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TESI DI LAUREA

The Effect of High Effective Treatments on Unconventional Clinical and Para-clinical Parameters in Patients with Multiple Sclerosis

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Abstract

Background:

Multiple sclerosis (MS) is a chronic, immune-mediated disease of the central nervous system (CNS), characterized by demyelination, axonal damage, and neurodegeneration. MS is one of the leading causes of non-traumatic neurological disability in young adults, with a significant social and economic burden. Manifestations of the disease are highly heterogeneous, and depend mostly on the area where damage occurs, which arises from the inflammation. Among the most common sites of damage is the optic nerve. Damage to the optic nerve can trigger several alterations within the retinal layers such as reduction of thickness, volume and the formation of hyperreflective foci (HRF). These alterations are linked to both clinical disability and a higher disease load, like the presence of brain lesions, which are determined by the disease progression. In recent years, there has been a growing interest in comprehending more profoundly the significance and behavior of the alterations detected in the retinal layers and their possible implication on the disease progression.

Objective:

This study focuses on evaluating the effect of Ofatumumab and sphingosine-1-phosphate receptor (S1PR) modulators, on retinal layers thickness and volume in patients with relapsing-remitting MS (RRMS). Furthermore, this study also aimed to better understand the behavior of HRF, and to monitor the impact that the treatments had on their count.

Materials and methods:

To this prospective single-center longitudinal study 25 patients diagnosed with RRMS were recruited. The patients were divided into two treatment cohorts: 14 patients were treated with Ofatumumab, and 11 patients underwent a therapy with S1PR modulators (Siponimod, Ozanimod, or Ponesimod). Each patient underwent optical coherence tomography (OCT) imaging at baseline (T0) and approximately

six months later (T1). OCT scans were utilized to measure changes in the sectors of the peripapillary retinal nerve fiber layer (pRNFL) thickness, in macular layers volumes, as well as the HRF count in various inner-retinal layers. we compared the changes in thicknesses, volumes and HRF count in the various retinal layers between the two cohorts and across the six-month period.

Results:

The S1PR modulators cohort exhibited a significant reduction in the nasal inferior sector of pRNFL, as well as a decrease in total volume of the macular ganglion cell layer (GCL) and thinning of the GCL outer ring thickness. On the other hand, the Ofatumumab cohort did not show any significant changes in the parameters examined during the study period. Additionally, the S1PR modulators cohort demonstrated a significant decrease in HRF count in the intermediate capillary plexus (and area situated between INL and IPL). Finally, the comparison between the two treatment cohorts yielded a significant difference in the change of the total volume of GCL and the GCL outer ring thickness, where both parameters were decreased significantly more during the 6 months study duration in the S1PR modulators cohort.

Conclusions:

This study found that Ofatumumab was able to maintain stable peripapillary RNFL thickness and retinal layer volumes, likely due to its anti-inflammatory properties. In contrast, S1PR modulators were linked to reductions in thickness and volume, particularly in the GCL, which may indicate pseudo-atrophy or disease progression. Ofatumumab was more effective in preserving GCL outer ring thickness and total volume compared to S1PR modulators. Additionally, the reduction in HRF count in the ICP observed with S1PR modulators cohort further supports the theory of a microglial origin for HRF and its connection to the blood-retina barrier (BRB).

Riassunto

Presupposti dello studio:

La sclerosi multipla (SM) è una malattia cronica immunomediata del sistema nervoso centrale (SNC), caratterizzata da demielinizzazione, danno assonale e neurodegenerazione. La SM è una delle principali cause di disabilità neurologica non traumatica nei giovani adulti, con un notevole impatto socio-economico. Le manifestazioni della malattia sono molto eterogenee e dipendono prevalentemente dall'area in cui si è verificato il danno, che deriva dall'infiammazione. Tra le sedi più comuni di danno vi è il nervo ottico. Il danno al nervo ottico può innescare diverse alterazioni all'interno degli strati retinici, come la formazione di foci iperriflettenti (HRF), la riduzione dello spessore e del volume. Queste alterazioni sono legate sia alla disabilità clinica sia ad un carico maggiore di malattia, come la presenza di lesioni cerebrali, dovuti alla progressione della malattia. Negli ultimi anni è cresciuto l'interesse a comprendere più a fondo il significato e il comportamento delle alterazioni rilevate negli strati retinici e la loro possibile implicazione nella progressione della malattia.

Scopo dello studio:

Questo studio si concentra sulla valutazione dell'effetto di Ofatumumab e dei modulatori del recettore della sfingosina-1-fosfato (S1PR) sullo spessore e sul volume degli strati retinici nei pazienti con SM recidivante-remittente (SMRR). Inoltre, questo studio mirava anche a comprendere meglio il comportamento delle HRF ed a monitorare l'impatto che i trattamenti avevano sulla loro conta.

Materiali e metodi:

In questo studio longitudinale prospettico monocentrico sono stati reclutati 25 pazienti con diagnosi di SMRR. I pazienti sono stati suddivisi in due coorti di trattamento: 14 pazienti sono stati trattati con Ofatumumab e 11 pazienti sono stati sottoposti ad una terapia con modulatori S1PR (Siponimod, Ozanimod o Ponesimod). Ogni paziente è stato sottoposto a tomografia a coerenza ottica (OCT)

al basale (T0) e circa sei mesi dopo (T1). Le scansioni OCT sono state utilizzate per misurare i cambiamenti nei settori dello spessore dello strato delle fibre nervose retiniche peripapillari (pRNFL), nei volumi degli strati maculari e nel numero delle HRF nei vari strati retinici interni. Abbiamo confrontato i cambiamenti degli spessori, dei volumi e della conta delle HRF nei vari strati retinici tra le due coorti ed il loro andamento nell'arco dei sei mesi.

Risultati:

La coorte dei modulatori S1PR ha mostrato una riduzione significativa del settore nasale inferiore del pRNFL, nonché una diminuzione del volume totale dello strato delle cellule ganglionari (GCL) maculare ed un assottigliamento dello spessore dell'anello esterno del GCL. Dall'altra parte, la coorte Ofatumumab non ha dimostrato cambiamenti significativi nei parametri esaminati durante il periodo di studio. Inoltre, la coorte dei modulatori S1PR ha dimostrato una diminuzione significativa della conta delle HRF nel plesso capillare intermedio (area situata tra INL e IPL). Infine, il confronto tra le due coorti di trattamento ha prodotto una differenza significativa nella variazione del volume totale e dello spessore dell'anello esterno del GCL, dove entrambi i parametri sono diminuiti maggiormente nella coorte dei modulatori S1PR.

Conclusioni:

Questo studio ha rilevato che Ofatumumab è stato in grado di mantenere stabili lo spessore del RNFL peripapillare ed i volumi degli strati retinici, probabilmente grazie alle sue proprietà antinfiammatorie. Al contrario, i modulatori S1PR sono stati collegati a delle riduzioni dello spessore e del volume, in particolare nel GCL, che potrebbero indicare una pseudo-atrofia oppure una progressione della malattia. Ofatumumab è stato più efficace nel preservare lo spessore dell'anello esterno del GCL ed il suo volume totale rispetto ai modulatori S1PR. Inoltre, la riduzione del numero di HRF nell'ICP osservata con la coorte dei modulatori S1PR supporta ulteriormente la teoria di un'origine microgliale dell'HRF e il suo collegamento alla barriera emato-retinica (BRB).

1. Introduction

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory, immune-mediated disease of the central nervous system (CNS) characterized by a demyelination, axonal loss and neurodegeneration. It is the most common chronic inflammatory disease of the CNS, affecting over 2 million people worldwide.[1] It is also the leading non-traumatic disabling condition among young adults,[2] imposing a significant social burden and financial cost that closely correlate with the severity of the disease. [3]

The etiology of MS is complex and multifactorial. In addition to a genetic predisposition, exposure to a number of environmental factors, such as levels of vitamin D or ultraviolet B light (UVB) exposure, Epstein–Barr virus (EBV) infection, obesity and smoking, also play a role in the onset of the disease.[4] The pathogenesis involves an autoimmune response against the central nervous system (CNS), leading to the formation of demyelinating lesions in the brain and spinal cord, that can be detected on MRI that have an important diagnostic value.

The clinical expression of MS is notably heterogeneous and characterized by fully or partially reversible episodes of neurological disability, usually lasting days to weeks. Typical manifestations may encompass monocular visual loss due to optic neuritis, fatigue, limb weakness or sensory loss due to transverse myelitis, double vision due to brainstem dysfunction, or ataxia due to a cerebellar lesion. The most common form of MS is relapsing remitting with many patients after typically 10-20 years from the disease onset then develop a progressive clinical course and eventually present with impaired mobility and cognition.

Currently, there is no cure that is capable of reversing or preventing the neurological deterioration of patients. However, there are more than a dozen disease-modifying medications that are capable of reducing the frequency of relapses and limit the accumulation of white matter lesions found on MRI. [1]

1.1.1 Epidemiology

MS is the most frequent demyelinating disease in high-income countries with a global prevalence of 2.8 million people affected, which equates to a ratio of 1 case in 3000 worldwide and 1 case in 300 people in countries with high prevalence of the disease.[5] It's global prevalence is quite heterogeneous: from high levels in North America and Europe (>100/100,000 inhabitants) to low rates in Eastern Asia and sub-Saharan Africa (2/100,000 population). The global median prevalence of MS has increased from 30/100,000 in 2008 to 33/100,000 in 2013, according to a report by the MS International Federation. In Europe, in particular, a North-South prevalence gradient has been described for the distribution of the disease (higher in the North, lower in the South).[6] Such increase in incidence and prevalence was also seen in Veneto region in Italy, where the number of patients with MS has continuously increased since 1960.[7]

The most frequent onset age of the disease is between 20-40 years, nonetheless in 5% of cases a late-onset MS is encountered, in which symptoms begin after the age of 50, as well as an early-onset in patients under the age of 18.[8]

MS is more common in females with a ratio of 3:1 approximately, however the ratio between females and males in the early 1900s was evenly distributed. This relative increase of incidence might be a reflection of the increasing number of women smoking.[2]

Moreover, due to the correlation between vitamin D levels and the risk of developing MS, a latitudinal prevalence gradient of patients diagnosed with MS emerges. In support of that observation, in countries where UVB exposure (which stimulates cutaneous vitamin D production) is greater, the prevalence of MS is smaller.[2]

1.1.2 Risk Factors

MS seems to develop predominantly in genetically susceptible populations as a result of environmental exposures, hence it is unlikely that the disease results from one causative event. Genetic predisposition is believed to be an indispensable factor in developing MS as shown by genetic epidemiological studies. [9] Variations in the human leukocyte antigen (HLA) genes are notably known to be associated with MS, like the HLA-DRB1*15. Heterozygotes for HLA-DRB1*15:01, which represents a common genetic risk factor in populations of northern European origin, have an odds ratio (OR) of MS >3 and homozygotes >6, with additional effects from class II risk alleles (HLA-DRB1*03:01 and HLA-DRB1*13:03) and class I protective alleles, including HLA-A*02:01, HLA-B*44:02 and HLA-B*38:01.[10]

Other possible genes that have a modest effect in the development of MS include the interleukin-7 receptor α (*IL7RA*), interleukin-2 receptor α (*IL2RA*), and C-type lectin-domain family 16 member A (*CLEC16A*).[11]

Environmental exposure seem to have a greater influence in development of MS. Such assumption is inferred from migration studies consistently support MS being secondary to an environmental exposure. Adult migrants from low risk countries to Europe are at low risk of developing MS; however, children born to migrants in Europe are at a greater risk.[12]

EBV infection appears to be ubiquitous among MS patients, with >99% of individuals affected have been found to have been infected with EBV compared with approximately 94% of age-matched controls.[13] The leading hypothesis for the connection between EBV infection and MS includes a cross reaction of the immune response to the infection with myelin antigens in genetically susceptible individuals.[13] Furthermore, individuals with high titers of anti-EBV antibodies seem to have a greater risk of developing MS compared with those with low titers.[9] It is hypothesized that the relationship is also temporal, with plasma antibody titers against the EBV nuclear antigen 1 (EBNA1) increasing several years prior to the onset of neurological symptoms of MS.[14]

Smoking has also been established as a considerable risk factor as seen in a retrospective meta-analysis showing an OR for developing MS of 1.51 (95% CI

1.24–1.83) compared to non-smoking individuals. A dose dependent relation between smoking and MS risk was found as well. [15,16]

The latitudinal gradient of MS is believed to be explained by differences in sun exposure and vitamin D levels. As shown in epidemiological studies in countries with a low sunlight exposure, and therefore lower levels of vitamin D, there is an increased risk of MS.[17]

Additionally, there is a growing body of evidence indicating a potential association between air pollution and the development of MS. Several studies have identified a correlation between MS and air pollutants, including PM (that comprise solid particles and liquid droplets, which can include acids, metals, soil, and dust), gaseous pollutants, and heavy metals. The role of air pollution on the prevalence and incidence of MS is a matter of controversy with some hypotheses being however supported by past studies. There are 2 main hypotheses, that attempt to explain the role of air pollutants as a risk factor for MS. First, air pollutants is associated with the increase of epithelial wall's permeability and oxidative stress, that can trigger inflammation and the activation of an autoimmune T-cell mediated response in the CNS. Second, air pollution might trigger epigenetic changes linked to pro-inflammatory cytokine's production. [18]

1.1.3 Immunopathology

An indispensable component regarding the cause of MS is attributed to the role of CD4+ T cell in the determination of the inflammatory response found in MS. The inflammation model that can mimic MS lesions can be observed in the experimental autoimmune encephalitis (EAE).[19] EAE is induced by immunizing an animal with a cerebral myelin antigen and subsequently transferring the previously immunized T helper 1 (Th) or Th17 of the affected animal to an healthy individual, thus inducing EAE in the latter one. Hence, it is believed that an autoreactive sensitized Th cells are capable of inducing an inflammatory cascade, which then results in the typical CNS damage, such as demyelination, gliosis and axonal loss that we also observe in MS. Nevertheless, clinical trial data suggest that, by contrast with EAE, targeting CD4 T-cell function in MS might not be of therapeutic benefit, as patients who received anti-CD4 antibody showed no clinical improvement.[20] Th subpopulations are thought to have a different role in the etiology of MS. For example, Th-1 cells are thought to play a significant role as the prime drivers of the autoimmune process occurring in MS, nonetheless, therapy targeting interleukin 12 (an important cytokine in the differentiation process of Th-1 cells) was found to be not beneficial in phase 2 clinical trials.[21] Other Th cells were also found to be abnormal in patients with MS compared with healthy individuals, such as T_{reg} cells that resulted to be functionally impaired,[22] or Th17 cells, that on the other hand, were found to be enriched. [23]

