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Exploring promising biomarkers in multiple sclerosis – Valentina Mazziotti
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ai miei genitori

Sommario

La sclerosi multipla (SM) è una malattia infiammatoria cronica del sistema nervoso centrale (SNC) che colpisce 2.3 milioni di persone nel mondo, di cui circa 122.000 in Italia. Si tratta di una patologia multifattoriale con esordio tipicamente in età giovane-adulta, la cui causa è ancora sconosciuta. Un ruolo fondamentale nella patogenesi della SM, sembra essere svolto da processi infiammatori a carico del SNC associati a fenomeni neurodegenerativi con conseguente alterazione o perdita delle funzioni sensoriali, motorie e cognitive. Le caratteristiche dell'infiammazione e del danno assonale che si manifestano nella SM, delineano differenti fenotipi di malattia, riconducibili a due forme principali: una forma recidivante-remittente e una forma progressiva. Le due forme di SM si distinguono in base al decorso di malattia. La prima è caratterizzata da un'alternanza di episodi acuti di malattia alternati a periodi privi di sintomi; la seconda, da una disabilità persistente e progressiva, causa di una graduale e irreversibile invalidità. Non esistono, a oggi, strumenti clinici e neuroradiologici prognostici per la SM. Di conseguenza, non poter identificare, sin dalla diagnosi, pazienti con un alto rischio di presentare un decorso di malattia più grave, rende la scelta terapeutica difficile per il clinico e rischiosa per il paziente. In questo scenario, identificare nuovi marcatori diagnostici, prognostici e predittivi di malattia, attraverso l'utilizzo di tecnologie molecolari avanzate, è di fondamentale importanza. Lo scopo di questa tesi è stato quello di identificare nuovi biomarcatori correlati a un alto rischio di attività di malattia, attraverso un'analisi esplorativa dei livelli liquorali di specifiche citochine e chemochine pro-infiammatorie associate all'attivazione delle cellule linfocitarie e microgliali, alla base dei meccanismi di patogenesi della SM, ed indagare il ruolo prognostico di diversi biomarcatori riconosciuti in SM, la cui validità è ancora dibattuta (CXCL13, IgM, Nf-L, PVALB), valutando l'associazione tra infiammazione intratecale (meningea e liquorale) e periferica. I risultati ottenuti hanno permesso di identificare un profilo liquorale associato a un alto rischio di attività di malattia, caratterizzato da alti livelli di fattori rilasciati alle cellule T (IFNγ e TNFα), B (LIGHT, APRIL, CXCL13, CXCL12, LIGHT) e microgliali (sCD163) attivate. Inoltre, l'analisi di questi biomarcatori, in combinazione con la valutazione di parametri clinici e di risonanza, ha permesso di confermare la compartimentalizzazione dell'infiammazione a livello intratecale in pazienti SM ad alto rischio di attività di malattia, già al momento della diagnosi. Infine, data la recente pandemia da COVID-19, è stato svolto uno studio specifico sulla risposta anticorpale e cellulo-mediata da parte pazienti SM trattati con diverse terapie immunosoppressive (cladribina, fingolimod e ocrelizumab). Lo studio mostrato in questa tesi, ha dimostrato un diverso effetto del vaccino in pazienti trattati con diversi trattamenti: i pazienti in terapia con ocrelizumab hanno mostrato bassi livelli di produzione anticorpale in risposta al vaccino e, al contrario, una risposta cellulo-mediata preservata, associabile alla produzione di diversi mediatori molecolari dell'infiammazione.

Abstract

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), affecting 2.3 million people worldwide, of which approximately 122,000 are in Italy. MS represents a multifactorial disease, typical of in young adulthood, whose cause is still unknown.
An important role in the MS pathogenesis, seems to be played by inflammatory processes affecting the CNS associated with neurodegenerative phenomena with consequent alteration or loss of sensory, motor and cognitive functions. The characteristics of the inflammation and axonal damage that occur in MS, determine different disease forms: a relapsing-remitting form and a progressive form. The two forms of MS are distinguished according to the course of the disease. The first is characterized by an alternation of acute episodes of disease, alternating with periods without symptoms; the second, consists in a persistent and progressive disability.
To date, there are no prognostic clinical and neuroradiological tools for MS. Consequently, it is not not being able to identify, since from the time of diagnosis, patients with a high risk of presenting a more severe disease course, makes the therapeutic choice difficult for the clinician and risky for the patient. In this scenario, identifying new diagnostic, prognostic and predictive markers of disease, through the use of advanced molecular technologies, is of fundamental importance.
The aim of this thesis was to identify new biomarkers associated with a high risk of disease activity, through an exploratory analysis of the CSF levels of specific cytokines and pro-inflammatory chemokines associated with the activation of lymphocytes and microglial cells underlying the pathogenesis mechanisms of MS, and investigate the prognostic role of several biomarkers recognized in MS, whose validity is still debated (CXCL13, IgM, Nf-L, PVALB), evaluating the association between intrathecal (meningeal and CSF) and peripheral inflammation. The results obtained allowed to identify a CSF profile associated with a high risk of disease activity, characterized by high molecules levels released by activated T (IFNγ and TNFα), B (LIGHT, APRIL, CXCL13, CXCL12, LIGHT) and microglial cells (sCD163). Furthermore, biomarkers analysis, in combination with clinical and radiological evaluation, confirms an early compartmentalized intrathecal inflammation at the level, in MS patients at high risk of disease activity, already at the time of diagnosis.
Finally, given the recent COVID-19 pandemic, a specific study on the antibody and cell-mediated response by MS patients treated with different immunosuppressive therapies (cladribine, fingolimod and ocrelizumab) was included in this thesis. The study demonstrated a different vaccine effect in patients treated with different treatments: patients receiving ocrelizumab showed an impaired antibody production and, on the contrary, a preserved cell-mediated response, associated with an inflammatory mediator's production.

Contents

Abbreviations list

CNS: central nervous system
CD4+ = Cluster of Differentiation 4+
CD8+ = Cluster of Differentiation 8+
CD20+ = Cluster of Differentiation 20+
CIS = Clinically Isolated Syndrome
CLs = Cortical Lesions
CNS = central nervous system
CRF = Case Report Form
CSF = Cerebro-Spinal Fluid
CCL- = Chemokine (C-C motif) Ligand-
CXCL- = Chemokine (C-X-C motif) Ligand-
EAE = experimental autoimmune encephalomyelitis
EDSS = Expanded Disability Status Scale
GM = Grey Matter
IL- = Interleukin-
Ig- = Immunogloblulin-
MRI = Magnetic Resonance Imaging
MS = Multiple Sclerosis
NAWM = Normal Appearing White Matter
NF-L = Neurofilaments light chain
PPMS = Primary Progressive Multiple Sclerosis
PV+ = Parvalbumin-Positive
PVALB = Parvalbumin
RRMS = Relapsing Remitting Multiple Sclerosis
sCD14 = soluble Cluster of Differentiation 14
sCD163 = soluble Cluster of Differentiation 163
SEL = Slowly Expanding Lesions
SPMS = Secondary Progressive Multiple Sclerosis
sTNFR1 = soluble TNF Receptor 1
sTNFR2 = soluble TNF Receptor 2
SWI = Susceptibility Weighted Imaging
TNF = Tumor Necrosis Factor
WM = White Matter
3T = 3 Tesla

1. Introduction

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic immune-mediated disease of the central nervous system (CNS), affecting 2.3 million people worldwide (Browne et al., 2014), with ratios of women to men of 2.3–3.5:1 (Harbo et al., 2013; Compston and Coles, 2002). MS is the most common cause of non-traumatic neurological disability in young adults that typically presents during the ages of 20–40 years (Koch-Henriksen and Sørensen, 2010). Life expectancy is around 25 years following diagnosis, but is nearing that of the general population (Confaxreux and Compston, 2004). MS prevalence is increasing and varies according to geographic areas: is highest in North America, Western Europe and Australasia (>100 cases per 100,000 population) and lowest in countries centered on the equator (<30 cases per 100,000 population) (GBD 2016 Multiple Sclerosis Collaborators, 2019).

The clinical course of MS is extremely variable: for most MS patients (approximately 80–85%), clinical onset is characterized by relapsing and then remitting neurological deficits, a condition referred to as relapsing-remitting MS (RRMS) (Li et al., 2018), that often will convert to the secondary progressive MS (SPMS), characterized by a progressive worsening of neurologic function and by an accumulation of disability over time, in the absence of remissions (Lublin and Reingold, 1996). About 15% of MS patients exhibit a gradual and steady progression and an unremitting increase of disability since the onset, described as primary progressive multiple sclerosis (PPMS) (Li et al., 2018; Lublin and Reingold, 1996). RRMS has an onset at a younger age (~30 years) when compared to PPMS (~40 years) and affects mostly women (nearly three times more often than men; Kamm et al., 2014).

Despite these distinctions, all clinical forms of MS appear to reflect the same underlying disease process characterized by two pathological hallmarks: 1) inflammation with demyelination, and 2) neurodegeneration (Hauser and Cree, 2020). The traditional view that inflammation is the cause of axonal and neuronal degeneration in MS brain has been challenged (Frischer et al., 2009).

Although inflammation is typically associated with relapses, and neurodegeneration with progression, it is now recognized that both pathologies are present in essentially

all patients across the entire disease course (Kamm et al., 2014) Therefore, the association between inflammation and neurodegeneration in all lesions and disease stages of multiple sclerosis (Doinikow, 1915; Ferguson et al., 1997; Kuhlmann et al., 2002; Trapp et al., 1998).

1.1.1 Etiology and Pathogenesis

The etiology of MS as a multifactorial disease, involving genetic, exogenous and immunological factors, is currently unknown. Complex genetics and environmental factors determine individual susceptibility to disease development and the determination of different disease subtypes (Veljkovic et al., 2018), namely neurological dysfunction and variable subsequent improvement, and progressive forms, progressive worsening of neurologic function and unremitting increase of disability (Lublin and Reingold, 1996; Inglese et al., 2011). Despite the exact aetiological factor has not been fully elucidated, alterations of immune system responses seem to play a key role in the pathogenesis of MS (Compston and Swingler, 1988). In the early (acute) stage of MS, lymphocytes (predominantly T-cells) are activated in the peripheral circulation by processed peptides that mimic some CNS antigens (e.g., myelin-associated glycoprotein, myelin oligodendroglia glycoprotein, and proteolipid protein) (Veljkovic et al., 2018). In addition to self-reactive T cells, in the last 10 years several experimental and clinical studies revealed that inflammation B-cell mediated in the CNS has been shown to be crucial in MS pathology. Indeed, B cells are involved in MS by different mechanisms, such as presentation of antigens to T cells, production of intrathecal Immunoglobulins (Igs), known as OCBs, and secretion of cytokines (i.e. TNF, IFNγ IL-6, IL-10, IL-34, IL-35 and GM-CSF) and other pro-inflammatory factors like lymphoid chemokines (i.e. CXCL10, CXCL12, CXCL13) in the cerebrospinal fluid (CSF) of MS patients (Inglese et al., 2011; Villar et al., 2014). Cytokines, chemokines, and also nitric oxide (NO), reactive oxygen species (ROS), glutamate, and free radicals production induced by T and B cells infiltrates in the cerebral parenchyma B cells activation, act on different types of glial cells, including astrocytes, microglia, and oligodendrocytes, resulting in myelin, oligodendrocyte, and neuronal damage (Kalafatakis et al., 2021). Viral infections of

the CNS, including Epstein-Barr, the virus that causes infectious mononucleosis, and autoimmune disorders, such as thyroid disease, pernicious anemia, psoriasis, type 1 diabetes or inflammatory bowel disease, are associated with an increased risk of developing the disease (Dendrou et al., 2015). The other prominent environmental factors that contribute to disease development are: smoking (Ascherio and Munger, 2008), obesity, ultraviolet light exposure, vitamin D status (Ascherio et al., 2014) and family history. Specifically, genetic predisposition accounts for only 30% of the explainable risk of MS (Dendrou et al., 2015). Indeed, studies conducted in twins, who carry a nearly identical genetic load, show that if one monozygotic twin has MS, the other has only a 25% risk of developing MS; moreover, in dizygotic twins, this risk decreases to 5% (Ascherio et al., 2012). Therefore, MS is thought to result from the combination of a genetic predisposition with certain environmental exposures.

1.1.2 Clinical symptoms and Diagnosis

Clinical symptoms of MS include fatigue, mobility impairments, weakness, balance impairments, stiffness and spasms, memory and other cognitive problems, visual changes, and dizziness (Motl et al., 2008).

The number of symptoms increases with increasing Expanded Disability Status Scale (EDSS) level. EDSS is the main method of measuring disability in MS on a scale from 0 (normal) to 10 (death due to MS) and monitoring changes in the level of disability over time (Kurtzke, 1983), considering both neurological impairments and functional aspects of the disease. MS symptoms depend on the presence of lesions occurring within the CNS. Diagnosis requires objective evidence of inflammatory CNS injury and often additional details of dissemination of the disease process “in space and time”, i.e. affecting more than one CNS location with evolution over time (Hauser et al., 2020). The main tests used to support diagnoses are magnetic resonance imaging (MRI) and CSF analysis (Hauser et al., 2020).

1.1.2.1 MRI assessment

Immunological abnormalities have consistently been observed in MS patients, resulting in widespread demyelination and axonal degeneration within the CNS.

Neural inflammation, demyelination, remyelination, neurodegeneration, and glial scar formation occur either focally or diffusely throughout the white and gray matter of the brain and spinal cord. The resulting disability manifests at the level of motor, sensory, autonomic and neurocognitive functions. Magnetic resonance imaging (MRI) allows to identify damage cerebral areas, through the detection of specific lesions: gadolinium-enhanced T1 brain lesions show active areas of ongoing inflammation, as the increase occurs due to increased permeability of the blood brain barrier, while T2 lesions show older or inactive lesions (van Waesberghe et al., 1998). The majority of lesions are found in the brain, particularly in the periventricular white matter, cerebellum, brainstem, and optic nerves (Pierson et al., 2012).

Many patients exhibit lesions in the spinal cord as well as the brain, whereas 2–10% of patients exhibit inflammation in the spinal cord and optic nerves without extensive involvement of the brain (Nociti et al., 2005; Pierson et al., 2012).

Since 2001, MRI has been formally included in the diagnostic work-up for patients suspected of having MS (Filippi and Rocca, 2011). The detection of white matter, grey matter and/or spinal cord lesions, demonstrating both dissemination in space and time is currently included in the diagnostic criteria for MS (Rovira et al., 2015; Thompson et al., 2018). Therefore, MRI provides objective measures to monitor disease activity and to assess treatment efficacy (Filippi and Rocca, 2011).

1.1.2.2 CSF analysis

Many of the diagnostic and prognostic biomarkers of MS are molecules involved in the immune responses, such as self-reactive antibodies, cytokines, chemokines and some of which have been selected as specific targets of therapeutic strategies (Lublin et al., 1996; Li et al., 2018). More specifically, MS diagnosis often relies on the demonstration of oligoclonal bands (OCBs) in the CSF, thus making them an hallmark of MS and allowing an earlier, more sensitive, and more specific diagnosis (Thompson et al., 2018). The presence of OCBs in the CSF, and not in paired serum samples, is found in >90% of MS patients (Davenport and Keren, 1988) and indicates the intrathecally synthesized IgG, namely reflects a highly immune response by activated B cells in the CNS. IgG, IgM and IgA antibodies are major contributors to the OCBs formation in the CSF (Lolli et al., 1999; Ziemssen et al., 2019) and intrathecal IgM

synthesis at diagnosis has been linked to more rapid and severe disease progression and higher probability of converting to SSMS in RRMS patients (Villar et al., 2002). Indeed, IgM i are the only Igs capable of recognizing myelin lipids and OCBs IgM are involved in demyelination processes and also in axonal injury (Villar et al., 2005), the main source of disability in MS patients (Mead et al., 2002). In addition, the presence of meningeal infiltrates, often aggregated in B-cell ectopic lymphoid-like follicles, is associated to increased CSF levels of cytokines and chemokines (CXCL13, CXCL10, IL6, IL10, TNF and LTα), associated with B cells recruitment and activity, cortical pathology and rapid disease progression (Farina et al., 2017).

1.1.3 Treatments

The exact cause of MS is not known and there is currently no cure. Moreover, the lack of known biological targets in progressive MS, hinders DMT development (Hollen et al., 2020). However, disease-modifying therapies (DMTs) have dramatically changed the treatment of MS over the last two decades (Al-Sakran et al., 2019). DMTs modify the course of MS through suppression or modulation of immune function (Hauser et al., 2020). Injectable disease-modifying therapies such as interferon beta preparations and glatiramer acetate were the first two DMTs approved 20 years ago for the treatment of MS (La Mantia et al., 2022). Mechanisms of action of interferon beta include: regulation of immune cell activation and proliferation through downregulating expression of MHC molecules on antigen-presenting cells, inhibition of T-cell proliferation, induction of anti-inflammatory cytokine shifts, blocking trafficking of inflammatory cells across the blood-brain barrier to CNS (Kieseier, 2011). Efficacy of glatiramer acetate is considered due to inhibition of the immune response to myelin basic protein and possibly other myelin antigens (La Mantia et al., 2022). Oral treatment options for disease-modifying therapy in relapsing multiple sclerosis have substantially increased over the past decade with four approved oral compounds now available: fingolimod, dimethyl fumarate, teriflunomide, and cladribine (Derfuss et al., 2020). Fingolimod is the first approved oral therapy for MS that acts as an immunomodulator: it does not inhibit T- or B-cell activation, but it reduces the migration of autoreactive lymphocytes into the CNS, acting as an agonist of the sphingosine-1-phosphate receptor and sequestering lymphocytes in lymph nodes

(Potenza et al., 2016). Dimethyl fumarate exerts anti-inflammatory and cytoprotective effects through activation of the antioxidative transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway (Schulze-Topphoff et al., 2016). Teriflunomide is the active metabolite of leflunomide, an immunosuppressant used in rheumatoid arthritis that inhibits dihydroorotate dehydrogenase, an enzyme involved in pyrimidine synthesis, blocking lymphocytes proliferation (Hauser et al., 202). Cladribine is a deoxyadenosine analogue prodrug that preferentially depletes lymphocytes, reducing peripheral T- and B-cell levels (Deeks et al., 2018). In addition to lymphocyte depletion, cladribine may impact dendritic cells (Singh et al., 2013) and microglia (Singh et al., 2013).

Current therapies are effective during the early RRMS stage of the disease, while patients with progressive disease do not respond to current immunomodulatory treatments (Hollen et al., 2020), except for Ocrelizumab, a humanized monoclonal anti-CD20 antibody, the only FDA-approved treatment (on 2017) that has been found to be effective also on PPMS, and for Siponimod, a selective S1P modulator, approved for active SPMS. Mechanisms of action of Ocrelizumab consists in the blockage of the intrathecal B cell activity which may stop intrathecal chronic inflammatory conditions, while Siponimod, the first oral medication for SPMS, effects are similar, but more selective, compared to Fingolimod (Sabsabi et al., 2022).

Although there are different MS treatments, these are mainly aimed at modulating the inflammatory response acting in different ways, blocking lymphocyte recruitment, proliferation or activation, while they have no effect on remyelination and confer only partial protection against the neurodegenerative component of MS. This is the reason why they are effective for relapsing and not for progressive forms of the disease. The aim of this thesis is in fact to find potential new biomarkers predicting the course of the disease in order to identify any new pharmacological targets.

1.2 Immune mechanisms involved in Multiple Sclerosis

CNS controls the functions of all the organs in the human body, through neuronal connectivity and neuronal signal transmission (Kempuraj et al., 2016). The perception of signals and the processing of complex responses is due to the presence in the CNS

tissue of highly specialized cells, such as neurons, excitable cells responsible for the reception and transmission of nerve impulses, and the glial cells (microglia, oligodendrocytes/Schwann cells, astrocytes), non-excitable cells that provide structural support, functional, immune and trophic to neuronal cells. CNS is particularly sensitive to damage associated with inflammatory processes, due to its limited regenerative and neurogenic abilities (Moisse and Strong, 2006): unlike other cells, once the neurons are damaged or degenerated, they are unable to be repaired or regenerate (Ransohoff et al., 2016). Given the high vulnerability, the nervous system is protected, both structurally, by one skull, both immunologically, from the blood-brain barrier (BBB) and limits pathogens entry into the CNS and reduces cell exchanges immune (monocytes and leukocytes) and inflammatory mediators (free radicals, cytokines and chemokines) with the bloodstream.

MS is characterized by immune dysregulation, which results in the infiltration of the CNS by immune cells, triggering demyelination, axonal damage, and neurodegeneration (Rodríguez et al., 2022). Increased BBB permeability, a characteristic phenomenon of the early stages of MS disease, allows entry of inflammatory cells and mediators in the CNS, which augment neuroinflammation (Kempuraj et al., 2016), enter into the brain, activate microglia, astrocytes, and neurons to release additional inflammatory mediators contributing to the enhancement of neuroinflammation and neurodegeneration (Kempuraj et al., 2016; Russo et al., 2015). Chronic neuroinflammatory processes affecting CNS determine the onset and progression of MS. Specifically, increased proinflammatory mediators (such as IL-1β, Il-6, IL-8, GM-CSF, TNF-α, IFN-ɣ), released from cells of both adaptive and innate immunity, lead to oligodendrocyte death and degeneration of the myelinated neurons in MS patients (Kempuraj et al., 2016).

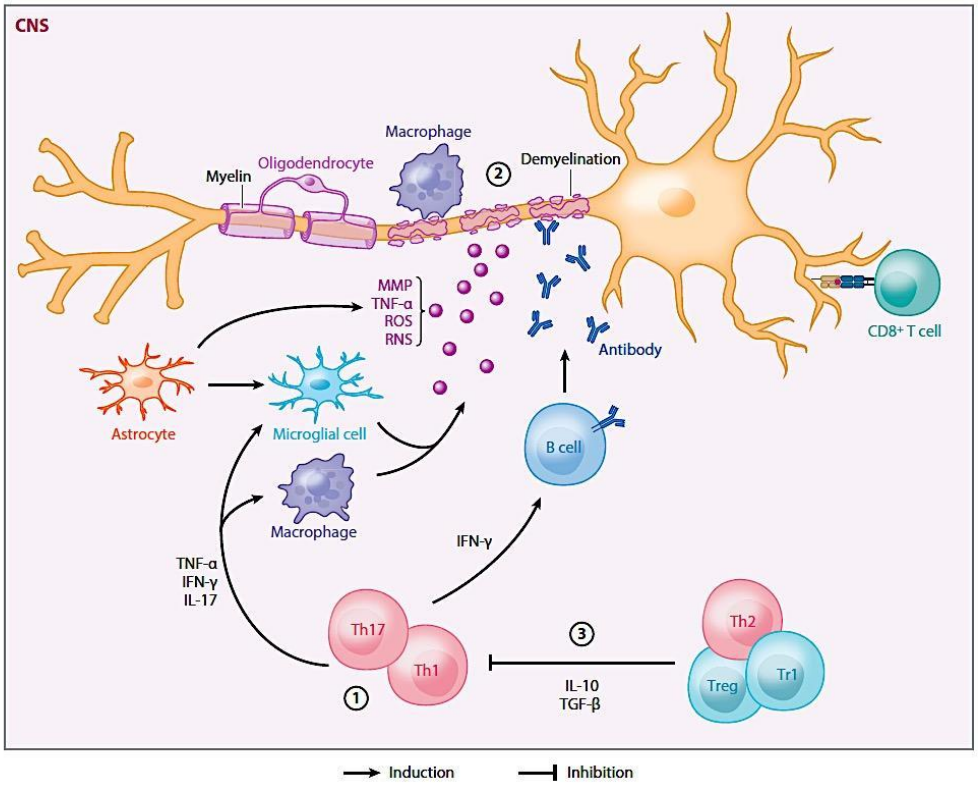

Figure 1: Pathogenetic immunological mechanisms of MS: (1) Pathogenic Th subsets (Th1 and Th17) secrete proinflammatory cytokines such as TNF-α, IFN-γ, and IL-17, which induce microglia and macrophage activation, supported also by astrocyte response. This response causes further pathogenic behavior led by macrophages, B cells, and cytotoxic T cells; (2) Soluble neurotoxic molecule production such as MMPs, TNF-α, ROS, and RNS, which are secreted by astrocytes, macrophages, microglia and CD8+ T cell, driven myelin and axonal damage; (3) Tregs limited inflammation secreting immunoregulatory cytokines such as IL-10 and TGF-β.*"The Immune Response in Multiple Sclerosis" