review of similar investigations. Some of these variables will be obtained from the clinical history of the patients.

- **Gender**: It has been demonstrated that women have a better prognosis than men as far as disability is concerned. It will be considered as a qualitative dichotomous variable.

- **Age at onset**: although in short term it has a better prognosis, infantile-MS ends up having a worse prognosis as these patients acquire a higher EDSS score earlier compared to those diagnosed during adulthood. It will be expressed as a quantitative discrete variable measured in years.
- **Ethnicity**: As MS has a latitude gradient in prevalence and incidence, not every ethnicity has the same risk of MS. They will be presented in 5 categories as follow: Caucasian, Asian, Black African, Arabs and Others. They will be presented as qualitative nominal
- **Diet** (vitamin D levels): Vitamin D levels may affect the course of the disease and it is necessary its consideration too as low levels can have a part in the natural history of MS. It will be presented as a quantitative continue. Its measurement will be included with a routine blood test.
- **Smoking**: itself it's a risk factor for the development of the disease and confers the disease a worse prognosis too. It will be presented as a dichotomous variable after asking the patient whether they smoke or not.

6.5. *Follow-up plan*

In this project, as it was previously said, a cohort of patients that comes from the BioEM study will be used. This study started back in the 2011 and blood samples, among others, of MS patients have been collected since. As currently are available laboratory techniques (PBMC and flow cytometry) to analyse cryogenised samples, we can study retrospectively the CD19+ levels. Besides, as routine follow-up of MS patients, it is performed annually a brain MRI and neurological assessment. Thus, we can assess the evolution of the disease through time in these patients using their clinical history and radiology reports.

On the other hand, as MS is such a heterogeneous disease its evolution cannot be accurately predicted and, reviewing articles that carried out similar studies, it has been proposed a minimum follow-up period of 4 years to evaluate the relation between the initial CD19 levels and the outcome of the disease.

Considering the above said, we will have patients with a follow up time of six years and patients who have only a follow-up of months. And to recruit the cohort of almost 200 patients from the BioEM study and to not lose any patient, it will be needed to continue the follow-up at least 4 more years from now. This is the reason why this is an ambispective study, because data from 6 years ago will be used along with the data from the next 4 years.

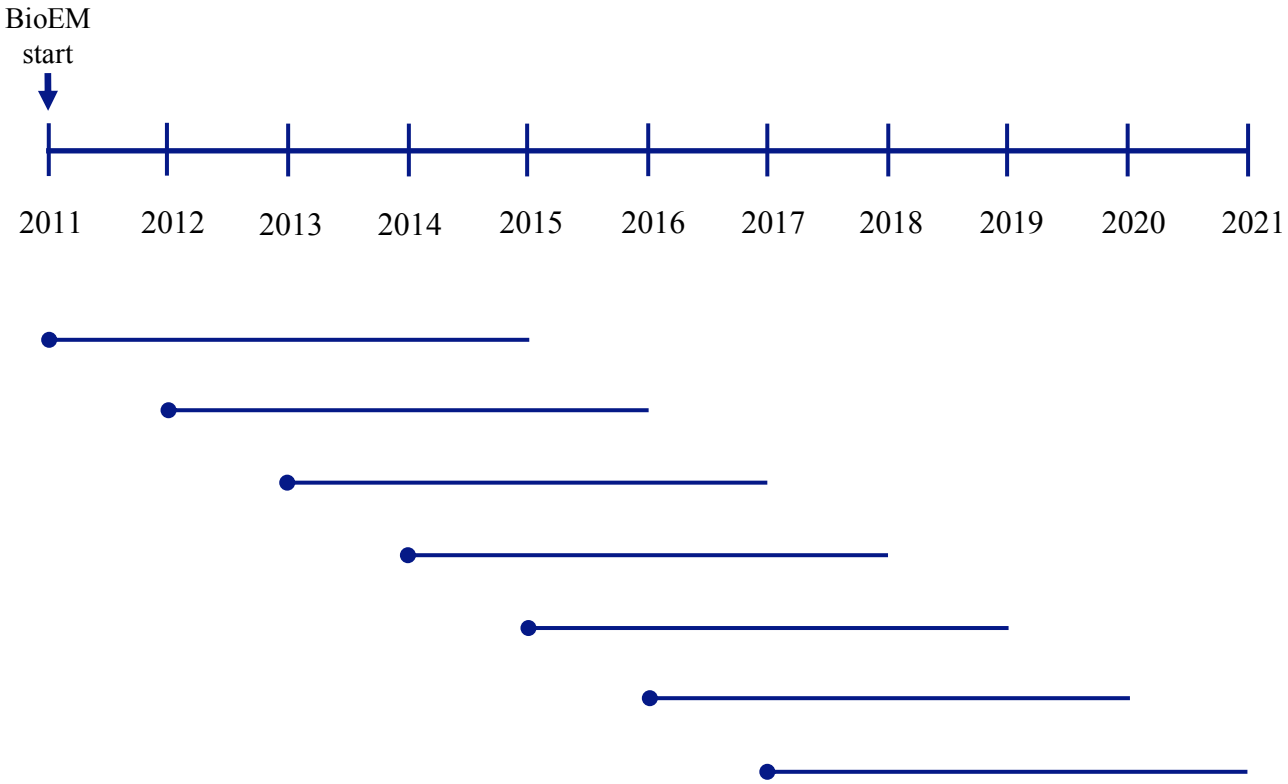


Fig. 9 Chart of the minimum follow-up time depending on the year of diagnosis.

7. STATISTICAL ANALYSIS

There will be 3 different levels of data analysis after all the collecting process:

UNIVARIATE DESCRIPTIVE ANALYSIS:

The categorical variables will be expressed as percentages (%) and proportions and will be represented with bar charts. Quantitative variables will be described as mean ± standard deviation (SD) if they follow a normal distribution and with median and interquartile range (25-75) if they do not follow a normal distribution and represented in box-plots.

BIVARIATE ANALYSIS

In this bivariate analysis, it will be compared the independent variable with the dependent ones. Dichotomous categorical variables will be compared in a contingency table and evaluated using the chi-square, χ^2, test or Fisher's exact test.

To evaluate CD19 levels between different clinical forms, the student-t test for independent data will be used if there is a normal distribution and the Mann-Whitney test if not.

Non-parametric Spearman's correlation coefficient will be used to assess the possible correlation between the B cells expressing CD19 levels and the EDSS.

To evaluate the discriminatory capacity of the biomarker it will be constructed a ROC curve. It will determine the optimum cut-off point.

MULTIVARIATE ANALYSIS

To evaluate the association between EDSS and CD19 levels, adjusted by potential confounders such as the covariates described above, it will be performed a multiple linear regression model.

All tests above mentioned, will be two-sided and p values <0.05 will be considered significant and $p<0.001$ will be considered highly significant. All the statistical analysis of the variables will be performed using the Statistical Package for the Social Sciences programme (SPSS) 19.0. And the collected information will be saved in an Excel document.

8. ETHICAL CONSIDERATIONS

The research protocol will be presented and submitted for consideration and approval by the Clinical Research Ethical Committee (CEIC, "Comitè Ètic d'Investigació Clínica") of the Institut d'Assistència Sanitària de Girona (IAS)– "Hospital Universitari Dr Josep Trueta" before the study begins.

This protocol will be conducted in accordance with the ethical principles established by the World Medical Association in the Declaration of Helsinki of Ethical Principles for Medical Research Involving Human Subjects (last updated in the 64th WMA General Assembly, Brazil in October 2013).

As in this study biological samples are used, it will be respected the "*Ley Orgánica 14/2007 de Investigación Biomédica*". Also, the "*Real Decreto 1716/2011*" must be respected, which establishes the basic requisites for the authorization and functioning of the biobanks for biomedical research purposes and the management of biological samples of human origin and regulate the functioning and organization of the "*Registro Nacional de Biobancos para Investigación Biomédica*".

The project will follow the "*Ley Orgánica 15/1999, de 13 de diciembre, de Protección de Datos de Carácter Personal*", all patient data obtained during the study will be confidential and only used with the purpose of the research and the anonymity of the patients will be guaranteed. To respect and guarantee the confidentiality of the patients, the investigators do not have access to individual confidential data, the patients will be codified on the database to maintain their anonymity and the data will be analysed anonymously. Patients will always be allowed to modify or destroy any of their collected data.

Also, it will follow the "*Ley 41/2002 Básica Reguladora de la Autonomía del Paciente y de Derechos y Obligaciones en materia de información y documentación clínica*" in which it is collected that all patient interested on being part of the study and who meet the criteria to participate in it, will be asked to sign voluntarily the informed consent. Before being included, they will receive all the appropriate information about the study through a personal conversation with the research stuff and the use of the information sheet.

No conflicts of interest with any part or organ is related to this study.

9. STUDY LIMITATIONS

Some limitations can be found in this study:

- This is a pilot study and therefore it will be used a sample of patients from a concrete area (Girona) that may be not representative of the population of patients of MS and the results may not be extrapolated to them.

- More disadvantages derive from the study design. Characteristically cohorts' studies require years to be performed. A consequence of this is the possible loss of patients during the project, either by death, geographical reasons or lack of attendance provoking follow-up biases. To avoid the withdrawal of patients, one phone call will be made to each patient the week before the appointment. Also, the investigator will ensure the motivation of the patients asking about their thoughts on the project and if they would change something to improve their adherence to it.

- Another drawback of the study is that sample handling and analysis process may influence the results leading to an information bias. Because of that, comparisons between different biomarkers measurements are at risk of being inaccurate.

- Related to the previous limitation and related too to the retrospective aspect of the study, as it couldn't have been controlled the moment of the day of the sample collection, there may exist differences in the results of the levels of CD19 between samples that otherwise wouldn't have existed, as it has been demonstrated that the levels of lymphocytes can vary depending on the time of the extraction (40,56).

- MS, as previously mentioned, has a heterogeneous pathophysiology which still must be clarified and there may exist several factors yet unknown that may affect the results of this study acting as confounders factors.

10. CLINICAL AND HEALTH SYSTEM IMPACT

The information obtained from this study will provide valuable information to improve the knowledge about multiple sclerosis. It will be further proof that in the pathophysiology of the disease, B cells are involved and it can lead a new line of research for the cause, prognosis and treatment of the disease.

If the hypothesis is confirmed it will be settled the determination of CD19+ as a potential biomarker for the prognosis of the disease. With this knowledge, once the patient comes to the visit and the physician gives the diagnosis of the disease, they would have a tool to give the patient a prognosis of the course of the disease with less uncertainty than it was previously done.

Besides, the outcomes of this study might have important implications regarding clinical practice: Neurologists may choose one therapy that would better fit the patient needs according the results of their CD19+ B cells levels. Depending on whether a more aggressive or indolent course is expected, a better adjustment in the treatment can reduce the burden of the disease and doing this, it can be prevented the neurological disability progression measured with the EDSS score. Different dimensions of the disease would benefit from this: first, the patient would have a better quality of life. Second, the national health system can save resources and money as it has been demonstrated that as the EDSS score is increased, the amount of money required per patient increases greatly too. A review that analysed the costs of the disease in Spain has settle that the total mean annual cost per patient is 33.456€. This amount can vary depending on the EDSS score of the patient, for example, an EDSS score below 4 has a cost of 1.803€ per patient, this quantity rises to 19.833€ when the patient has a EDSS score between 4-7 and above that the cost rises to 38.224€ (57).

So, as it can be seen, if we have a tool that allow the physicians a better therapy election, we may have a better control of the disease and it leads to a reduced burden of disease and less disability, saving money to the National Health System and improving the patients' quality of life.

11. FEASIBILITY

11.1. *Research team:*

The team will be formed by:

- The principal investigator (PI) will be a neurologist from the Santa Caterina Hospital who will coordinate the entire project; participate in the follow-up of the patients; interpret the statistical analysis; write the final paper and present the results.
- Neurologists (Nrl) from the Neuroimmunology and Multiple Sclerosis Unit who will follow up the patients, perform the pertinent lumbar punctures, make the neurological disability assessment according the Kurtzke EDSS score, interpret the results of the clinical and paraclinical tests plus all the routine follow up of these patients.
- Neuroradiologists (Rx) from the hospital who will perform the neural axis MRI and its interpretation and their report.
- Laboratory staff (Lab) who will pick the samples from the Biobanc and will carry out the immunophenotyping of the B cells in the peripheral blood to calculate the % of CD19 in every patient.
- A statistician (Sta) who will make the statistical analysis of the results obtained.

11.2. *Work plan*

The study will collect data from back 2011 and will continue until 2021 for the complete follow-up of the cohort of patients. As we can obtain the data retrospectively, we can run a 10-year study in half the time. It will follow the next stages:

Stage 0: **Literature review and protocol design**: This stage consists on literature review, final protocol design and proposal of the same to the Comitè Ètic d'Investigació Clínica (CEIC) for its evaluation and acceptance. This stage is preview to last 4 months, but it will depend on the time the CEIC takes to approve the study.

Stage 1: **Preparation and coordination stage**: In this stage, organization and informative meetings will be held between the main investigators and the rest of the research team. In here every detail of the project will be explained. At the end of the meeting everybody must know their implication in the study and the tasks to which they are in charge of. Any doubt any member of the staff might have about the project should be cleared in this moment.
Besides the coordination of the personnel, it will be created a joint database for the compilation of the clinical, analytical and radiological information that will be later obtained.
This stage will last 1 month.

Stage 2: **Data collection:** During this phase, it will be collected the clinical, analytical and radiological information of every patient from their clinical histories and reports and then it will be entered in a database specifically created for this study. This stage will last 4 months.

Stage 3: **Sample selection and processing:** Laboratory staff will choose the samples that meet the criteria and the will proceed to their analysis. This process will last 8 months and it will be simultaneous to the data recovery.

Stage 4: **Follow-up and compilation of the new information**: The follow up of the patients that required it will continue until the year 2021, when all the patients will have had a follow-up period of the minimum 4 years required.
Annually, as routine clinical practice, a MRI will be performed along with a neurological disability assessment. The new data, besides being saved in the clinical course of the patient, it will be saved in the database of the study. The follow-up will be done at the same time as the sample processing.

Stage 5: **Data analysis and interpretation of the results and article elaboration**: In a first step the statistician will analysed the given data. Then he will hand the results to the investigators for their interpretation. From these results, the principal investigator will draft a conclusion and write the final article. This stage will take 4 months.

Stage 6: **Publication and dissemination of results**: The main investigator will present the study's results in a prestigious neurology publication. The final article will be sent, with the intent of acceptance and publication. The results will be also presented in nationals and internationals congresses of the specialty. The final stage will last up to 2 months.

11.3. *Chronogram of the study:* See ANNEX VII

The starting date in the chronogram is suggested to be the first of March of 2017. Any adjustment of the beginning of the project can be made following the schedule previously defined whilst its length is respected.

12. BUDGET

Materials, visits and procedures that are included in the routine clinical practice will not be contemplated in the budget of this project.

BUDGET ESTIMATED FOR SAMPLE PROCESSING			
Description	**Cost per unit**	**# of patients**	**Total**
PBMC	15 €	200	3000 €
Flow Cytometry analysis	30 €	200	6000 €
			TOTAL: 9.000€

BUDGET ESTIMATED FOR STAFF COSTS:		
Description	**Estimated time**	**Total**
Laboratory staff	25€/h x 600h	15.000€
Statistical analysis	30€/h x 3h/day; 2 days/week x 4 weeks	720€
		TOTAL: 15.720 €

TOTAL STUDY COST:	
Description	**Total**
Sample processing	9.000 €
Staff cost	15.720 €
Article scientific revision and publication	1.500 €
MS national and international meetings	2.000 €
	TOTAL COST OF THE STUDY: 28.220 €

13. BIBLIOGRAPHY

1. Milo R, Miller A. Revised diagnostic criteria of multiple sclerosis. Autoimmun Rev. 2014;13(4–5):518–24.

2. Evans C, Beland SG, Kulaga S, Wolfson C, Kingwell E, Marriott J, et al. Incidence and prevalence of multiple sclerosis in Europe: A systematic review. Neuroepidemiology [Internet]. 2013;40(3):195–210. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3856596/pdf/1471-2377-13-128.pdf

3. Alonso A, Hernán MA. Temporal trends in the incidence of multiple sclerosis: A systematic review. Neurology [Internet]. 2008; Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4109189/pdf/129.pdf

4. Zarranz JJ. Neurología. 5th ed. Barcelona: Elservier; 2013. 451-462 p.

5. Zuvich R, Mccauley J, Pericak-Vance M, Haines J. Genetics and Pathogenesis of Multiple Sclerosis. Natl Institutes Heal [Internet]. 2009;21(6):328–33. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2789848/pdf/nihms141392.pdf

6. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: The role of infection. Ann Neurol. 2007;61(4):288–99.

7. Benito-León J. Are the prevalence and incidence of multiple sclerosis changing? Neuroepidemiology [Internet]. 2011;36(3):148–9. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3095836/pdf/ned0036-0148.pdf

8. Multiple Sclerosis International Federation. Atlas of MS 2013: Mapping Multiple Sclerosis Around the World. Mult Scler Int Fed [Internet]. 2013;1–28. Available from: https://www.msif.org/wp-content/uploads/2014/09/Atlas-of-MS.pdf

9. Kurtzke JF. Multiple sclerosis in time and space ± geographic clues to cause. Available from: https://pdfs.semanticscholar.org/d522/df170c2f692afa82c79794e82c18399dad2a.pdf

10. Compston A, Coles A. Multiple Sclerosis. 2010;48(2):1–9.

11. McAlpine D, Compston A. McAlpine's Multiple Sclerosis. 4th ed. Churchill Livingstone Elsevier; 2005.

12. Álvarez-Cermeño JC, Arroyo González R, Casanova Estruch V, Comabella López M, García Merino JA, Hernández Pérez MÁ, et al. Guía Oficial de Práctica Clínica en Esclerosis Múltiple. 2014;11–95.

13. Ewing C, Bernard CCA. Insights into the aetiology and pathogenesis of multiple sclerosis. Immunol Cell Biol [Internet]. 1998;76:47–54. Available from: http://www.nature.com/icb/journal/v76/n1/pdf/icb19986a.pdf

14. Ramagopalan S V., Dobson R, Meier UC, Giovannoni G. Multiple sclerosis: risk factors, prodromes, and potential causal pathways. Lancet Neurol. 2010;9:727–39.

15. Hafler DA, Compston A, Sawcer S, Lander ES, Daly MJ, De Jager PL, et al. Risk Alleles for Multiple Sclerosis Identified by a Genomewide Study. n engl j med [Internet]. 2007;3579357(9):851–62. Available from: http://www.nejm.org/doi/pdf/10.1056/NEJMoa073493

16. Pender MP. The Essential Role of Epstein-Barr Virus in the Pathogenesis of Multiple Sclerosis. Hypothesis Neurosci. 2011;17(4):351–67.

17. Mouhieddine TH, Darwish H, Fawaz L, Yamout B, Tamim H, Khoury SJ. Risk factors for multiple sclerosis and associations with anti-EBV antibody titers. Clin Immunol. 2015;158:59–66.

18. Pierrot-Deseilligny C, Souberbielle J-C, To C. Is hypovitaminosis D one of the environmental risk factors for multiple sclerosis? Brain [Internet]. 133:1869–88. Available from: http://www.medscape.com/viewarticle/725412

19. Dutta R, Trapp BD. Pathogenesis of axonal and neuronal damage in multiple sclerosis. Neurology. 2007;68:22–31.

20. Ciccarelli O, Barkhof F, Bodini B, Stefano N De, Golay X, Nicolay K, et al. Pathogenesis of multiple sclerosis: Insights from molecular and metabolic imaging. Lancet Neurol. 2014;13:807–22.

21. Loma I, Heyman R. Multiple Sclerosis: Pathogenesis and Treatment. Curr Neuropharmacol [Internet]. 2011;9:409–16. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3151595/pdf/CN-9-409.pdf

22. Robles Cedeño R. Identificación de genes de susceptibilidad en esclerosis múltiple. Descripción clínica y análisis genético de una extensa familia de etnia gitana (Estudio EMGypsy). Universitat de Girona; 2016.

23. Peterson LK, Fujinami RS. Inflammation, Demyelination, Neurodegeneration and Neuroprotection in the Pathogenesis of Multiple Sclerosis. Natl Institutes Heal. 2007;184(1–2):37–44.

24. Correale J, Gaitán MI, Ysrraelit MC, Fiol MP. Progressive multiple sclerosis: from pathogenic mechanisms to treatment. Brain. 2016;1–20.

25. Piancone F, Saresella M, Marventano I, Rosa F La, Zoppis M, Agostini S, et al. B Lymphocytes in Multiple Sclerosis: Bregs and BTLA/CD272 Expressing-CD19+ Lymphocytes Modulate Disease Severity. Nature. 2016;1–11.

26. Disanto G, Morahan JM, Barnett MH, Giovannoni G, Ramagopalan S V. The evidence for a role of B cells in multiple sclerosis. Neurology [Internet]. 2012;78(11):823–32. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3304944/pdf/znl823.pdf

27. Moreno Torres I, García-Merino A. Anti-CD20 monoclonal antibodies in multiple sclerosis. Expert Rev Neurother [Internet]. 2016;7175(October 2016):1–13. Available from: https://www.tandfonline.com/doi/full/10.1080/14737175.2017.1245616

28. Habib J, Deng J, Lava N, Tyor W, Galipeau J. Blood B cell and regulatory subset content in multiple sclerosis patients. J Mult Scler (Foster City) [Internet]. 2015;2(2):1–12. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4484600/pdf/nihms-699201.pdf

29. Krumbholz M, Derfuss T, Hohlfeld R, Meinl E. B cells and antibodies in multiple sclerosis pathogenesis and therapy. Nat Publ Gr. 2012;8(11):613–23.

30. Kinzel S, Weber MS. B Cell-Directed Therapeutics in Multiple Sclerosis: Rationale and Clinical Evidence. CNS Drugs. 2016;

31. Confavreux C, Vukusic S. Natural history of multiple sclerosis: A unifying concept. Brain [Internet]. 2006; Available from: http://brain.oxfordjournals.org/content/brain/129/3/606.full.pdf

32. Lublin FD, Reingold SC. Defining the clinical course of multiple sclerosis. Neurology. 1996;46:907–11.

33. Lublin FD, Reingold SC, Cohen JA, Cutter GR, Sørensen PS, Thompson AJ, et al. Defining the clinical course of multiple sclerosis: The 2013 revisions. Neurology [Internet]. 2014;83:278–86. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4117366/pdf/NEUROLOGY2013555623.pdf

34. Okuda DT, Mowry EM, Beheshtian A, Waubant E, Baranzini SE, Goodin DS, et al. Incidental MRI anomalies suggestive of multiple sclerosis: The radiologically isolated syndrome. Neurology. 2009;72:800–5.

35. Miller D, Barkhof F, Montalban X, Thompson A, Filippi M. Clinically isolated syndromes suggestive of multiple sclerosis, part I: Natural history, pathogenesis, diagnosis, and prognosis. Lancet Neurol. 2005;4(5):281–8.

36. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 Revisions to the McDonald criteria. Ann Neurol [Internet]. 2011;69(2):292–302. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3084507/pdf/ana0069-0292.pdf

37. Freedman MS, Thompson EJ, Deisenhammer F, Giovannoni G, Grimsley G, Keir G, et al. Recommended Standard of Cerebrospinal Fluid Analysis in the Diagnosis of Multiple Sclerosis A Consensus Statement. Available from: http://jamanetwork.com/journals/jamaneurology/fullarticle/788592

38. Villar LM, Masjuan J, Sadaba MC, Gonzalez-Porque P, Plaza J, Bootello A, et al. Early differential diagnosis of multiple sclerosis using a new oligoclonal band test. Arch Neurol [Internet]. 2005;62(4):574–7. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15824255

39. D'Ambrosio A, Pontecorvo S, Colasanti T, Zamboni S, Francia A, Margutti P. Peripheral blood biomarkers in multiple sclerosis. Autoimmun Rev. 2015;

40. Katsavos S, Anagnostouli M. Biomarkers in Multiple Sclerosis: An Up-to-Date Overview. Mult Scler Int. 2013;340508(20).

41. Villar LM, Sádaba MC, Roldán E, Masjuan J, González-Porqué P, Villarrubia N, et al. Intrathecal synthesis of oligoclonal IgM against myelin lipids predicts an aggressive disease course in MS. J Clin Invest [Internet]. 2005;115(1):187–94. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC539201/pdf/JCI0422833.pdf

42. Villar LM, Casanova B, Ouamara N, Comabella M, Jalili F, Leppert D, et al. Immunoglobulin M oligoclonal bands: Biomarker of targetable inflammation in primary progressive multiple sclerosis. Ann Neurol. 2014;76(2):231–40.

43. Rovira A, Tintoré M, Álvarez-Cermeño JC, Izquierdo G, Prieto JM. Recommendations for using and interpreting magnetic resonance imaging in multiple sclerosis. Neurología. 2010;25(4):248–65.

44. Haider L, Zrzavy T, Hametner S, Höftberger R, Bagnato F, Grabner G, et al. The topograpy of demyelination and neurodegeneration in the multiple sclerosis brain. Brain. 2016;139(3):807–15.

45. Bot JC, Barkhof F. Spinal-Cord MRI in Multiple Sclerosis: Conventional and Nonconventional MR Techniques. Neuroimaging Clin N Am. 2009;19(1):81–99.

46. Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. N Engl J Med. 2000;343(13):938–52.

47. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: An expanded disability status scale (EDSS). Available from: http://www.neurology.org/content/33/11/1444.full.pdf

48. García Merino A, et al. Consenso para el tratamiento de la esclerosis múltiple 2016. Neurología. 2016;1–7.

49. Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, et al. New Diagnostic Criteria for Multiple Sclerosis: Guidelines for Research Protocols. Ann Neurol. 1983;13(3):227–31.

50. Mowry EM. Natural History of Multiple Sclerosis: Early Prognostic Factors. Neurol Clin. 2011;29:279–92.

51. Tremlett H, Zhao Y, Rieckmann P, Hutchinson M. New perspectives in the natural history of multiple sclerosis. Neurology. 2010;74:2004–15.

52. Renoux C. Natural History of Multiple Sclerosis: Long-Term Prognostic Factors. Neurologic Clinics. 2011.

53. Scalfari A, Knappertz V, Cutter G, Goodin DS, Ashton R, Ebers GC. Mortality in patients with multiple sclerosis. Neurology [Internet]. 2013;81(2):184–92. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3770174/pdf/WNL205174.pdf

54. Housley WJ, Pitt D, Hafler DA. Biomarkers in Multiple Sclerosis. Clin Immunol. 2015;

55. Caruana P, Lemmert K, Ribbons K, Kea R, Lechner-Scott J. Natural killer cell subpopulations are associated with MRI activity in a relapsing- remitting multiple sclerosis patient cohort from Australia. Mult Scler J. 2016;1–9.

56. Wipfler P, Heikkinen A, Harrer A, Pilz G, Kunz A, Golaszewski SM, et al. Circadian rhythmicity of inflammatory serum parameters: A neglected issue in the search of biomarkers in multiple sclerosis. J Neurol. 2013;221–7.

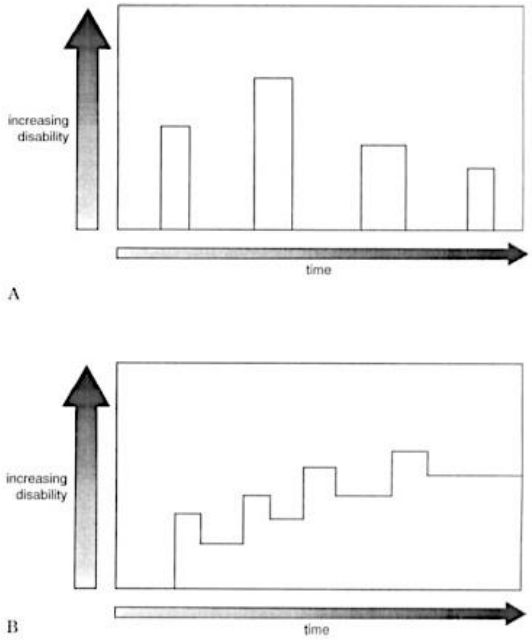
57. Kobelt G, Berg J, Lindgren P, Izquierdo G, Sánchez-Soliño O, Pérez-Miranda J, et al. Costs and quality of life of multiple sclerosis in Spain Original Papers. Eur J Heal Econ. 2006;7:65–74.

58. Oksenberg JR, Baranzini SE. Multiple sclerosis genetics - is the glass half full, or half empty? Nat Rev Neurol [Internet]. 2010;6(8):429–37. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20625377

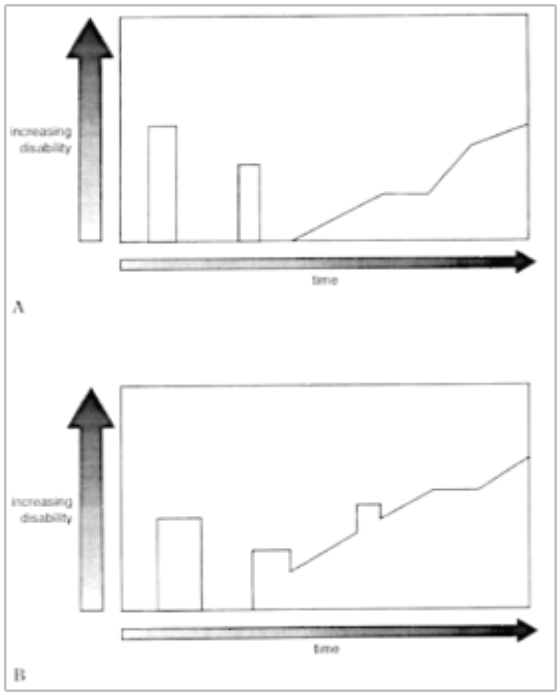
59. Friese MA, Schattling B, Fugger L. Mechanisms of neurodegeneration and axonal dysfunction in multiple sclerosis. Nat Rev Neurol [Internet]. 2014 Apr;10(4):225–38. Available from: http://www.nature.com/nrneurol/journal/v10/n4/abs/nrneurol.2014.37.html

14. ANNEXES

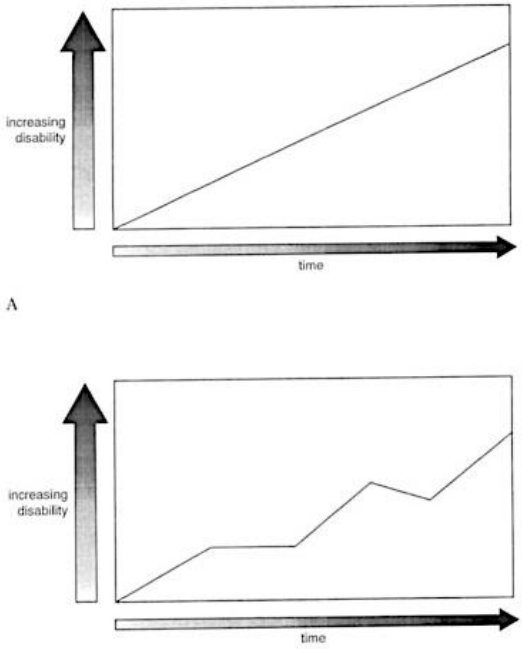
ANNEX I Clinical courses of Multiple Sclerosis. Extracted from (32).



RRMS: acute attacks with (A) full recovery or (B) with sequelae and residual deficit upon recovery



SPMS: It begins with a RRMS form followed by progression of variable rate (A) that may also include occasional relapses and minor remissions (B).



PPMS: progression of disability from onset, without plateaus or remissions (A) or with occasional plateaus and temporary minor improvements (B)

ANNEX II Revised 2010 McDonald Criteria for the diagnosis of Multiple Sclerosis (36)

Clinical Presentation	Additional Data Needed for MS Diagnosis
≥2 attacks[a]; objective clinical evidence of ≥2 lesions or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack[b]	None[c]
≥2 attacks[a]; objective clinical evidence of 1 lesion	Dissemination in space, demonstrated by: ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)[d]; or Await a further clinical attack[a] implicating a different CNS site
1 attack[a]; objective clinical evidence of ≥2 lesions	Dissemination in time, demonstrated by: Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time; or A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or Await a second clinical attack[a]
1 attack[a]; objective clinical evidence of 1 lesion (clinically isolated syndrome)	Dissemination in space and time, demonstrated by: For DIS: ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)[d]; or Await a second clinical attack[a] implicating a different CNS site; and For DIT: Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time; or A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or Await a second clinical attack[a]
Insidious neurological progression suggestive of MS (PPMS)	1 year of disease progression (retrospectively or prospectively determined) plus 2 of 3 of the following criteria[d]: 1. Evidence for DIS in the brain based on ≥1 T2 lesions in the MS-characteristic (periventricular, juxtacortical, or infratentorial) regions 2. Evidence for DIS in the spinal cord based on ≥2 T2 lesions in the cord 3. Positive CSF (isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index)

If the Criteria are fulfilled and there is no better explanation for the clinical presentation, the diagnosis is "MS"; if suspicious, but the Criteria are not completely met, the diagnosis is "possible MS"; if another diagnosis arises during the evaluation that better explains the clinical presentation, then the diagnosis is "not MS."

[a]An attack (relapse; exacerbation) is defined as patient-reported or objectively observed events typical of an acute inflammatory demyelinating event in the CNS, current or historical, with duration of at least 24 hours, in the absence of fever or infection. It should be documented by contemporaneous neurological examination, but some historical events with symptoms and evolution characteristic for MS, but for which no objective neurological findings are documented, can provide reasonable evidence of a prior demyelinating event. Reports of paroxysmal symptoms (historical or current) should, however, consist of multiple episodes occurring over not less than 24 hours. Before a definite diagnosis of MS can be made, at least 1 attack must be corroborated by findings on neurological examination, visual evoked potential response in patients reporting prior visual disturbance, or MRI consistent with demyelination in the area of the CNS implicated in the historical report of neurological symptoms.

[b]Clinical diagnosis based on objective clinical findings for 2 attacks is most secure. Reasonable historical evidence for 1 past attack, in the absence of documented objective neurological findings, can include historical events with symptoms and evolution characteristics for a prior inflammatory demyelinating event; at least 1 attack, however, must be supported by objective findings.

[c]No additional tests are required. However, it is desirable that any diagnosis of MS be made with access to imaging based on these Criteria. If imaging or other tests (for instance, CSF) are undertaken and are negative, extreme caution needs to be taken before making a diagnosis of MS, and alternative diagnoses must be considered. There must be no better explanation for the clinical presentation, and objective evidence must be present to support a diagnosis of MS.

[d]Gadolinium-enhancing lesions are not required; symptomatic lesions are excluded from consideration in subjects with brainstem or spinal cord syndromes.

MS = multiple sclerosis; CNS = central nervous system; MRI = magnetic resonance imaging; DIS = dissemination in space; DIT = dissemination in time; PPMS = primary progressive multiple sclerosis; CSF = cerebrospinal fluid; IgG = immunoglobulin G.

ANNEX III

Kurtzke Expanded Disability Status Scale (EDSS)

- ❑ 0.0 - Normal neurological exam (all grade 0 in all Functional System (FS) scores*).
- ❑ 1.0 - No disability, minimal signs in one FS* (i.e., grade 1).
- ❑ 1.5 - No disability, minimal signs in more than one FS* (more than 1 FS grade 1).
- ❑ 2.0 - Minimal disability in one FS (one FS grade 2, others 0 or 1).
- ❑ 2.5 - Minimal disability in two FS (two FS grade 2, others 0 or 1).
- ❑ 3.0 - Moderate disability in one FS (one FS grade 3, others 0 or 1) or mild disability in three or four FS (three or four FS grade 2, others 0 or 1) though fully ambulatory.
- ❑ 3.5 - Fully ambulatory but with moderate disability in one FS (one grade 3) and one or two FS grade 2; or two FS grade 3 (others 0 or 1) or five grade 2 (others 0 or 1).
- ❑ 4.0 - Fully ambulatory without aid, self-sufficient, up and about some 12 hours a day despite relatively severe disability consisting of one FS grade 4 (others 0 or 1), or combination of lesser grades exceeding limits of previous steps; able to walk without aid or rest some 500 meters.
- ❑ 4.5 - Fully ambulatory without aid, up and about much of the day, able to work a full day, may otherwise have some limitation of full activity or require minimal assistance; characterized by relatively severe disability usually consisting of one FS grade 4 (others or 1) or combinations of lesser grades exceeding limits of previous steps; able to walk without aid or rest some 300 meters.
- ❑ 5.0 - Ambulatory without aid or rest for about 200 meters; disability severe enough to impair full daily activities (e.g., to work a full day without special provisions); (Usual FS equivalents are one grade 5 alone, others 0 or 1; or combinations of lesser grades usually exceeding specifications for step 4.0).
- ❑ 5.5 - Ambulatory without aid for about 100 meters; disability severe enough to preclude full daily activities; (Usual FS equivalents are one grade 5 alone, others 0 or 1; or combination of lesser grades usually exceeding those for step 4.0).
- ❑ 6.0 - Intermittent or unilateral constant assistance (cane, crutch, brace) required to walk about 100 meters with or without resting; (Usual FS equivalents are combinations with more than two FS grade 3+).

❑ 6.5 - Constant bilateral assistance (canes, crutches, braces) required to walk about 20 meters without resting; (Usual FS equivalents are combinations with more than two FS grade 3+).

❑ 7.0 - Unable to walk beyond approximately 5 meters even with aid, essentially restricted to wheelchair; wheels self in standard wheelchair and transfers alone; up and about in wheelchair some 12 hours a day; (Usual FS equivalents are combinations with more than one FS grade 4+; very rarely pyramidal grade 5 alone).

❑ 7.5 - Unable to take more than a few steps; restricted to wheelchair; may need aid in transfer; wheels self but cannot carry on in standard wheelchair a full day; May require motorized wheelchair; (Usual FS equivalents are combinations with more than one FS grade 4+).

❑ 8.0 - Essentially restricted to bed or chair or perambulated in wheelchair, but may be out of bed itself much of the day; retains many self-care functions; generally has effective use of arms; (Usual FS equivalents are combinations, generally grade 4+ in several systems).

❑ 8.5 - Essentially restricted to bed much of day; has some effective use of arm(s); retains some self-care functions; (Usual FS equivalents are combinations, generally 4+ in several systems).

❑ 9.0 - Helpless bed patient; can communicate and eat; (Usual FS equivalents are combinations, mostly grade 4+).

❑ 9.5 - Totally helpless bed patient; unable to communicate effectively or eat/swallow; (Usual FS equivalents are combinations, almost all grade 4+).

❑ 10.0 - Death due to MS.

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*Excludes cerebral function grade 1.

Note 1: EDSS steps 1.0 to 4.5 refer to patients who are fully ambulatory and the precise step number is defined by the Functional System score(s). EDSS steps 5.0 to 9.5 are defined by the impairment to ambulation and usual equivalents in Functional Systems scores are provided.

Note 2: EDSS should not change by 1.0 step unless there is a change in the same direction of at least one step in at least one FS.

ANNEX IV Biobanc storage information sheet and consent form

*Catalan and Spanish versions would be available if requested.

USE OF BIOLOGICAL SAMPLES AND CLINICAL DATA FOR RESEARCH PROJECTS AND COLLECTION IN THE BIOBANC

In the University Hospital of Girona Dr Josep Trueta (HUGJT) and other adscript hospitals, besides the patient care, it is carry out biomedical research. The aim of this investigation is to progress in the knowledge of diseases and their prevention, diagnosis and treatment. This biomedical research requires collecting clinical data and biological samples from patients and healthy donors to analyse and obtain conclusions with the purpose of acquiring better knowledge of the diseases and progress toward their diagnosis or treatment.
The samples and clinical data obtained for the diagnosis or control of the diseases, once used for this objective, are useful and necessaries for the research too. In fact, many scientific advances recently acquire in medicine come from this type of studies.

We ask for your authorization for the cession of the biological samples and the clinical information associated to continue with the biomedical research, once this project has finished.

According to the "*Ley 14/2007 de Investigación Biomédica," "Ley Orgánica 15/1999 de Protección de Datos Personales*" and their development rules, we demand you to read carefully this information sheet and its consent form for your sign, if you agree in participating in this proposal.

AIM OF THE RESEARCH: progress in the knowledge of the diseases

The aim of the research is to increase our knowledge of the diseases. The samples, clinical and analytical data and image tests will be used for biomedical research. All of this allows the progression in the expertise of the prevention, diagnosis, prognosis and treatment of the diseases.

PARTICULAR CONSIDERATIONS OF THE PROJECT:

The *Unitat de Neuroimmunologia i Esclerosi Multiple* (UNIEM), coordinated by Dr. Lluís Ramió i Torrentá is composed by health professionals, teachers and research specialists, it is dedicated to a global and entire care of patients who suffer immunological diseases with the involvement of the central nervous system. Besides the day by day care work, it also focuses their efforts in the biomedical research related to this type of pathologies.
Multiple sclerosis is a complex autoimmune disease of unknown cause, in which environmental and genetic factors are involved in its development. Little is known in the moment of the diagnose, the clinical course that the disease will acquire in every patient. That is why the medical community, and the general society, have interest in the improvement of the diagnosis, prognosis, follow-up and treatment of these patients.

<u>BIOLOGICAL SAMPLES AND ASSOCIATED INFORMATION</u>: once finalised this research study they will be stored and conserved in the IDIBGI Biobanc till their extinction.

You can decide, if once the project comes to an end, the clinical data collected and the spare biological samples from this project are guarded and conserved in the IDIBGI Biobanc (bank of biological samples), until their extinction.
This Biobanc is a non-profit institution inscribed to the "*Registro Nacional de Biobancos*" dependent of the "*Instituto de Salud Carlos III*" with the reference B.0000872, that hosts the collections organised by the biological samples and associated information in the conditions and guaranties of security demanded the before mentioned legislation and the behavioural codes approved by the Ethic Committees.
The mentioned samples and their information are available for those investigators that solicit them to the Biobanc.
Any research study in which it is requested the use of these data or samples must get the approval of the "*Comité d'Ética de la Investigació Clínica*" (CEIC), that will ensure the highest ethical and legal standards of the studies. Moreover, the scientific committee of the Biobanc will guarantee that the projects are of scientific excellence. The biomedical investigation is now a global phenomenon, so that occasionally these samples may be given to research groups from outside the nation, as long as they meet the requisites of the Spanish legislation and the corresponding approves it.
In case that there is the need of extra sample, the health institution will contact you to ask for a new collaboration. In this case, you will be informed of the motives and your consent will be demanded.

<u>DATA PROTECTION AND CONFIDENTIALITY:</u> the samples are stored coded.

The personal data collected will be obtained, manage and stored fulfilling in every moment the right of secret, according the current legislation of personal information.
The identification of the biological samples of the Biobanc will undergo a codification process. To every sample it will be assigned an identification code that will be used by the investigators. Only the authorised personnel of the Biobanc and the personnel authorised by Dr. Lluís Ramió i Torrentá are allowed to relate your identity with the mentioned codes. Through this process the investigators that apply for the samples to the Biobanc will not know any of the data that may reveal your identity. In the same way, although the obtained results of the research that uses your samples is published in scientific journals, your identity will not be given. In these studies, in which the results do not contemplate potentially useful results for your health, and according the corresponding Ethics Committee, the samples and data can be anonymized, this is, there will be no opportunity to associate your sample to your identity.
Your samples and clinical data associated will be part of the Biobanc's archive, inscribed to the "*Agencia de Protecció de Dades*" under the responsibility of the "*Institut d'Investigació Biomédica de Girona*" (IDIBGI).

You can exercise your rights of access, revoke, cancelation and objection, as well as obtain information about the use of your samples and associated data, heading to:

BIOBANC ADDRESS
Avinguda de França s/n Hospital Universitary de Girona Dr Josep Trueta
17007 Girona
Biobanc@IDIBGI.org Tlf: 972 940 282

ALTRUIST CARACTER OF THE DONATION: The cession of your biological samples to the IDIBGI Biobanc is free.

The donation has an altruistic character; therefore, you will not obtain neither in the present or in the future any economic benefit from it, and will not have any right over any possible commercial benefit from de discoveries that may be achieved as a result of the biomedical research.

VOLUNTARY PARTICIPATION: your refusal will NOT have any impact in your current or future health care.

Your participation is completely voluntary. If you sign the consent form, you will confirm that you want to participate. You can deny your participation or retire your consent in any posterior moment from the sign without having to give any reason and it will not have any repercussion in your health care, current or future.

ASSOCIATED COSTS AND RISKS: your donation will not cost you any money.

The collection of the sample will not cost you any money to you. Any procedure will be performed exclusively to obtain the samples for the research without your explicit consent.

CONSENT REVOKING: if you decide to sign this consent you can also cancel it freely. This will lead to the destruction of your samples.

If in the future you desire to nullify your consent, the biological samples will be destroyed and their associated data will be retired from the Biobanc. You can also request the anonymization of the samples, so this way the relation between the samples and your identity will be eliminated. The consequences of this cancelation or anonymization wouldn't be extended to the research that it is being done. If you want the cancelation of the consent, you must request it in writing to the IDIBGI Biobanc Address previously mentioned.

<u>INFORMATION ABOUT THE RESULTS OF THE RESEARCH</u>: you will be provided information if you claim it

In case you expressly request it, the Biobanc can give information about who are the investigations in which your samples are being used and the global results of these investigations, except in the case of cancelation or anonymization.
The used methods in the biomedical research are normally different from the approved for the clinical practice, so they cannot be considered to have clinical value for yourself. Despite this fact, in case these investigations provide information that are clinically or genetically relevant for you and interest the health of yours or your family, it will be communicate to you if it is considered necessary. In the same way, there may be obtained important information for your family. In this case, you will decide if you want or not share this information. If so, you must sign it at the end of this sheet.
If you do not want to share this information, you have to consider that according the law, when the information obtained is necessary to avoid a severe damage for the health of your biological family, an experts Committee will evaluate your case and will decide if it is convenient inform those affected or their legal representative.

Please, ask the health personnel that has communicated this information to you any doubt you might have, now or in the future, about this consent. In the same way, you can share your doubts with your physician, who will get in contact with the authorised health personnel.

Thank you for your collaboration.

Biobanc IDIBGI

We appreciate your unselfish collaboration with the science and medicine. This way, you are collaborating to conquer the diseases and helping many current and future sick people.

USE OF BIOLOGICAL SAMPLES AND CLINICAL DATA FOR RESEARCH PROJECTS AND COLLECTION IN THE BIOBANC

If you have understood the information provided in the information sheet, solved any doubt you might have had and decided to collaborate with the IDIBGI Biobanc and in the terms explained above, read and sign the following form:

Who signs the present document authorises the HUGJT and/or other adscript hospitals to incorporate the blood and CSF samples into the Biobanc once this research project is finished and which can be transferred from the same to develop projects of biomedical research, as long as they are approved by the competent Ethics Committee.

This authorization is given after been informed verbally and having read the attached information about the informed consent for the collection of clinical and analytical data, imaging tests and surplus biological samples for biomedical research.

I agree with:

1. I authorise to give, once this study has concluded, the exceeding sample and all the associated information from it to the IDIBGI Biobanc:
   YES ☐ NO ☐
2. I authorise the cession of the biological sample and the clinical information associated for their use in research:
   National: YES ☐ NO ☐ International: YES ☐ NO ☐
3. I would like to be informed about the results derived from the investigations that may be relevant and applicable for my health or my family's health:
   YES ☐ NO ☐ Tlf./email……
4. I authorise to be contacted in case more information or additional biological samples are needed:
   YES ☐ NO ☐ Tlf./email……
5. I have expressed my wish that the following exceptions from the aim and method of the investigation are respected:

   ……
   ……

DONOR	INFORMANT	☐ WITNESS/TUTOR☐
Name Surname DNI Age Signature	Name Surname DNI Signature	Name Surname DNI Relationship with the donor: Signature

In Girona, __ __/ __ __/ __ __ __ __

ANNEX V Chronogram of the study

ACTIVITIES	Personnel	2017										2018		2019	2020	2021					2022	
		Mr	Ap	My	Jn	Jl	Ag	Sp	Oc	No	De	Jan-Mr	Ap-De	Jan-Dec	Jan-Dec	Jan-Ag	Se	Oc	No	De	Ja	Fe
Stage 0																						
▪ Literature review	PI																					
▪ Protocol design	PI																					
▪ Presentation, evaluation and approval of the CEIC	PI																					
Stage 1																						
▪ Meetings and coordination with the personnel. ▪ Elaborating the database	All members																					
Stage 2																						
▪ Old data compilation	PI, Nrl																					
Stage 3																						
▪ Sample selection and processing	Lab																					
Stage 4																						
▪ Follow up ▪ Data compilation	PI, Nrl, Rx																					
Stage 5																						
▪ Statistical analysis	Sta																					
▪ Interpretation of the statistics	PI																					
▪ Article elaboration	PI																					
Stage 6																						
▪ Results publication and dissemination	PI																					