Although the involvement of T cell mediated immunity is widely accepted to have an important impact on the development of MS, humoral immunity seem to have a substantial role as well. As detected in many cerebral spinal fluid (CSF) samples taken from MS affected individuals, there is a consistent intrathecal synthesis of IgG, generating CSF oligoclonal bands, indicating a significant abnormal B-cell-related processes active within the brain parenchyma. [9]

1.1.4 Pathophysiology

The activation of autoreactive T-cells in MS is attributed to two mechanisms: immune cross-reactivity with foreign antigens and the recognition of CNS auto-antigens that leak to cervical lymph nodes. In the process of molecular mimicry, that causes an autoimmune autoreactive response, antigen presenting cells (APC) are exposed to foreign antigens in various organs such as the intestines, lungs or skin. Subsequently to the capture of those antigens, APC then migrate to lymph nodes and trigger the activation of T cells, which later might present also an affinity to CNS auto-antigens. Following their activation, these auto-reactive T cells might migrate to the CNS causing tissue damage.[24] (Figure 1)

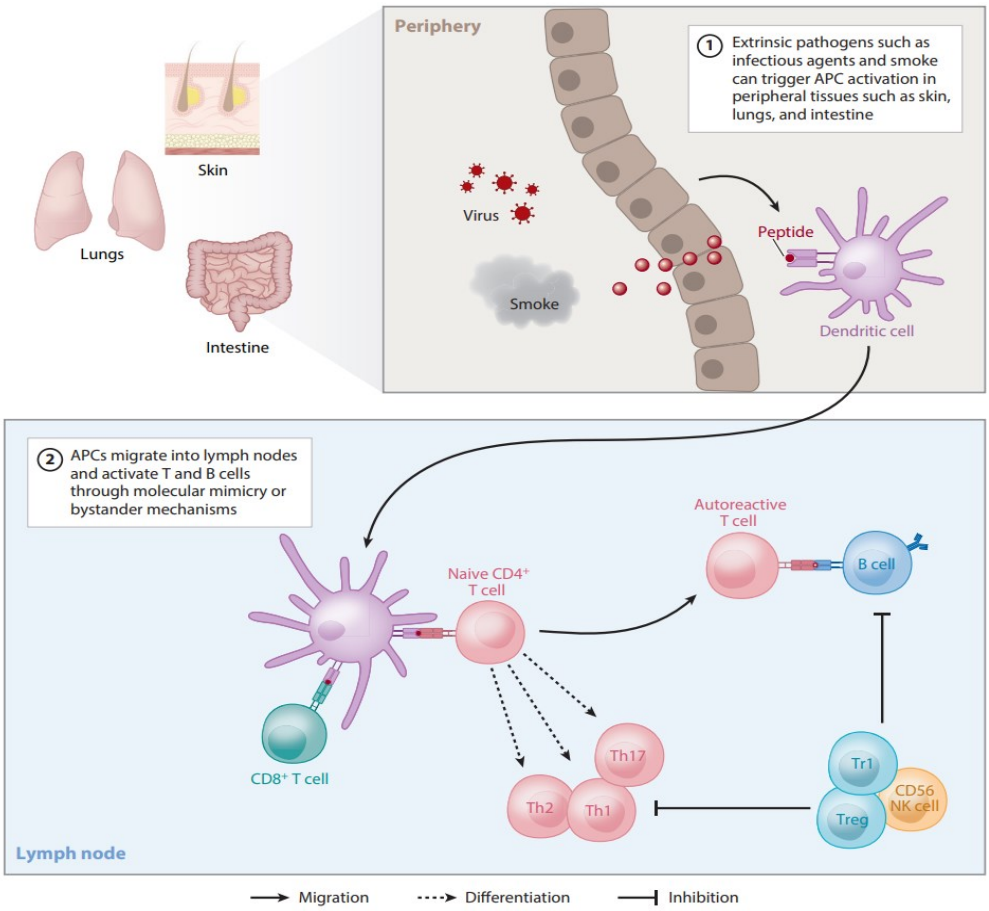

Figure 1. *The activation of autoreactive T-cells in peripheral lymph nodes following the presentation of cross-reactive antigens derived from pathogens by APCs. Abbreviations: NK, natural killer; Tr1, type 1 regulatory T cell; Treg, regulatory T cell.*

The function of the blood brain barrier (BBB) is another important factor in the lesion formation in the CNS. One of the roles of the BBB is to limit the passage of

circulating cells and large molecules, transforming the CNS into a relatively immunologically privileged site. Thus, it is thought that the activation of autoreactive T-cells triggers the expression of adhesion molecules that facilitate the migration of T-cells into the CNS. Additionally, chemokines produced by endothelial cells in proximity to damaged areas in the CNS stimulate the activation of integrins, which are capable of interacting with ligands found on the surface of the endothelial cells, promoting the immune cell rolling and, as a consequence, infiltration to the CNS. In fact, natalizumab, a monoclonal antibody that targets the interaction between the T-cells and the endothelial cells (by blocking VLA-4 found on the surface of T-cells), prevents lymphocyte entry into the CNS and suppresses disease activity.

Following the infiltration to the CNS, T-cells get restimulated by residing cells, like dendritic cells, microglia and others. After their reactivation, the T-cells undergo a clonal expansion of CD4+ T-cells (mainly Th1 and Th17), which are responsible of the production of proinflammatory cytokines. Then numerous residing and immune cells get stimulated causing inflammation and consequently damage to the CNS. This inflammation process is characterized by several mechanisms, including the production of neurotoxic and oligotoxic mediators, humoral and cytotoxic immune response. The inflammation is later on contrasted by an anti-inflammatory response, in which we can observe the action of T-reg cells among other cells involved in the reduction of the inflammatory response. Indeed, via the production of immunosuppressive cytokines such as IL-10 and FoxP3, T-reg cells are believed to have a crucial role in the induction of the remission of the inflammatory process.[24]

MS lesions can appear throughout the CNS and are most easily recognized in the white matter as focal areas of demyelination, inflammation, and glial reaction due to the inflammation. Even though lesions may be more evident in the white matter, demyelination can affect the gray matter as well with lesions often being perivascular. Demyelination can be distinguished by different patterns. The most common patterns involve a perivascular and parenchymal T-cell infiltration, that can be accompanied with immunoglobulin and complement deposition, on a background of mononuclear phagocytes. As a consequence to the demyelination, oligodendrocytes are able to initiate a remyelination process of damaged axons.

Nonetheless, the remyelination process is limited, and the original myelin thickness is never achieved again. The extent of the remyelination is dependent on several factors. For example, imaging studies have indicated that lesions that form in younger individuals may repair more effectively. [1,24] (figure 2)

The optic nerve represents yet another target of the damage caused during relapses in MS. Retinal damage can be assessed in vivo by optical coherence tomography (OCT), which shows, substantial thinning of the retinal nerve-fiber and ganglion cell layers, due to injury to axons in the optic nerve. [25]

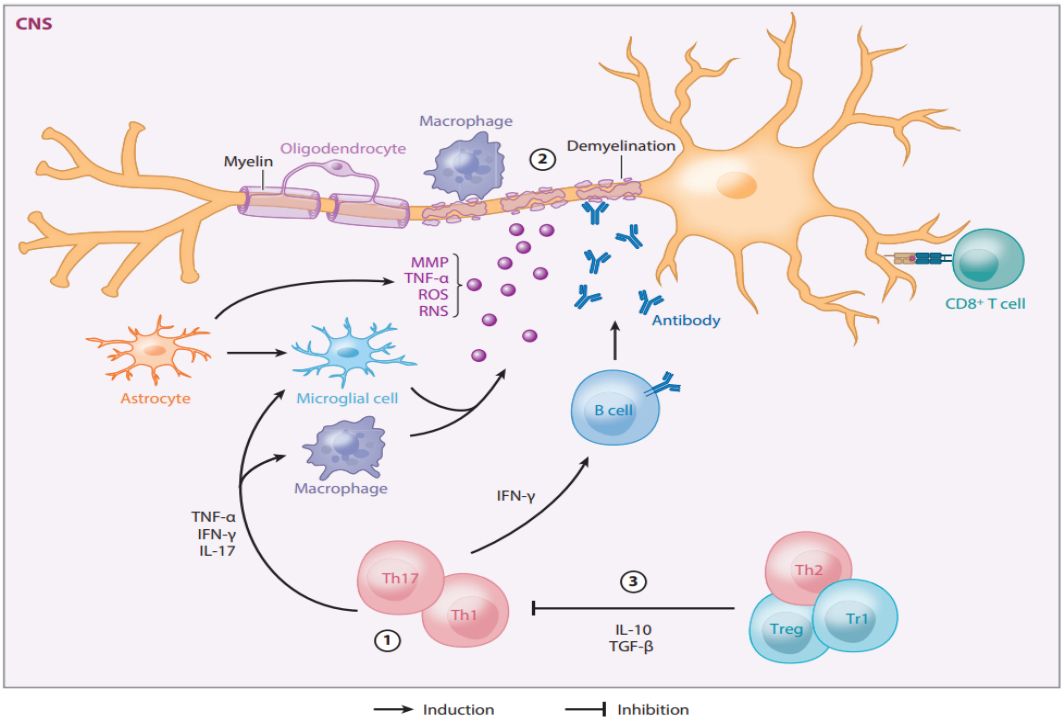

Figure 2. *The CNS damage driven by activated immune cells in the CNS. (1) Pathogenic Th cells secrete proinflammatory cytokines such as TNF-α, IFN-γ, and IL-17, which induce microglia and macrophage activation. Such immune cell activation and recruitment are supported by astrocyte activity, which potentiates the immune response by cytokine and chemokine secretion. (2) Numerous mechanisms drive myelin and axonal damage, principally soluble neurotoxic molecule production such as MMPs, TNF-α, ROS, and RNS, which are secreted by astrocytes, macrophages, and microglia, as well as activated CD8+ T cell cytotoxicity, ADCC, and complement. (3) Local CNS inflammation associated with MS relapses is reduced by FOXP3+ Tr1 Tregs via the secretion of immunoregulatory cytokines such as IL-10 and TGF-β and additional mechanisms. Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; IFN, interferon; IL, interleukin; MMP, matrix metalloproteinase; RNS, reactive nitrogen species; ROS, reactive oxygen species; TGF, transforming growth factor; TNF, tumor necrosis factor; Tr1, type 1 regulatory T cell.*

1.1.5 Clinical Presentation

The clinical presentation of MS is considered highly heterogeneous and depends on the location of the lesions within the CNS. The demyelinating lesions cause a range of different clinical consequences which in turn result in neurological dysfunction. Nevertheless, there are certain manifestations that are considered more typical in patients with MS. One example is given by the optic nerve involvement which represents a common target of the inflammatory process leading to optic neuritis, observed in approximately 70% of patients during the course of the disease. It is characterized by a partial or total visual loss in one eye with a central scotoma, dyschromatopsia and pain within the orbit that is worsened by eye movement. The onset of optic neuritis may also predict the onset of MS in some individuals. To this end, several MRI criteria have been established in order to predict the conversion to clinically definite MS with high sensitivity and specificity. Furthermore, MRI evidence of dissemination in space and time can now enable MS diagnosis at presentation in some patients with acute optic neuritis.[26] (figure 3)

Other lesions can be found in the spinal cord (leading to myelitis), brainstem, cerebellum (leading to brainstem and/or cerebellar syndromes) or the cerebral hemispheres (cerebral hemispheric syndrome). In RRMS these episodes last for ≥24 hours and occur in the absence of fever, infection or clinical features of encephalopathy (for example, altered consciousness or epileptic seizures).[27]

Diagnosis requires dissemination in space and time
Dissemination in space • At least one lesion visible on T2-weighted scan in at least two of four locations: juxtacortical, periventricular, infratentorial, and spinal cord Dissemination in time • A new T2 lesion or gadolinium-enhancing lesion visible on a follow-up MRI scan when compared with a previous scan (which is thought to be the baseline scan) obtained at any time after the onset of symptoms; or • An MRI scan showing both gadolinium-enhancing and non-enhancing lesions that do not cause clinical signs (ie, asymptomatic lesions)

Figure 3. *Diagnosis of MS in MS-optic neuritis (2010 McDonald MRI criteria).*

Additionally, the occurrence of sensory symptoms is reported to be a common manifestation during episodes of clinical relapse. These symptoms may include paresthesia, Lhermitte sign (an electric shock radiating down the spine or into the limbs with flexion of the neck), impairment of vibration and light touch sensitivity. Such symptoms can also get worse with rising of body temperature (known as Uhthoff phenomenon). Other symptoms include pain (that varies heavily in location, quality and duration), headache, fatigue, respiratory symptoms (such as shortness of breath and cough) and dizziness.[28]

Motor symptoms resemble those of an upper motor neuron lesion, like the positivity to pathological reflexes (Babinski sign), rigidity and more pronounced reflexes. The brainstem and cerebellar impairment may also lead to a ataxia, gait imbalance, slurred speech, dysphagia, pathological ocular movement as well as diplopia. Furthermore, autonomic dysfunction might occur and include sexual dysfunction, bladder dysfunction and constipation.[27]

Finally, affective disturbances are a notable cause of disability as well. Major depressive disorder (MDD) is among the most prevalent comorbidities in MS, with a lifetime prevalence of approximately 50%.[29] Data shows a higher rate of MDD in patients with MS compared to other neurologic disorders. MDD symptoms typically associate with a progressive MS course, leading to severe consequences on cognitive performance and worsening physical disability. In addition to MDD, anxiety disorders represent yet another major cause of disability, leading to significantly more fatigue, pain and sleep problems, which worsen with the co-occurrence of depression. Anxiety disorders are more common in females, and are often related to a younger age, as well as, an early MS onset and diagnosis. Disability is also highly influenced by cognitive dysfunction. Like all symptoms of MS, cognitive dysfunction is characterized by high variety between patients, although the most frequent cognitive impairment involve cognitive processing speed, memory, acquiring and retrieving information, attention and executive function. Cognitive deficits can occur in the early stages of multiple sclerosis, even in the absence of other neurological deficits, and worsen over time. [29–31]

1.1.6 Clinical Course

In the past, MS subtypes were classified by U.S. National Multiple Sclerosis Society (NMSS) into 4 phenotypes:

1. Relapsing remitting MS (RRMS): characterized by episodes of acute worsening of neurological function followed by a full or partial recovery of neurological capability without evidence of progression of the disease.
2. Primary progressive MS (PPMS): steadily worsening neurologic function from the beginning without any distinct relapses or remissions.
3. Secondary progressive MS (SPMS): progressive course of the disease following an initial relapsing remitting course, with or without relapses.
4. Progressive relapsing MS (PRMS): steadily worsening neurologic function from the beginning with occasional relapses.

In recent years this classification was reevaluated, and certain factors were added. Every phenotype was further described as active/inactive and worsening/stable. The disease was considered active when evidence of new relapses, new gadolinium enhancing lesions and/or new or enlarging T2 lesions on MRI over a specified time period was detected. On the other hand, the disease was defined as worsening when increased disability was confirmed over a specified time period following a relapse. Furthermore, 2 new disease courses were added to the classification:

1. Radiologically isolated syndrome (RIS): identifies patients with incidentally found MRI abnormalities highly suggestive of demyelination in the absence of clinical signs or symptoms.
2. Clinically isolated syndrome (CIS): describes a first clinical event highly suggestive of demyelinating CNS disease but not yet meeting dissemination in time for diagnosis of MS. The presenting symptoms in CIS show similar characteristics to symptoms in MS, and involve the optic nerve, cerebellum, spinal cord or brainstem.[32]

1.1.7 Diagnosis

With regard to the diagnosis of MS neither a pathognomonic clinical feature nor a diagnostic test have yet been identified. Furthermore, it's heterogeneous clinical and radiological manifestations, which differ between patients and change within individual patients over time, contribute to the misdiagnosis of MS, that to this day constitute an issue in clinical practice. The diagnosis of MS relies on the integration of clinical manifestation, imaging, and laboratory findings, as summarized in the McDonald diagnostic criteria for MS. (figure 4)

MRI plays an important role in the diagnosis of MS, and nowadays, it is recommended that all patients should undergo MRI of the brain and the spinal cord (if there are findings suggesting an involvement of the spinal cord). MRI may aid with confirming eventually the diagnosis of MS, by demonstrating the dissemination in space and time as mentioned, as well as exclude MS mimics.[2]

The fundamental feature necessary to make a diagnosis of MS is the determination of the dissemination of a focal neurological disease in space and time. Dissemination in space can be determined by demonstrating at least two lesions on T2-weighted MRI in at least two MS-typical sites (Periventricular, (juxta)cortical, infratentorial, and spinal cord). On the other hand, for the establishment of the dissemination in time, it is sufficient at a given time, to define at least one typically located MS lesion with gadolinium enhancement, in addition to other non-enhancing T2 lesions.[33,34]

Oligoclonal bands (OCB) of the CSF, which represent a substantial component in the evaluation of the inflammatory processes circumscribed to the CNS, have been important in the diagnosis of MS for many years, and recently became a part of the latest diagnostic criteria as well. Though, the presence of OCB are not specific for MS, in the appropriate clinical setting, especially when the diagnosis of the condition is uncertain, evidence of OCB in the CSF may support the diagnosis. In addition, negative CSF findings constitute a high negative predictive value, hence excluding the diagnosis of MS.[34–36]

CSF examination therefore is strongly recommended in the following situations:

1. When clinical and radiological evidence is insufficient to support a diagnosis of MS, particularly if initiation of disease-modifying therapies is being considered
2. When clinical, imaging, or laboratory features are atypical of MS.
3. In populations in which MS is less common (eg, children or older individuals).[34]

	MacDonald 2010 (relapsing–remitting MS)	MacDonald 2017 (relapsing–remitting MS)
DIS	*Either* (i) Objective clinical evidence of ≥2 lesions or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack involving a different CNS site *or* (ii) ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, spinal cord); *symptomatic lesions in patients with brainstem or spinal cord syndromes are excluded*	*Either* (i) Objective clinical evidence of ≥2 lesions or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack involving a different CNS site *or* (ii) ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, spinal cord)
DIT	*Either* (i) ≥2 attacks separated by at least 1 month *or* (ii) simultaneous presence of *asymptomatic* gadolinium-enhancing and non-enhancing lesions at any time *or* (iii) a *new* T2 and/or gadolinium-enhancing lesion on follow-up MRI irrespective of its timing with reference to a baseline scan	*Either* (i) ≥2 attacks separated by at least 1 month *or* (ii) simultaneous presence of *asymptomatic* gadolinium-enhancing and non-enhancing lesions at any time *or* (iii) a *new* T2 and/or gadolinium-enhancing lesion on follow-up MRI irrespective of its timing with reference to a baseline scan *or* (iv) *demonstration of CSF-specific OCBs (as a substitute for DIT)*
MacDonald 2010 criteria for primary progressive MS		
(i) 1 year of disease progression (retrospectively or prospectively determined) *and* (ii) 2 out of 3 of	Evidence of DIS in the brain based on ≥1 T2 lesion in at least one area characteristic for MS (periventricular, juxtacortical, infratentorial) *and/or* evidence of DIS in the spinal cord based on ≥2 T2 lesions in the cord *and/or* positive CSF (OCBs on isoelectric focusing and/or elevated IgG index)	

***Figure 4**: The 2010 McDonald Diagnostic criteria with the 2017 revision for relapsing–remitting and primary progressive MS. Abbreviations: DIS, dissemination in space; DIT, dissemination in time; OCB, oligoclonal band.*

1.2 Therapy

MS treatment focuses mainly on reducing disease activity, managing symptoms, and improving quality of life. Treatment strategies include:

1. Treatment of acute attacks: characterized by the emerging of new symptoms or either the appearance of new lesions detected on an MRI (also when patients are asymptomatic). First-line treatment are glucocorticoids (such as methylprednisolone), providing short-term clinical benefit by reducing the severity and shortening the duration of attacks. In case of refractory response to glucocorticoids, second line treatment consists of plasmapheresis, IV immunoglobulin (IVIG), and adrenocorticotropic hormone (ACTH) which showed to possess direct anti-inflammatory effects and immunomodulatory activity.[37–39]
2. Symptomatic treatments: refers to pharmaceutical and physical therapies that target symptoms arising as a result of CNS damage. It is useful to encourage attention to a healthy lifestyle, including maintaining an optimistic outlook, a healthy diet, and regular exercise as tolerated. Vitamin D supplementation may also be considered, in light of the fact that vitamin D deficiency represents a risk factor for the disease.[38]
3. Disease modifying therapies (DMT): consist of immunomodulatory and immunosuppressant agents.

Disease modifying therapies

DMTs encompass numerous agents, varying from injectable DMTs (interferons and glatiramer acetate) and oral DMTs (such as sphingosine-1-phosphate receptor modulators, fumarates, teriflunomide) to monoclonal antibody DMTs (natalizumab, ocrelizumab, ofatumumab, alemtuzumab). They primarily exert an anti-inflammatory activity during the relapsing phase of MS, promoting the modulation of the immune system through various mechanisms. Those mechanisms include the sequestration of lymphocytes, T_H1/T_H2 shift, interference with DNA synthesis in lymphocytes, depletion of immune cells, and/or changes in cytokine secretion pattern. As a consequence, they are able to alter the course of MS by

reducing the risk of relapses, decreasing disease activity as assessed on MRI scans, and/or slowing the accumulation of MS symptoms that interfere with daily life.[38]

The agent is selected based on a combination of patient factors (age, comorbidities, plans for pregnancy), disease factors (number and location of lesions) and patient preferences (medication side effects versus efficacy).[40] The number of DMT agents, as well as their early use in the course of the disease, have increased over the past years aiming to prevent long-term disability. Eventually, due to the high number of DMTs available, the management of patients became more complex over the years. Currently two therapeutic approaches are available in the clinical setting:

1. Step-up approach (or escalation strategy): Consists of starting with a first line, modestly effective initial agent, and then escalating to a more effective medication, if the patient's relapse rate has not changed when compared with the pre-treatment period.
2. Step-down approach (or induction strategy): Involves starting with a high-efficacy treatment, which may be stepped back with a less effective DMT following a period of disease stability.[40,41]

Because of their immunosuppressive effects, continuous monitoring for adverse effects is indispensable for DMTs. Some (such as natalizumab and alemtuzumab) require a specific risk evaluation mitigation strategy. Most DMTs are associated with an increased risk for infection, which are typically urinary tract infections, upper respiratory tract infections, and pneumonias. [42]

Treatment is normally life-long once a patient has initiated a treatment with DMT, unless breakthrough disease or adverse effects occur that require a medication switch. However, several observational studies have suggested that older individuals undergoing injectable or oral DMTs who have been stable clinically and radiographically for an extended period (≥4 years) have a low reoccurrence of disease activity and may benefit from treatment discontinuation. [43,44]

1.2.1 Ofatumumab

Ofatumumab (Kesimpta®) is a fully human monoclonal antibody currently used in the treatment of RRMS. Ofatumumab has a 20 mg s.c. monthly dosing regimen, which has been approved by FDA (August 2020) and EMA (March 2021) for the treatment of active relapsing MS forms. It was the first anti-CD20 therapy which patients can self-administer at home via subcutaneous injection, offering convenience and ease of use. Clinical trials showed that ofatumumab is more effective than other DMTs, like teriflunomide, in reducing the annual relapse rate, MRI-detected lesion activity, and disability progression.[46] Overall, currently approved anti-CD20 monoclonal antibodies (rituximab, ocrelizumab, and ofatumumab) consistently lead to a dramatic reduction of clinical relapses and MRI disease activity together with a significant limitation of disability worsening and brain atrophy progression.[45] Ofatumumab also demonstrated a generally manageable safety profile, with infections (such as upper respiratory tract and urinary tract infections) and injection-related reactions being the most common adverse effects. [46] Though the exact mechanism of action of Ofatumumab is not yet fully understood, it is known that the FAB portion of ofatumumab selectively binds to CD20, a transmembrane phosphoprotein expressed on B lymphocytes. This binding leads to B cell (and T cell) depletion via complement-mediated $CD20^+$ B cell lysis and antibody-dependent cell-mediated cytotoxicity. The ability to reduce B cell levels is the core to its efficacy in managing the disease symptoms and progression, because of their notable role in the pathogenesis of MS. [45,46]

As a part of the broader category of anti-CD20 therapies, ofatumumab represents a promising option for personalized treatment approaches in MS, with potential for further optimization in dosing and combination therapies to enhance patient outcomes. Overall, ofatumumab is considered a valuable treatment option as a part of the arsenal of DMTs currently available for MS.[45,46]

1.2.2 Sphingosine-1-phosphate receptor modulators

Sphingosine 1-phosphate receptor (S1PR) modulators are another class of DMT currently used in the treatment of MS, that are administered orally once daily. These drugs target the S1P signaling pathway, which plays a significant role in lymphocyte trafficking and numerous cellular processes in the CNS. S1P modulators bind to 1 of the 5 subtypes of S1P receptors, resulting in internalization of the receptor and sequestration of lymphocytes in lymph nodes, thus reducing the number of circulating lymphocytes in peripheral blood and limiting their migration into the CNS. Moreover, these drugs are capable of crossing the blood-brain barrier (BBB), potentially having some direct effects on various cells in the CNS (including neurons, astrocytes, oligodendrocytes, and microglia) expressing S1P receptors. As a result of the interaction with these cells, S1PR modulators are able to regulate neuroinflammation, demyelination, and potentially promote remyelination. The efficacy of S1PR modulators in reducing relapse rates is documented in clinical trials, demonstrating reduced MRI detected lesions, as well as potentially slowing disability progression in patients with RRMS. [42,47,48]

There are currently four FDA-approved S1PR modulators for MS treatment:

1. Fingolimod: Targets S1PR subtypes 1, 3, 4, and 5
2. Siponimod: Targets S1PR subtypes 1 and 5
3. Ozanimod: Targets S1PR subtypes 1 and 5
4. Ponesimod: Targets only S1PR subtype 1

S1PR modulators generally have a manageable safety profile, yet they still present several adverse effects such as: transient bradycardia and atrioventricular conduction block in correspondence with treatment initiation (through the binding to the subtype 1 receptor on cardiac myocytes), increased risk of infections, macular edema (due to increased vascular permeability), elevated liver enzymes, hypertension and potential risk of cutaneous malignancies. Hence, patients treated with S1PR modulators should undergo monitoring with a complete blood cell count; measurement of serum transaminase and total bilirubin levels; testing for varicella zoster virus (VZV) antibodies; electrocardiogram; and ophthalmologic

examination of the fundus for macular edema prior to initiation. The more selective S1PR modulators (siponimod, ozanimod, and ponesimod) may have a reduced risk of certain side effects compared to the less selective fingolimod. [42,47]

Nowadays, research is focusing on the optimization of dosing strategies, development of new S1PR modulators with improved selectivity profiles, and exploring combination therapies in order to enhance treatment efficacy. There is also interest in investigating the potential of S1PR modulators in other neurological and autoimmune conditions. [49]

1.3 The optic pathway in patients with multiple sclerosis

1.3.1 The anatomy of the retina

The retina is the innermost layer in the eye. It is responsible for the visual processing that turns light energy from photons into three-dimensional images. The retina itself consists of six different cell lines divided into ten different layers, each playing a specific role in creating and transmitting vision. The different cell types perform a particular role and form functional circuits that specialize in detecting specific variations and movements of light.

As anticipated previously, the retina is a layered structure with ten distinct layers of neurons interconnected by synapses. The layers from the front anterior of the head towards the posterior pole of the head are as follows:

1. Inner limiting membrane (ILM)
2. Nerve fiber layer (NFL)
3. Ganglion cell layer (GCL)
4. Inner plexiform layer (IPL)
5. Inner nuclear layer (INL)
6. Outer plexiform layer (OPL)
7. Outer nuclear layer (ONL)
8. External limiting membrane (ELM)
9. The layer of rods and cones
10. Retinal pigment epithelium (RPE)

Within these layers of the retina, there are different types of cells with specific roles that help processing incoming light. The six different cell types in the retina include:

1. Rods: The most predominant cell type, which specialize in registering low-light levels, thus helping to create a black and white vision known as scotopic vision.

2. Cones: The type of cells responsible for photopic vision, which involves color vision at varying light levels. They are mostly concentrated in the macula, in which the fovea is found. The central fovea contains neither rods nor even synapses, but only 100% cones which have an unobstructed view of the incoming light.

3. Retinal ganglion cells: They are the main output neuron of the retina, but also considered a third class of photoreceptors that are also photosensitive. They help transmit both image-forming and non-image forming information that functions in the physiological processes of the circadian rhythm, modulation of melatonin release, and regulation of pupil size.

4. Bipolar cells: Bipolar cells are second-order long-projection neurons, that receive visual inputs from photoreceptors and projects their axons onto retinal ganglion cells.

5. Horizontal cells: The type of cells that aid in modulating information transfer between bipolar cells and photoreceptors and are involved with helping eyes adjust to both bright light and low light conditions.

6. Amacrine cells: Characterized by great diversity and normally release inhibitory neurotransmitters. This diversity among amacrine cells allows them to form dedicated functional microcircuits that allow the retina to detect different shades and movements of light in particular directions.

1.3.2 Retinal layers thickness in patients with MS

During the course of MS, the anterior visual pathway is known to be a common site of damage. Damage at this site, even in the absence of lifelong visual symptoms, has been observed since the earliest pathological descriptions of MS and it seems to reflect also CNS inflammation outcomes.[50,51]
The retina shares many structural and functional features with the CNS. One of them is the blood-retina barrier (BRB), which acts similarly to the blood-brain barrier and makes retinal microenvironment a specific interstitial space in terms of soluble molecules and ions. Moreover, the immunologic trafficking into and out from the retina mirrors as well the CNS dynamics, and therefore is prone to react to systemic or local pathologic processes. This reactivity might be the underlying cause for the alteration of retinal layer thickness during CNS inflammation or therapies acting on the BRB. [51]

High resolution quantitative retinal imaging technologies, like Heidelberg retinal tomography, scanning laser polarimetry and optical coherence tomography (OCT) have increased our understanding of retinal injury in MS. They have firmly established the association between RNFL and macular thinning thickness and MS pathology. (figure 5) In particular, the largest and most robust differences between the eyes of people with MS and control eyes were found in the peripapillary RNFL (pRNFL) and macular GCIPL (a combined measurement of GCL and IPL).[25,50] pRNFL thickness was shown to correlate inversely with disease duration and the grade of disability and OCT measurements of the retinal layers showed high sensitivity and specificity for detecting disease activity. Furthermore, pRNFL thinning was also found to be associated with higher Expanded Disability Status Scale (EDSS) scores. Moreover, it was also observed that achievement of no evident disease activity (NEDA) with disease modifying treatment is related with less marked atrophy of the pRNFL longitudinally. Consistent with the observation from the RNFL, GCIPL atrophy seem to reflect as well disease activity and appears to be the most severe in patients with history of optic neuritis. In addition, the retinal GCL complex is the thickest in the macula, and due to the fact that most of the MS related damage includes the macula, the macular GCIPL (mGCIPL) may represent

a good biomarker for neurodegeneration in the visual pathway in MS. [25] Also, the reduction of mGCIPL thickness after optic neuritis has a prognostic value for long-term visual outcome. [52] The inner nuclear layer (INL) seem to be also an important site of inflammation as the occurrence of inflammatory cells in the INL of MS patients was described by histological post-mortem retinal analysis. Furthermore, the appearance of microcystic macular oedema (MMO) in the INL by OCT analysis was found to be correlated with disease severity. Further studies confirmed that INL volume correlated with inflammatory disease activity, as higher INL volumes were associated with an increase in T_2 and gadolinium-enhancing (Gd+) lesion load in cerebral MRI, annualized relapse rate, and higher EDSS score.[53] These findings suggest that the incorporation of OCT measurements and monitoring retinal changes could be used in clinical practice as a valuable tool for evaluating disease progression, assess treatment efficacy, and potentially predict outcomes in MS patients.[25,53–55]

It is important to note that, retrobulbar demyelination and inflammation underlie the acute visual dysfunction seen in acute optic neuritis, and patient who underwent an episode of acute optic neuritis presented a more pronounced RNFL atrophy compared to patients with MS that never had an episode of optic neuritis. However, MS patients who do not have a history of clinically evident acute optic neuritis still present RNFL and macular atrophy compared to healthy individuals. This potentially argues against the proposition that optic nerve inflammation underlies all the changes seen in the retina.[25,50] The RNFL atrophy in the absence of optic neuritis has brought researchers to the hypothesis of a trans-synaptic degeneration. According to that hypothesis, a damage to the posterior optic pathway would translate into a retinal atrophy, and vice versa, a damage to the anterior optic pathway could suggest a damage to the posterior visual structures.[56]

Additionally, due to the predominant presence of demyelinated axons in the RNFL, it is more likely that the RNFL atrophy reflects better the axonal loss rather than the demyelination process seen in MS, hence making the RNFL atrophy a viable marker for axonal damage.[57] The axonal loss, by contrast with demyelination, is an irreversible process and an important cause of sustained disability. Therefore, a validated tool for monitoring of axonal loss is important.[58]

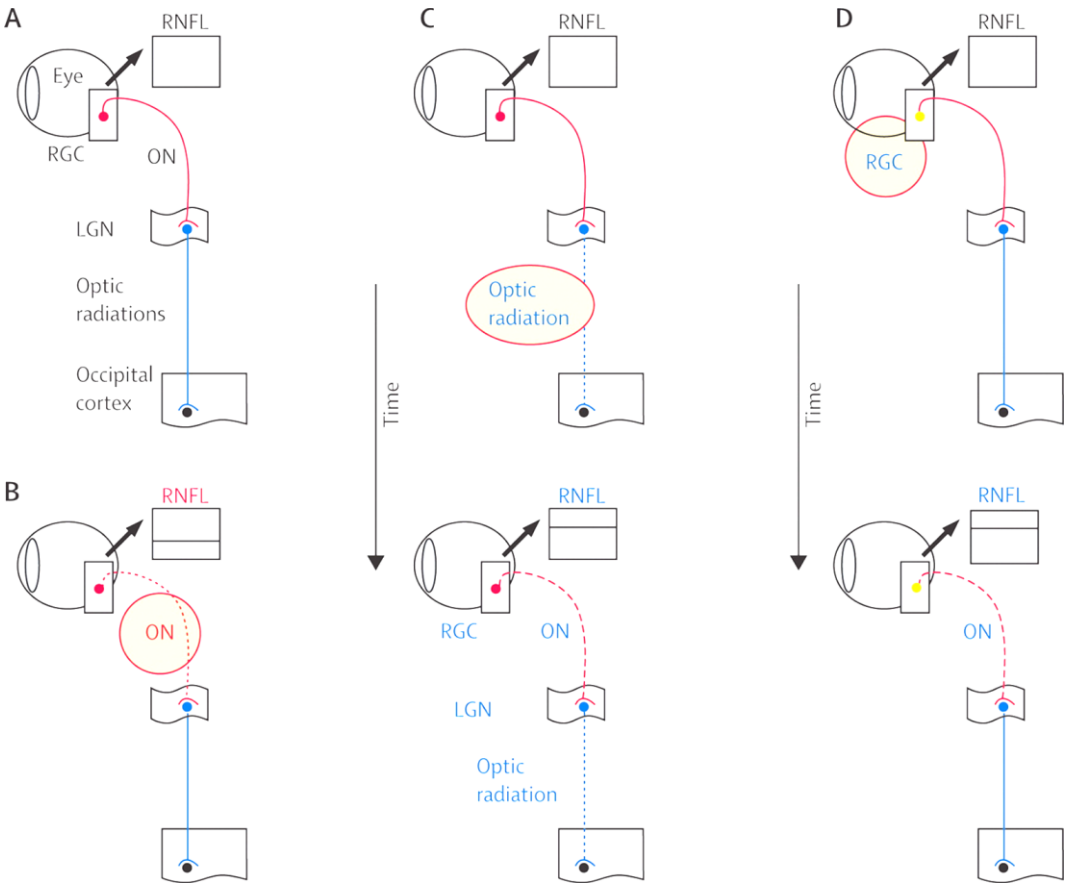

***Figure 5**. A model of the presumed relation between RNFL thickness and MS pathology*

(A) A simplified sketch of the human visual pathway. The unmyelinated axons of the Retinal ganglion cells (RGCs) form the RNFL (grey inlay), then continue to the optic disc, and leave the orbit. Once the axons pass the sclera they become myelinated and form the optic nerve (ON). They are called the optic tract after passing through the chiasma where the temporal fibers cross (not shown). The optic tract winds its way around the midbrain and enters the lateral geniculate nucleus (LGN), where all of these axons form synapses. Finally, the axons fan out through the deep white matter (optic radiations) to reach the occipital cortex. (B) In MS, optic neuritis directly causes acute axonal loss in the ON (red dotted line), leading to thinning of the RNFL (small grey box). (C) MS lesions within the optic radiations (blue dotted line) do not immediately result in RNFL thinning. This outcome is thought to be a chronic consequence of trans-synaptic axonal loss through the LGN. With time, trans-synaptic axonal degeneration causes a smaller amount of axonal loss in the ON (red dashed line), with a quantifiable degree of RNFL loss (grey box). (D) Progressive loss of RGCs (yellow dot) is a probable result of chronic changes in the anterior visual pathways themselves in MS, and causes a small amount of RNFL loss (grey box). Note that (C) and (D) both occur in the absence of optic neuritis. Taken from Petzold A et al., Lancet Neurol 2010.

1.3.3 Hyperreflective Foci

Hyperreflective foci (HRF) are small intraretinal, hyperreflective lesions that are detectable on structural linear Spectral-domain (SD) OCT scans in healthy individuals and in patients with both retinal and choroidal diseases. (figure 6) HRF have already been found to have a relevant role in several macular diseases, such as age-related macular degeneration, edema secondary to branch vein occlusion, and diabetic retinopathy.[59]

Currently, two hypotheses are considered with regard to the origin of the HRF. The first hypothesis suggests that they represent extravasated lipoproteins. In support of this hypothesis, a correlation between HRF count and two key parameters, namely globotriaosylsphingosine serum concentration and vessel tortuosity, was observed in Fabry disease (an X-linked inherited storage disorder caused by deficiency of lysosomal alpha-Galactosidase A). As the retina and in particular the macular area are highly vascularized and perfused, capillary dysfunction and concomitant endothelial glycosphingolipids deposition were described as a potential explanations of the HRF's origin.[60] The second describes the HRF as aggregates of activated proliferating microglial cells. In support of that hypothesis are the association with MRI parameters of cortical inflammation, the presence of HRF in pathologies which are not associated with retinal lipid deposition and the association between HRF and inflammatory markers (i.e., IL-8, V-CAM-1) in aqueous humor in patients with intractable macular edema. Additionally, in patients with RRMS, a correlation between the HRF count with CSF cytokines/chemokines and MRI parameters of both gray and white matter inflammation and degeneration was also detected. Finally, indirect evidence supports the hypothesis that these clusters of activated retinal microglia migrate close to the BRB probably in response to detrimental triggers. Nevertheless, the lack of available histological specimens of the human retina in vivo keeps the question on the origin and pathologic significance of HRF still open. [61,62]

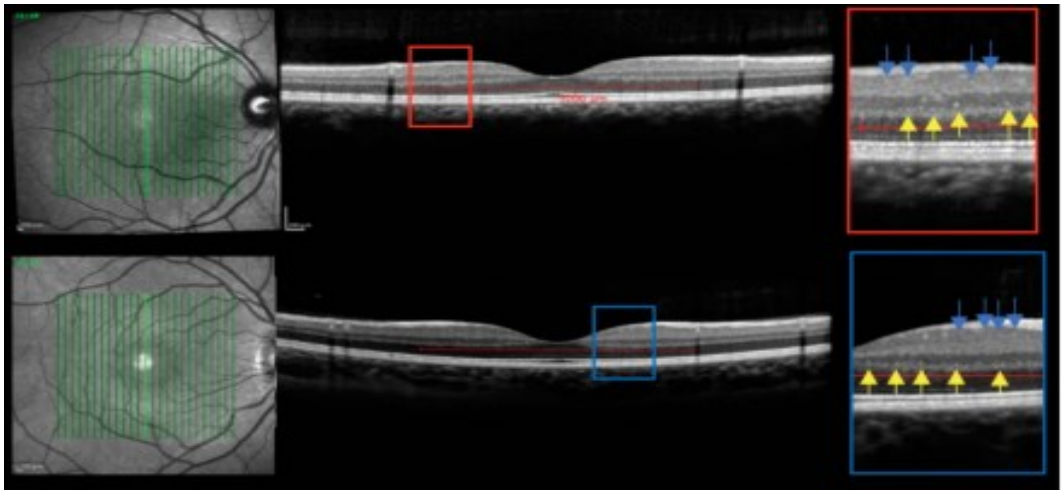

Figure 6 : *Macular scans and HRF visualization in RRMS (upper image) and healthy controls (lower image); INL foci are indicated by yellow arrows and ganglion cell and inner plexiform layer (GCIP) HRF by blue arrows.*

A study by Pilotto et al. showed that the presence of an increased number of HRF in the inner retina in the absence of retinal layer thinning or other signs of local pathologic features may indicate that in early RMS phases, microglial activation precedes any neurodegenerative process in the retina.[61]

The HRF count appear to be associated with INL volume as well as with cortical inflammation (especially in the gray matter), suggesting furthermore that the retina and the gray matter might share common immunopathogenic mechanisms. The change in the INL volume seem to further expand the association between the HRF count and INL microcystic macular edema (MME), which was also observed previously in patients with MS.[63] In MME the INL volume increases because of both an impairment of Müller cells to maintain retinal fluid homeostasis and an increased BRB permeability induced by microglial production of proinflammatory cytokines (IL-1 and IL-6) and inducible nitric oxide synthase. Due to the fact that, studies which supported the correlation between INL volume and HRF also excluded from their research patients with a history of optic neuritis, it is presumed that retinal microglial activation was not driven by optic nerve inflammation, but probably by local immunopathologic mechanism, hence suggesting that the retina is also a primary pathologic site in MS.[51,62] To conclude, the HRF count at baseline predicts the additional inflammatory events observed during the follow-up, indicating that they should be further explored as candidate prognostic biomarkers in MS.[51]

1.4 Clinical parameters

1.4.1 Expanded Disability Status Scale (EDSS)

During the past decades, the clinical severity and the functional deficits in MS, as well as the assessment of the effectiveness of treatments in clinical trials, were evaluated through a variety of instruments.

The most popular and widely used is Kurtzke's Expanded Disability Status Scale (EDSS).[64] The EDSS is a clinician-administered rating scale that assesses functional systems of the CNS. It is used for the evaluation of disease progression in patients with MS and for the assessment of the efficacy of therapeutic interventions in clinical trials. It consists of an ordinal rating system that ranges from 0 (indicating a normal neurological status) to 10 (which is death due to MS) at intervals of 0.5 points, when an EDSS of 1 is achieved. (figure 7) The lower end of the EDSS scale measures impairment on the basis of neurological examination, while the upper end of the scale (> EDSS 6) measures disability in people with MS.[65]

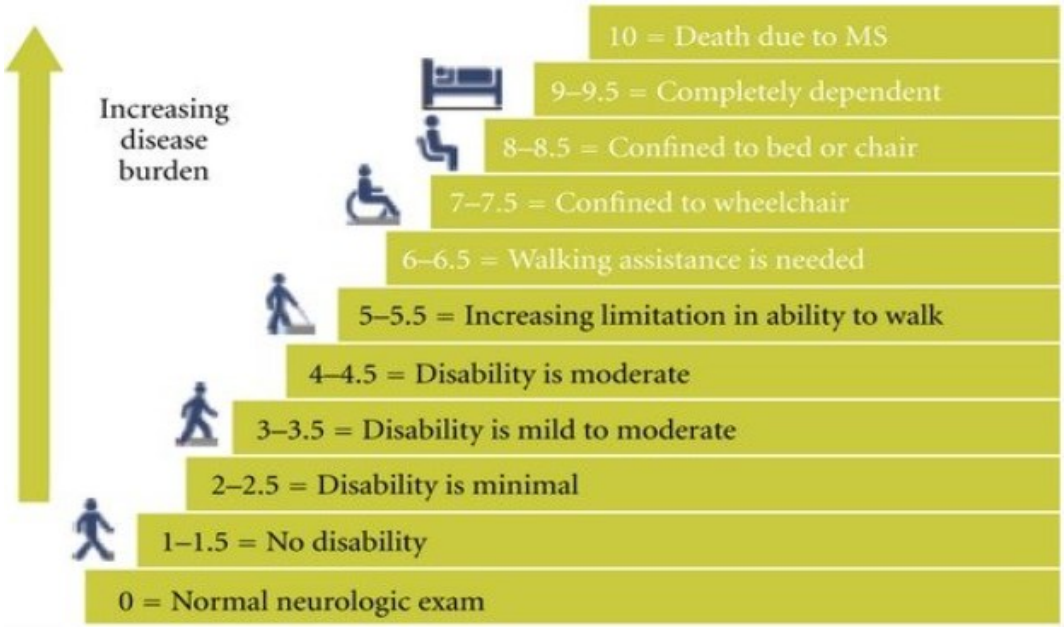

Figure 7: *The EDSS provides a total score on a scale that ranges from 0 to 10. The first levels 1.0 to 4.5 refer to people with a high degree of ambulatory ability and the subsequent levels 5.0 to 9.5 refer to the loss of ambulatory ability.*

1.4.2 No Evidence of Disease Activity (NEDA)

High-efficacy therapies (HETs) are being distinct from low- and moderate-efficacy DMTs because of their more robust impact on inflammation. This has led to a shift in disease management toward achieving the outcome assessment known as no evidence of disease activity (NEDA).

NEDA is a composite assessment based on both clinical and radiological criteria to evaluate the treatment efficacy of DMTs in patients with MS. The most common NEDA definition, NEDA-3, is composed of three related measures, namely no clinical relapses, no sustained disability progression (as defined by no increase in EDSS score), and no activity seen on MRI (i.e. new or enlarging T2 hyperintense lesions or gadolinium-enhancing lesions) during a specified time period, usually 3–12 months.

NEDA was predominantly used in clinical trials, however, recently there is also growing interest in its implementation as a tool to help patients and healthcare professionals in clinical decision making, when used as a treatment target. [66]

1.4.3 Progression Independent of Relapse Activity (PIRA)

Traditionally, in RRMS the accrual of irreversible disability is attributed to incomplete recovery from relapses, in contrast with progressive forms in which disability arise from relapse-independent mechanisms. Nonetheless, accumulating evidence suggests that progression unrelated to relapses is not restricted only to patients diagnosed with progressive forms of MS. Indeed, also in early phases of the disease, patients diagnosed with RRMS demonstrated already a substantial proportion of disability which was independent of relapse activity.[67] The disability that arises despite the lack of concomitant clinically evident relapses has been termed progression independent of relapse activity (PIRA) or silent progression, in contrast to relapse-associated disability worsening (RAW).

Currently, a uniform definition of PIRA doesn't exist. However, a recent review proposed a harmonized definition and diagnosis of PIRA, which is applicable both in RRMS and progressive MS, considering 4 determinants:[68]

1. baseline/reference score: the EDSS or individual measure taken as reference doesn't necessarily have to be the first chronologically, but it must be a roving baseline. A new reference score should be set every time the EDSS or individual measure of the composite is lower than the previous measure and confirmed at the following visit. The reference score should also be reset if a relapse causes residual disability.
2. Event score: an increase of EDSS or composite measure should only be considered for classification to PIRA, if it is not determined within 30 days before and 90 days after the onset of an investigator-reported relapse. For an increase of EDSS to be significant the following conditions should be met: an increase of EDSS score of 1.5 points or more from an EDSS of 0; an increase of 1.0 point or more from an EDSS of 1.0 to 5.0; or an increase of 0.5 point or more from an EDSS score of 5.5 or more. Moreover, a composite measure evaluation is recommended and should include:
    1. upper limb function: measured by Nine-Hole Peg Test (NHPT), which is widely considered a gold standard metric for manual dexterity.[69] Threshold: >20% decline compared to previous visit.
    2. walking speed: measured by the Timed 25-Foot Walk Test (T25FWT), that assesses mobility based on time and degree of assistance required when walking 25 feet as quickly as possible but safely. The T25FWT correlates strongly with other measures of walking and lower extremity function.[70] Threshold: >20% decline compared to previous visit.
    3. cognitive testing: information processing speed measured by Symbol Digit Modalities Test (SDMT), in which the patient is presented with a page headed by a key that pairs the single digits 1–9 with nine symbols. Rows below contain only symbols, the patient's task is to write or orally report the correct number in the spaces below. After completing the first 10 items with guidance, the subject is timed to determine how many responses can be made in

90 seconds. SDMT is considered the most sensitive metric of neurocognitive function in MS.[71] Threshold: ≥4 points or >10% decline compared to the previous visit.

3. Confirmation score: the confirmation visit should take place no earlier than 3 months, preferably 6, or 12 months after the initial disability increase and should not happen 30 days before and 90 days after the onset of an investigator-reported relapse.
4. Sustained score: the last visit of follow-up, sensibly at least 12 or even 24 months apart from start of PIRA. To be defined as sustained PIRA, the EDSS score defining PIRA should not improve beyond the requirement for a significant EDSS score increase compared to the baseline/reference score.

PIRA is the most frequent form of disability accumulation across various MS types, including CIS and RRMS.[72] Approximately 5% of RRMS patients experience PIRA annually. Therefore, based on that observation, the traditional distinction between relapsing and progressive MS stages has recently been challenged.

The prevalence of PIRA varies based on:

1. Definitions used (e.g., EDSS vs. composite measures).
2. Population under study (e.g., early vs. late MS/ the various MS phenotypes).
3. Length of follow-up.

The proportion of PIRA vs RAW increases with age, disease duration, and in particular in patients undergoing HET. The latter observation might be attributed to more effective suppression of acute relapse activity by DMT. The mechanisms underlying PIRA are thought to resemble those causing disability in progressive MS, as patients with RRMS and CIS exhibit comparable pathological features.[68]

2. Purpose of the study

The purpose of this single-center, cross-sectional pilot study is to:

1) Investigate the impact of high-efficacy therapies (HET) on the retinal layers and HRF in patients with MS.
2) Compare the retinal layers thickness, volumes and HRF between Ofatumumab and S1PR modulators treated patients.
3) Monitor the behavior of the HRF.

3. Materials and Methods

3.1 Study population

The study conducted is a prospective, single-center, longitudinal study. Patients diagnosed with RRMS were divided into 2 treatment cohorts:

1. Ofatumumab treatment cohort, which was composed of 14 patients.
2. S1PR modulators cohort, which was composed of 11 patients, treated with either Siponimod, Ozanimod or Ponesimod.

The 2 cohorts were recruited from September 2023 to February 2024, at the Day Hospital of the Neurological Clinic of the University Hospital of Padua.

Inclusion criteria were:

1. A confirmed diagnosis of RRMS according to the most recent McDonald criteria (the 2017 revision), with or without a positive history of optic neuritis (defined by patient's history, as described in the Optic Neuritis Trial)[73] and confirmed by a rigorous ophthalmic assessment.
2. Indication of Ofatumumab or S1PR modulators treatment in line with AIFA (Agenzia Italiana del Farmaco) criteria.
3. Acquisition of 2 OCT scans of which the first at the date of treatment initiation or no later than a maximum of one month after it (time T0), the second approximately 6 months after the beginning of the therapy (time T1).

Exclusion criteria were:

1. Any systemic disease capable of causing retinal alterations other than MS.
2. Severe ocular pathology (such as: severe myopia > -6 dp or axial eye length > 26 mm, severe hypermetropia > 5dp, cylinders > 3dp, optic disc drusen, cataract, ongoing or a history of glaucoma, or other causes of vision loss not attributable to MS) and toxic retinal damage.
3. A high-dose steroid therapy within 30 days prior to OCT acquisition.

The following data was then collected from the enrolled patients: date of birth, sex, date of diagnosis, date of treatment initiation with Ofatumumab or S1PR modulators respectively, as well as any previous therapies carried out. Finally, the dates of the various OCT acquisitions with the corresponding EDSS calculated at the routine examination carried out on the same occasion.

3.2 OCT image acquisition protocol

All patients underwent OCT with SPECTRALIS®HRA+OCT (Heidelberg Engeneering, Heidelberg, Germany), which combines SD-OCT technology with confocal laser scanning ophthalmoscopy (cSLO) with infrared wave (IR, 820 nm). TruTrack™ technology allows to actively track the eye while scanning all images at high speed (40000 scans/second). Thanks to the simultaneous use of a dual beam, eye movement tracking help also in reducing motion artefacts, background noise and in the variation of the trajectory over time. The result is a punctual correlation between fundus oculi and OCT scans, combined with improved image quality, and image stabilization for small shifts. OCT scans were acquired without the use of mydriatic agents in a darkened room, with natural light, by experienced operators.

The protocol encompassed two scans, a circular peripapillary and a macular volumetric scan.

A circular peripapillary scan of 3.4 mm diameter centered on the optic nerve head, as shown in figure 8, was used to measure RNFL thickness (μm), both globally (pRNFL) and by sector: temporal (T), superior temporal (ST), inferior temporal (IT), papillo-macular bundle (PMB), nasal (N), superior nasal (SN), inferior nasal (IN). Each scan was processed using ART (Automatic Real-Time), which increases image quality by reducing motion related artefacts and optimizing the signal to noise ratio. Images with an ART between 90 and 100 were considered valid.

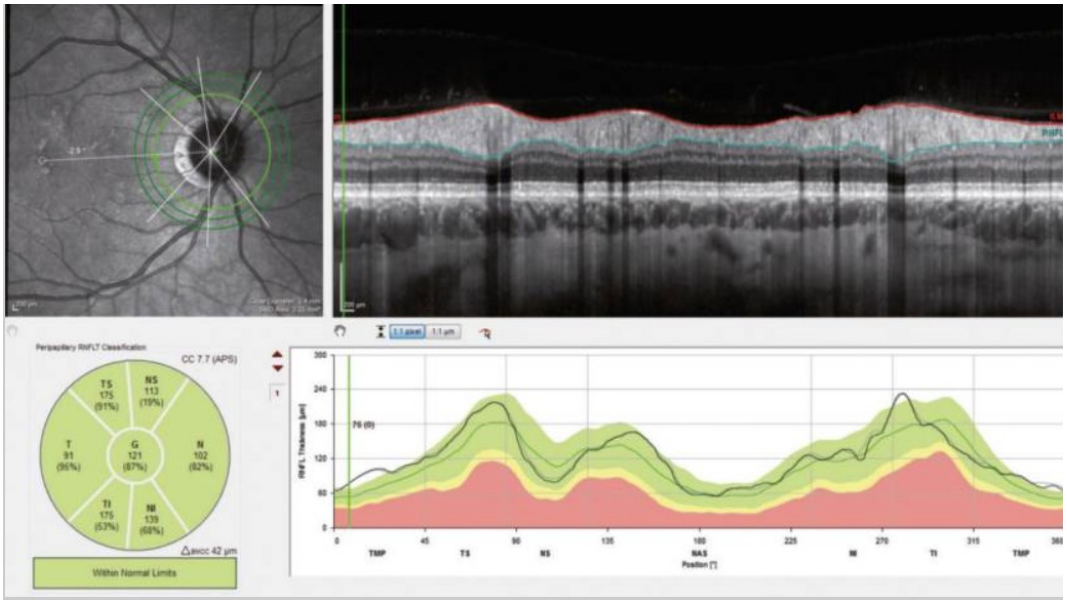

Figure 8: *Scan display screen for pRNFL thickness analysis.*

Macular volume scan of 20x20° automatically centered on the fovea obtained with 25 vertical B-scans, with a distance of 240 µm between B-scans, and ART 49. The software allows the assessment of the total macular volume (VM), calculated as the volume subtended by a surface defined by a circle with the fovea as it's center and a 2 mm radius. The Early Treatment Diabetic Retinopathy Screening (ETDRS) is a 9 sector map of the macular area divided into an outer and an inner ring. Both rings, with diameters of 3mm and 6 mm respectively, are each segmented into 4 quadrants (superior, inferior, nasal, temporal). Two numbers are displayed in each of the four quadrants: the black numbers represent the retinal thickness ratios, the red numbers the volume ratios. These numbers represent the ratio of the inner quadrant of the "1, 3, 6 mm ETDRS" to the outer quadrant. The numbers in the center are the thickness and volume ratios of the total inner ring to the total outer ring. Each macular scan was automatically segmented into the different retinal layers, obtaining the volumes (expressed in mm^3) and thicknesses of the following layers of interest:

A. Macular Retinal Nerve Fiber Layer (mRNFL)
B. Ganglion Cell Layer (GCL)
C. Inner Plexiform Layer (IPL)
D. Inner Nuclear Layer (INL)
E. Outer Plexiform Layer (OPL)

F. Outer Nuclear Layer (ONL)

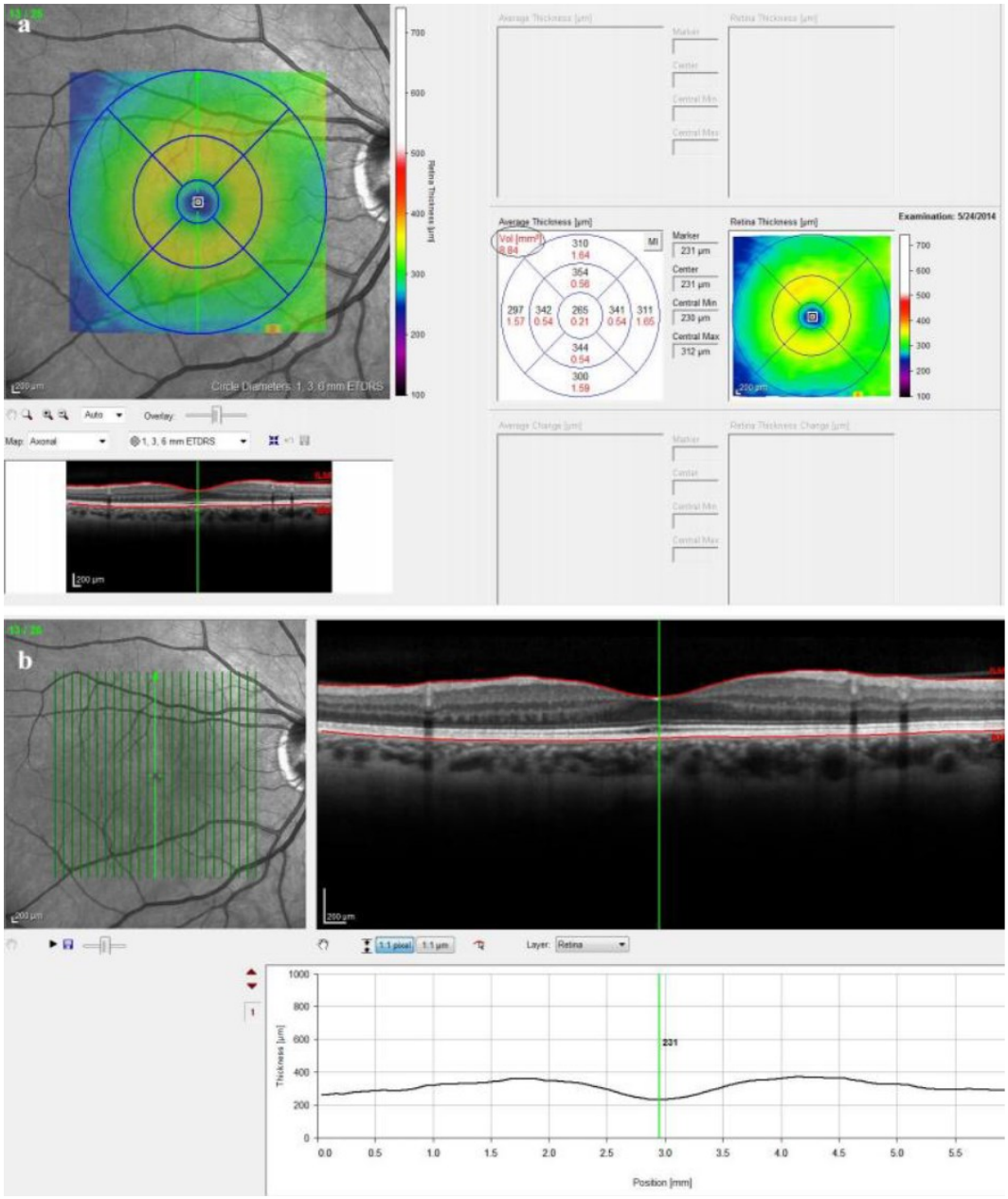

Figure 9: *Macular scan:* ***a)*** *9 sector ETRDS map* ***b)*** *The acquisition and segmentation display of the retinal layers.*

Utilizing the machine's software, all peripapillary and macular scans were segmented automatically. In particular, an automatic algorithm determines the thickness of the pRNFL, as well as the total volumes of the macular layers based on the chromatic differences in the grey scale corresponding to the reflectivity indices specific to each layer. Each SD-OCT scan was then reevaluated by an

experienced neurologist to apply manual segmentation correction when it was needed, thus ensuring the accuracy of stratification

3.3 Hyperreflective retinal foci (HRF)

In line with recent publications,[59,61] only the central linear macular scan, which passes through the fovea, was considered for counting of the HRF. They were counted in an area between two lines that are perpendicular to Bruch's membrane drawn at 1500 μm, both temporally and nasally from the center of the fovea. HRF were defined as isolated, small (<30 μm), punctiform elements with moderate reflectivity (resembling the RNFL's reflectivity) but without any posterior shadow (Figure 10). Their count was conducted in the inner retinal layers, and was performed separately in each of the following retinal areas: GCIPL, ICP (which is situated between IPL and INL), INL, DCP (which is situated between INL and OPL) and OPNL.

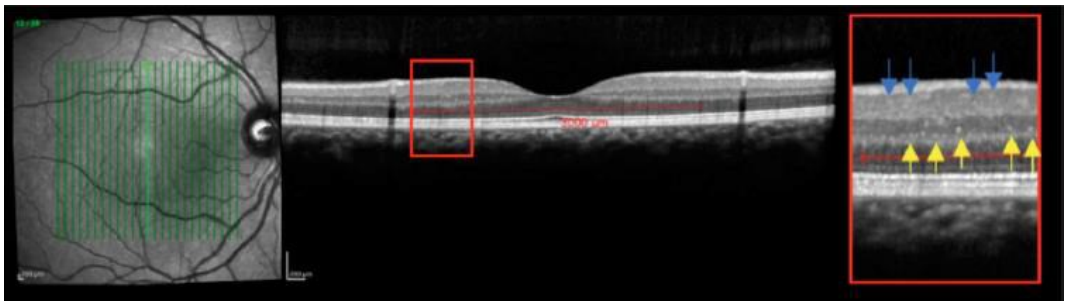

Figure 10: *A linear scan centered on the macula and passing through the fovea. HRF at in the INL are indicated by yellow arrows, while those within the GCL and IPL by blue arrows.*

3.4 Statistical Analysis

The results obtained from the statistical analysis are represented by the mean and standard deviation (± SD) for the continuous variables and by N (%) for the categorical variables. The analysis was conducted as a confrontation of the clinical parameters obtained at baseline (T0) and during follow-up (T1), as well as a confrontation of the change of these variables (from T0 to T1) between the two treatment cohorts. Variables such as HRF count and the pRNFL thickness were represented as the calculated mean in each retinal layer obtained from both eyes. These parameters were compared using either a two-tailed parametric T-test or the non-parametric Mann Whitney U-test based on their distribution. All statistical analyses were performed using Graphpad Prism software, and p values < 0.05 were considered statistically significant.

4. Results

4.1 Description of the study population

To this study, 25 participants were recruited (amounted to a total of 49 eyes that were examined) and divided into 2 treatment cohorts: the first, composed of 14 patients, underwent a therapy with Ofatumumab. The second, composed of 11 patients, underwent a therapy with S1PR modulators. Both cohorts were examined using an OCT scan both at baseline and (T0) after 6 months (T1).

The main clinical and demographic characteristics of the study population are summarized in Table I. The study population had a mean overall age of 37.36 years (±11.55), a mean age of 34.14 (±13.06) in the Ofatumumab group and a mean age of (41.45 ± 8.1) in the S1PR modulators group. A total of 16 women (64%) participated, 9 of them belonged to the Ofatumumab group and the resto to the S1PR modulators cohort. The mean disease duration, calculated as the time interval between the date of diagnosis and the date of initiation of either Ofatumumab or S1PR modulators therapy, stood at 77.2 months overall, for the Ofatumumab group the mean stood at 36.21 months and 129.4 months for the S1PR modulators group. The median EDSS at baseline was 1.5 overall (range 0-6.5) overall, for the Ofatumumab cohort the median stood at 1.0 (range 0-4.5) and 1.5 (range 1.0-6.5) for the S1PR modulators cohort. Moreover, 24% of patients (6/25) overall presented with a clinical onset of disease characterized by monocular optic neuritis.

Age (y)	Overall	37.36 ±11.55
	S1PR Modulators	41.45 ± 8.1
	Ofatumumab	34.14 ± 13.06
Female sex (%)	Overall	16 (64%)
	S1PR Modulators	7 (64%)
	Ofatumumab	9 (64%)
Mean of disease duration (m)	Overall	77.2 ± 92.99
	S1PR Modulators	129.4± 108.3
	Ofatumumab	36.21 ± 53.27
Basal EDSS (range)	Overall	1.5 (range 0-6.5)
	S1PR Modulators	1.5 (range 1.0-6.5)
	Ofatumumab	1.0 (range 0-4.5)
Optic neuritis (%)	Overall	6 (24%)
	S1PR Modulators	3 (27%)
	Ofatumumab	3 (21%)

Table I*: Demographic and clinical characteristics of the study population y: years, m: months EDSS: Expanded Disability Status Scale.*

Regarding previous therapies, as described in Table II, 16 patients out of 25 (64%) underwent other immunomodulatory therapies. The vast majority of them, 10 patients out of 16 (62.5%) are patients from the S1PR modulators cohort. On the other hand, in 36% of patients Ofatumumab or S1PR modulators therapy was administered as a first DMT therapy.

Number of previous treatments	**Number of patients (%)**		
	Overall	**Ofatumumab**	**S1PR modulators**
0	9 (36%)	8 (57%)	1 (9%)
1	7 (28%)	2 (14%)	5 (45%)
2	8 (32%)	4 (29%)	4 (36%)
3	1 (4%)	0	1 (9%)

Table II: *Number of previous treatment in the study population.*

An increase in the EDSS score of the study population during the observation period was registered in 5 patients (20%) but a progression of disability (expressed as worsening of EDSS ≥ 1 in patients with EDSS < 5.5, confirmed at 6 months) occurred in only one of them. EDSS score remained unchanged in 17 patients (68%) and decreased in 3 patients (12%).
Finally, MRI alteration was recorded only in 3 patients out of 25 (12%) compared to their control at baseline.

4.2 Analysis of OCT parameters

The following section describes the analysis of the changes in retinal layers volume and thickness at the peripapillary and macular area, as well as the modification of HRF detected in the inner retinal layers.

4.2.1 The effect of Ofatumumab and S1PR modulators on pRNFL

We initially observed the effect of Ofatumumab and S1PR modulators on the thickness of pRNFL which was divided into the various sectors. From an initial analysis obtained by calculating the mean of the various thicknesses recorded in each quadrant, of both eyes, at each time point, we obtain the data summarized in Table III.

	Mean thickness (± SD) (µm)					
	Ofatumumab T0	Ofatumumab T1	P value	S1PR modulators T0	S1PR modulators T1	P value
pRNFL-G	96.5 ± 7.47	96.11 ± 7.47	0.1956	91.18 ± 12.7	90.27 ± 12.9	0.0878
pRNFL-PMB	44.46 ± 6.35	43.43 ± 6.93	0.0852	43.41 ± 6.35	42.91 ± 8.32	0.2737
pRNFL-NTRatio	1.31 ± 0.26	1.32 ± 0.25	0.2926	1.39 ± 0.52	1.38 ± 0.52	0.8066
pRNFL-NS	114.3 ± 15.74	114.6 ± 15.88	0.6864	94.27 ± 15.84	93.18 ± 16.28	0.1451
pRNFL-N	75.18 ± 10.42	75.32 ± 9.7	0.7336	74.73 ± 15.54	73.91 ± 16.14	0.1368
pRNFL-NI	121 ± 15.58	120 ± 11.95	0.1393	107.9 ± 20.73	105.5 ± 20.41	**0.0025**
pRNFL-TI	133.1 ± 13.92	131.3 ± 14.71	0.0715	135 ± 27.11	134 ± 27.08	0.1005
pRNFL-T	58.46 ± 8.43	58.04 ± 8.6	0.1894	57.23 ± 11.46	57 ± 11.27	0.4374
pRNFL-TS	136.3 ± 13.94	136 ± 13.92	0.6633	129 ± 22.47	127.2 ± 22.17	0.0851

Table III: *Mean thickness in various sectors of the pRNFL at each time point. G=global, PMB=papillomacular bundle, NTRatio= nasal-temporal ratio, NS= nasal superior, N= nasal, NI=nasal inferior, TI=temporal inferior, T=temporal, TS=temoral superior.*

As described in figure 11. statistical significance change in thickness between follow-up and baseline was obtained in pRNFL-NI in the S1PR modulators cohort, where a tendency of thickness decrease was detected.

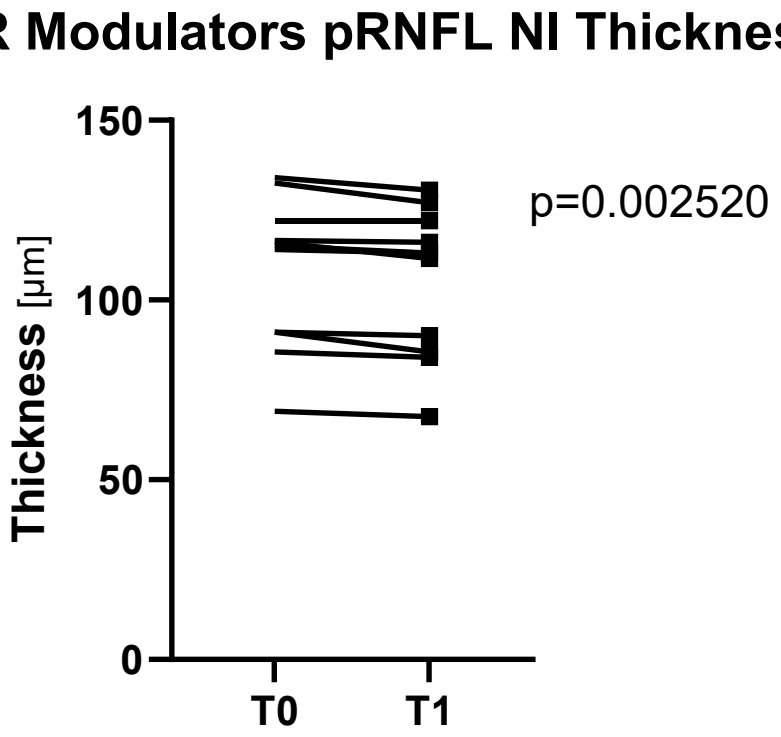

Figure 11: *comparison between T0 and T1 in pRNFL-NI in the S1PR modulators cohort*

4.2.2 The effect of Ofatumumab and S1PR modulators on macular volumes and thicknesses

We proceeded then with the analyses of the effect of Ofatumumab and S1PR modulators on macular volumes and thicknesses. We calculated, in a completely similar manner, the mean values recorded for each layer in both eyes, and at each time point, as shown in Table IV.

	Mean Volume (± SD) (mm^3)					
	Ofatumumab T0	Ofatumumab T1	P value	S1PR modulators T0	S1PR modulators T1	P value
mRNFL-TV	0.82 ± 0.11	0.82 ± 0.12	0.6166	0.79 ± 0.11	0.8 ± 0.11	0.2554
mGCL-TV	1.06 ± 0.1	1.07 ± 0.1	0.2807	0.98 ± 0.12	0.97 ± 0.12	**0.05**
mIPL-TV	0,9 ± 0.07	0.89 ± 0.08	0.3725	0,83 ± 0.08	0.83 ± 0.08	0.9187
mINL-TV	0.99 ± 0.06	0.99 ± 0.07	0.6696	0.96 ± 0.07	0.96 ± 0.06	>0.9999
mOPL-TV	0.81 ± 0.05	0.81 ± 0.07	0.6571	0.82 ± 0.05	0.81 ± 0.04	0.3893
mONL-TV	1.82 ± 0.17	1.81 ± 0.17	0.5103	1.79 ± 0.18	1.81 ± 0.19	0.1640

Table IV: *mean volume of each macular layer. TV=total volume*

In figure 12, we can notice a statistically significant reduction in total volume of GCL of the S1PR modulators cohort from the baseline total volume value to the value measured during follow-up.

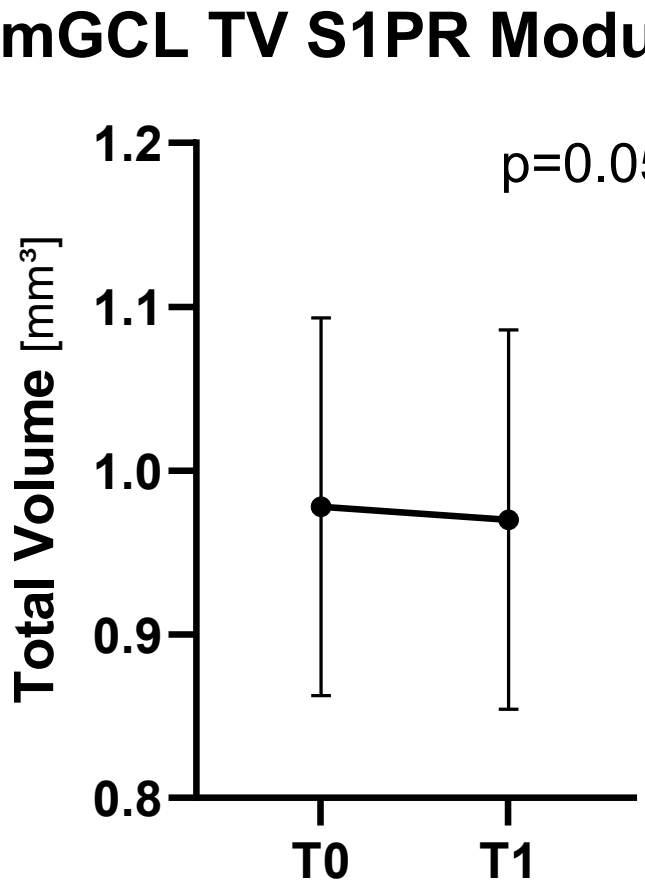

Figure 12: *comparison of the total volume of GCL between T0 and T1of the S1PR modulators cohort*

In calculating the thicknesses of the macular layers, we proceeded by calculating the mean values for each sector of both the inner and outer rings into which the each macular layer is subdivided, as described in Table V.

	Mean thickness (± SD) (µm)					
	Ofatumumab T0	Ofatumumab T1	P value	S1PR modulators T0	S1PR modulators T1	P value
mRNFL-OR	32.37 ± 4.6	32.46 ± 4.91	0.7489	31.1 ± 4.59	31.42 ± 4.61	0.1657
mRNFL-IR	19.5 ± 1.85	19.63 ± 2.1	0.6172	19.25 ± 1.9	19.47 ± 1.63	0.4102
mGCL-OR	35.43 ± 3.27	35.63 ± 3.14	0.1591	32.67 ± 3.7	32.28 ± 3.7	**0.0140**
mGCL-IR	47.83 ± 6.14	48.04 ± 6.13	0.6103	43.72 ± 6.552	43.49 ± 6.73	0.3782
mIPL-OR	29.46 ± 2.4	29.24 ± 2.59	0.2822	27.47 ± 2.45	27.38 ± 2.43	0.6051
mIPL-IR	40.77 ± 3.77	40.68 ± 4.01	0.7070	37.51 ± 4.06	37.83 ± 3.97	0.2400
mINL-OR	34.7 ± 1.85	34.8 ± 2.18	0.6441	33.08 ± 2.51	33.1 ± 2.42	0.8968
mINL-IR	39.01 ± 3.11	38.96 ± 3.58	0.8681	38.41 ± 2.86	38.16 ± 2.68	0.6552
mOPL-OR	27.7 ± 1.79	27.81 ± 2.11	0.5720	27.67 ± 1.71	27.51 ± 1.28	0.6229
mOPL-IR	32.02 ± 2.2	32.8 ± 3.4	0.2947	33.75 ± 3.2	33.01 ± 2.53	0.4621
mONL-OR	60.65 ± 5.37	60.38 ± 5.3	0.4994	59.64 ± 6.48	60.09 ± 6.29	0.1464
mONL-IR	73.4 ± 8.1	72.94 ± 8.04	0.5680	72.49 ± 7.34	73.6 ± 8.47	0.3208

***Table V*:** *mean thickness of each macular layer further divided into two rings. OR=outer ring, IR=inner ring*

In figure 13, we described the comparison of thickness values between baseline and follow-up of mGCL (which was further divided into 2 rings). A statistical significance reduction in thickness of the mGCL OR was observed, as well in the S1PR modulators cohort from the baseline value to follow-up.

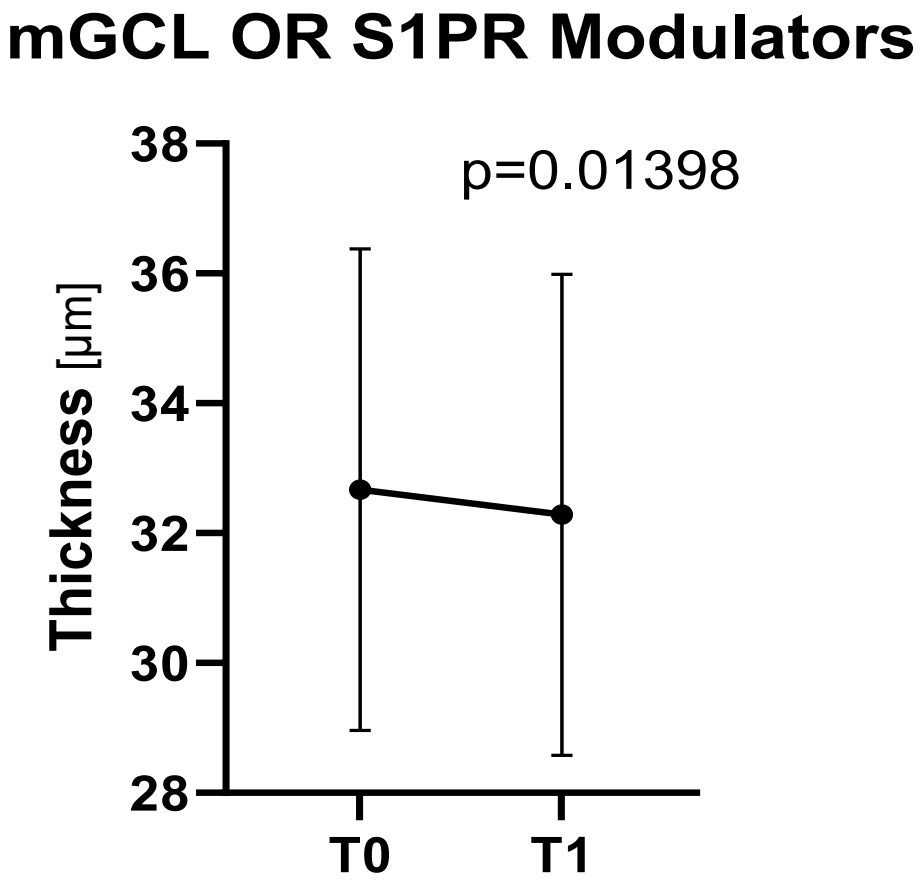

Figure 13: *comparison between T1 and T0 thickness values in each macular layers divided, further divided into outer and inner rings.*

4.2.3 The effect of Ofatumumab and S1PR modulators on HRF

The effect of these high effective therapies on hyperreflective foci (HRF) was also analyzed. We used the same method for the sample representation used earlier for the layer thicknesses and volumes, namely calculating the mean HRF count of both eyes in each retinal layer as shown in Table VI.

	Mean HRF count (± SD)					
	Ofatumumab T0	Ofatumumab T1	P value	S1PR modulators T0	S1PR modulators T1	P value
HRF GCIP	2.43 ± 1.25	2.14 ± 1.54	0.4673	3.59 ± 2.67	3.64 ± 2.19	0.9255
HRF ICP	2.79 ± 1.71	2.89 ± 1.62	0.8506	2.91 ± 2.12	2.05 ± 1.52	**0.0473**
HRF INL	0.21 ± 0.32	0.21 ± 0.32	>0.9999	0.36 ± 0.32	0.36 ± 0.5	>0.9999
HRF DCP	4.25 ± 2.29	4.86 ± 2.69	0.3285	5.09 ± 3.7	5.23 ± 3.33	0.8648
HRF OPNL	1.04 ± 0.99	1.07 ± 0.7	0,8796	0.77 ± 1.17	1.13 ± 0.45	0.1582

Table VI: *HRF count in each retinal layer at baseline and during follow-up. ICP= intermediate capillary plexus (transitional area between IPL and INL), DCP=deep capillary plexus (transitional area between INL and OPL)*

In figure 14, we are able to notice that there was statistically significant decrease in HRF count in the ICP area of the retinal in the S1PR modulators cohort.

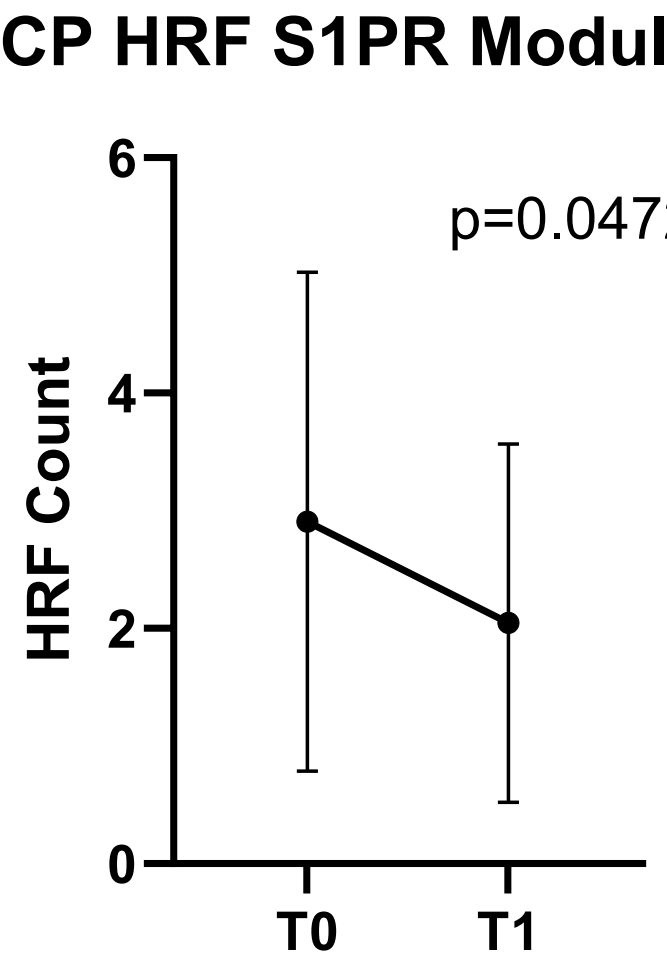

Figure 14: *HRF count at baseline and during follow-up of the S1PR modulators cohort in the ICP.*

4.2.4 Comparison between Ofatumumab and S1PR modulators

For the comparison between ofatumumab treated patients and S1PR modulators treated patients we calculated for each group the difference between values obtained at baseline and during follow-up, as well as the percentage difference obtained from dividing the value of the difference between baseline and follow-up and the baseline value. In Table VII we calculated the values mentioned earlier of the pRNFL thickness for each treatment cohort.

	Mean thickness (± SD) (µm)					
	Difference T1-T0 Ofatumumab	Difference T1-T0 S1PR modulators	P value	Percentage Difference T1-T0 Ofatumumab (%)	Percentage Difference T1-T0 S1PR modulators (%)	P value
pRNFL-G	-0.393 ± 1.077	-0.91 ± 1.59	0.2872	-0.4 ± 1.2	-1 ± 1.8	0.3393
pRNFL-PMB	-1.036 ± 2.080	-0.5 ± 1.432	0.4638	-2.3 ± 4.47	-1.2 ± 3.38	0.4622
pRNFL-NTRatio	0.0092 ± 0.032	0.002 ± 0.02	0.5017	0.75 ± 2.5	-0.7 ± 3.9	0.4030
pRNFL-NS	0.32 ± 2.913	-1.091 ± 2.289	0.2005	0.23 ± 2.4	-1.2 ± 2.4	0.1349
pRNFL-N	0.1429 ± 1,537	-0.455 ± 1.313	0.3151	0.08 ± 2.1	-1.22 ± 2.53	0.1724
pRNFL-NI	-1.036 ± 2.461	-2.318 ± 2.089	0.1809	-0.93 ± 2.2	-2.1 ± 1.8	0.1721
pRNFL-TI	-1.821 ± 3.473	-1.14 ± 1.83	0.7152	-1.4 ± 2.8	-0.87 ± 1.64	0.6089
pRNFL-T	-0.4286 ± 1.158	-0.318 ± 0.845	0.7933	-0.72 ± 2.1	-0.42 ± 1.72	0.7008
pRNFL-TS	-0.2857 ± 2.4	-1.909 ± 3.081	0.2794	-0.31 ± 1.9	-1.38 ± 2.5	0.3483

Table VII: *Difference and percentage difference values of Ofatumumab and S1PR cohorts in the various pRNFL sectors, obtained from baseline and follow-up values.*

As observed in table VII, the comparison between the two patient groups did not yield any statistically significant difference in the pRNFL thickness in the various sectors.

In table VIII, we described the difference and percentage difference values between the two treatment cohorts of the total volume for all macular layers, obtained in an identical manner to the earlier peripapillary thickness comparison.

	Mean Volume (± SD) (mm^3)					
	Difference T1-T0 Ofatumumab	Difference T1-T0 S1PR modulators	P value	Percentage Difference T1-T0 Ofatumumab (%)	Percentage Difference T1-T0 S1PR modulators (%)	P value
mRNFL-TV	0.004 ± 0.026	0.008 ± 0.018	0.6570	0.43 ± 3.47	0.98 ± 2.64	0.6637
mGCL-TV	0.005 ± 0.018	-0.008 ± 0.011	0.0422	0.56 ± 1.99	-0.76 ± 1.19	0.0498
mIPL-TV	-0.004 ± 0.014	0.001 ± 0.014	0.3992	-0.4 ± 1.6	-0.02 ± 1.62	0.6961
mINL-TV	0.002 ± 0.018	-0.0009 ± 0.021	0.7009	0.21 ± 1.88	0.14 ± 2.16	0.9304
mOPL-TV	0.004 ± 0.032	-0.01 ± 0.034	0.2965	0.52 ± 4.13	-1.14 ± 4.29	0.3364
mONL-TV	-0.009 ± 0.047	0.018 ± 0.035	0.1301	-0.45 ± 2.55	0.86 ± 1.94	0.1705

Figure VIII: *Difference and percentage difference values of Ofatumumab and S1PR cohorts of the total volume values in the various macular layers, obtained from baseline and follow-up values.*

In figure 15 we compared the difference, both absolute and in percentage between the two patient cohorts. As can be observed, a statistically significant difference in GCL, in both parameters (p=0.04218 for the difference parameter and p=0.04976 for the percentage difference parameter), between the Ofatumumab and S1PR modulators cohorts was achieved. The S1PR modulators cohort presented a decrease in total volume in GCL, which was found indeed to be statistically significant compared to the change in total volume of the same macular layer in the Ofatumumab cohort.

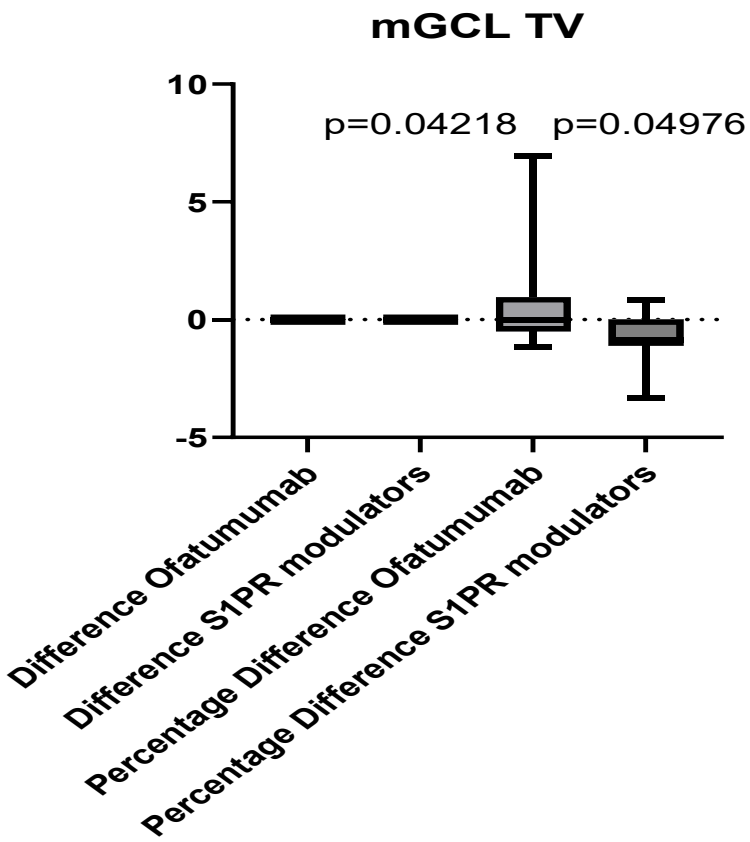

Figure 15: *Confrontation between Ofatumumab and S1PR modulars cohorts of the difference and percentage difference values of the total volume in mGCL.*

In table IX we calculated and summarized the difference and percentage difference values (obtained in the same manner as for the other two comparisons) of the thickness of each macular layer further divided into two rings.

	Mean thickness (± SD) (µm)					
	Difference T1-T0 Ofatumumab	Difference T1-T0 S1PR modulators	P value	Percentage Difference T1-T0 Ofatumumab (%)	Percentage Difference T1-T0 S1PR modulators (%)	P value
mRNFL-OR	0.09 ± 1.022	0.341 ± 0.689	0.4912	0.316 ± 3.522	1.14 ± 2.53	0.5181
mRNFL-IR	0.125 ± 0.913	0.284 ± 0.775	0.6489	0.58 ± 4.821	1.245 ± 4.45	0.7266
mGCL-OR	0.196 ± 0.492	-0.341 ± 0.444	**0.0096**	0.562 ± 1.521	-1.13 ± 1.37	**0.0084**
mGCL-IR	0.205 ± 1.471	-0.318 ± 0.717	0.4409	0.647 ± 3.971	-0.511 ± 1.67	0.5007
mIPL-OR	-0.214 ± 0.715	-0.068 ± 0.563	0.5840	-0.77 ± 2.418	-0.363 ± 1,948	0.6543
mIPL-IR	-0.089 ± 0.869	0.386 ± 0.778	0.1687	-0.154 ± 2,371	0.702 ± 2,06	0.3537
mINL-OR	0.098 ± 0.777	0 ± 0.562	0.7278	-0.263 ± 2.23	0.134 ± 1,64	0.8739
mINL-IR	-0.053 ± 1.184	-0.432 ± 1.646	0.5103	-0,095 ± 3.123	-0.23 ± 4.596	0.9325
mOPL-OR	0.116 ± 0.749	-0.205 ± 1.019	0.3735	0.439 ± 2.774	-0.514 ± 3.624	0.4634
mOPL-IR	0.786 ± 2.692	-0.648 ± 3.21	0.2368	2.667 ± 8.736	-2.21 ± 9.731	0.2007
mONL-OR	-0.277 ± 1.49	0.5 ± 0.919	0.1438	-0.466 ± 2.474	0.696 ± 1.47	0.1823
mONL-IR	-0.464 ± 2.966	1.182 ± 3.503	0.2159	-0.523 ± 3.93	1.516 ± 4,72	0.2503

Table IX: *Confrontation between Ofatumumab and S1PR modulars groups of the difference and percentage difference values of the thickness in each macular layer inner and outer ring.*

In figure 16 we can notice that, there has been a statistically significant difference in OR GCL thickness between the two treatment groups, where the Ofatumumab cohort presented greater thickness values compared to S1PR modulators cohort.

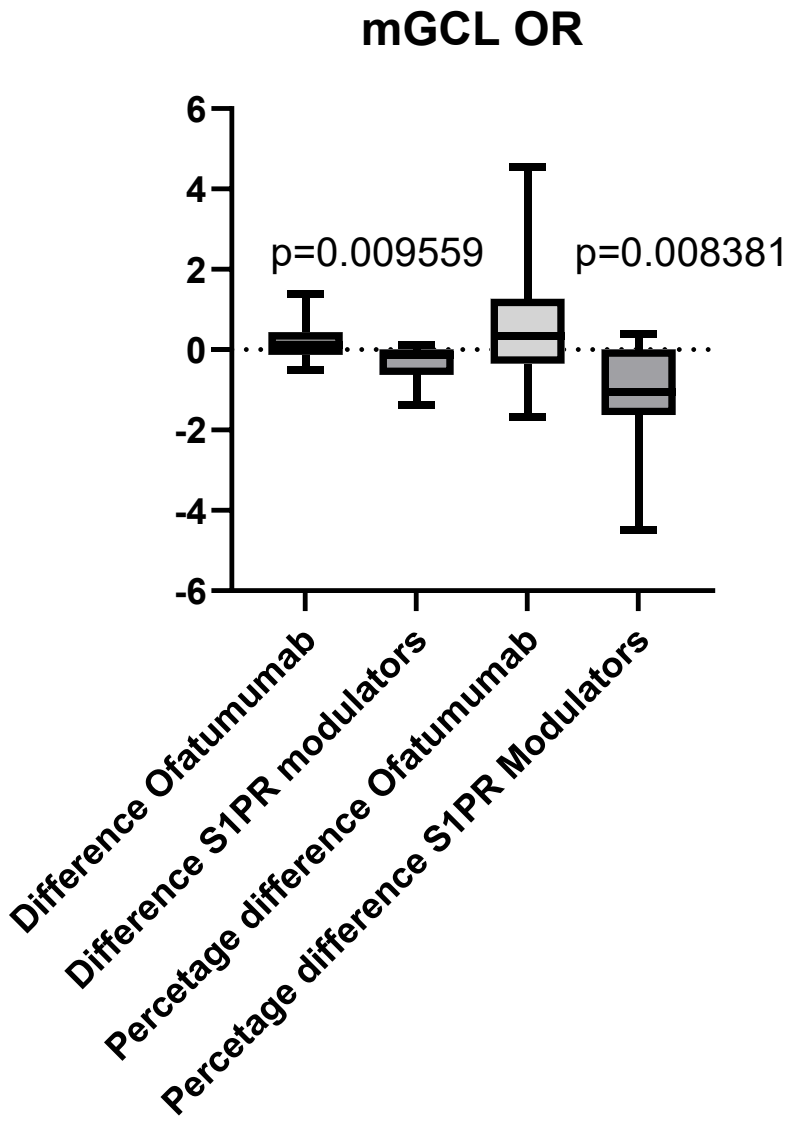

Figure 16: *Confrontation between Ofatumumab and S1PR modulars cohorts of the difference and percentage difference values of the thickness values of the mGCL outer ring.*

In Table X we calculated as well the difference between baseline and follow-up HRF count in each of the examined retinal layers for both treatment cohorts. As shown, no statistically significant difference between Ofatumumab and S1PR modulators treatment cohorts was achieved.

	Mean HRF count (± SD)		
	Difference T1-T0 Ofatumumab	Difference T1-T0 S1PR modulators	P value
HRF GCIP	-0.286 ± 1.31	0.046 ± 1.572	0.5712
HRF ICP	0.107 ± 2.086	-0.864 ± 1.27	0.1883
HRF INL	0 ± 0.277	0 ± 0.707	>0.9999
HRF DCP	0.607 ± 2.238	0.136 ± 2.589	0.6305
HRF OPNL	0.036 ± 0.865	0.364 ± 1.164	0.2011

Table X: *Difference values of HRF count of Ofatumumab and S1PR modulators treatment groups.*

5. Discussion

This prospective study was designed to evaluate the effectiveness of HET on unconventional clinical parameters in patients with MS, as well as to assess the difference in those parameters between patients treated with Ofatumumab and patients treated with S1PR modulators. In this study we monitored the changes that occur in the retinal layers as a consequence to the characteristic inflammatory process seen in MS. As emerged from several studies, changes in the retina, and in particular the thinning of retinal layers thickness, changes in retinal layers volumes are associated with the clinical picture of patients, EDSS score and eventually the response to therapy. [53–55] Moreover, the HRF count appears to be associated with INL volume as well as with cortical inflammation, and in particular with the presence of brain gadolinium-enhancing lesions. [51] Hence, we conducted this study in order to analyze those parameters in our study population. Furthermore, we monitored the behavior of the HRF located in the inner retina with the goal to explore their origin and significance. The results obtained showed, on the one hand, an overall stability during the observation period in the Ofatumumab cohort in terms of the volumes and thicknesses of the peripapillary area and the macula. On the other hand, the analysis conducted on the S1PR modulators cohort yielded several changes in peripapillary and macular thickness, macular layers volumes, and finally HRF count.

The effect of Ofatumumab on retinal layers volumes, thicknesses and inner-retinal layers HRF count

The analysis performed on the patients treated with Ofatumumab showed an overall stability in all parameters examined. This stability might be in line with the anti-inflammatory properties of the drug, which are able to contrast the natural progression of the disease, that normally results in the thinning of pRNFL, increase in HRF count in the inner-retinal layers, as well as a reduction of total volumes, and thicknesses of macular layers, except for the INL where we would expect to see a decrease in volume. In fact, the stability of INL may not be consistent with the observations made by Knier et al. [53], which showed a reduction of INL volume as a

response to therapy. This is due to the anti-inflammatory capabilities of the drug, that are able to reduce the inflammation occurring in the INL, and thus reducing it's volume. However, the relative short disease duration interval (defined as the amount of time passed from treatment initiation and the diagnosis) and the younger age of the patients underwent Ofatumumab treatment might also contribute to the overall stability of macular layers volumes and thicknesses. Moreover, the short duration of the study as well as the lower number of participants may be confounding factors when looking at the results, henceforth, it is necessary to conduct further studies in order to better understand the behavior of these parameters in patients undergoing Ofatumumab therapy.

The effect of S1PR modulators on retinal layers volumes and thicknesses

The results obtained from the analysis conducted on the S1PR modulators cohort showed a statistically significant reduction in thicknesses and volumes in pRNFL NI sector, GCL OR and GCL TV respectively. This reduction could be explained with the anti-inflammatory effect that the drug exerted on those layers, thus reducing inflammation and eventually the volume and thickness of those layers, and as a consequence generating pseudo-atrophy result. On the other hand, such reduction could also arise from the natural progression of the disease, as demonstrated by Petzold et al. However the relatively short duration of the observation period make this hypothesis less likely. In order to better comprehend the underlying cause of the observed results, it is necessary to continue monitoring those parameters for a longer period of time, and based on whether the volume and thickness continue to decrease or on the contrary remain stable, we could make a better deduction on the nature of the results seen.

The comparison between the two treatment cohorts

The results obtained from the comparison between the two treatment cohorts yielded a statistically significant difference regarding the volume and thickness of GCL TV and GCL OR respectively. This difference is line with the changes of those parameters observed earlier in the S1PR modulators cohort. From this

comparison, we can understand that Ofatumumab had a superior capability in maintaining the total volume of GCL and the thickness of the outer ring. However, it is still necessary to assess initially whether the changes observed earlier in the S1PR modulators cohort was indeed caused by the disease progression. Furthermore, the short duration of the study, as well as the relatively low number of participants require a longer observation period and larger cohorts to be performed, in order to strengthen our understanding of the results. Additionally, the lower mean age, the shorter disease duration, and the lower number of patients who underwent previous therapies in the Ofatumumab cohort may represent confounding factors that should be considered when interpreting the results.

The effect of S1PR modulators on HRF in the inner-retinal layers

The impossibility of obtaining histological specimens of the human retina in vivo keeps the question on the origin and pathologic significance of HRF still open. Nevertheless, indirect evidence, accumulated over the last decade, supports the hypothesis that these nodules are constituted by clusters of activated retinal microglia that migrate close to the blood-retinal barrier probably in response to detrimental triggers. [61,62] Results from our study seem to be in line with this hypothesis, as a statistically significant reduction of HRF count was detected in the ICP, where the presence of vessels is predominant. A recent study had also confirmed the ability of S1PR modulators to interact both with microglia and the BBB, thus regulating the BBB permeability, which provide additional therapeutic benefits in MS.[74] This capability seem to be also in line with the results we obtained from our study, where the reduction in HRF count was detected only in the S1PR cohort and not in the Ofatumumab Cohort, which exert it's anti-inflammatory properties by predominantly suppressing lymphocytes activity and vitality.[45,46] This observation may provide yet another evidence to support to the microglial origin of the HRF. However, establishing which factor determines the HRF formation, whether it is the microglia or the BRB, still remains unknown. Further studies are necessary to strengthen the hypothesis about the HRF origin, and to clarify better the determinants factors to their formation and behavior.

6. Conclusions

This study found that Ofatumumab treatment in MS patients led to stability in thickness and volumes in the peripapillary RNFL, and the various retinal layers respectively, likely due to its anti-inflammatory properties, while S1PR modulators were associated with statistically significant reductions in thickness and volume, especially in the GCL, possibly reflecting either pseudo-atrophy or disease progression. Ofatumumab showed greater effectiveness in preserving the GCL outer ring thickness and total volume compared to S1PR modulators. Additionally, the reduction in HRF in patients treated with S1PR modulators supports the hypothesis of a microglial origin for HRF, and is in line with the evidence of a close proximity between HRF and BRB. However, long-term studies are needed in order to confirm our observations, and to deduce the nature of the changes seen in patients treated with S1PR modulators.

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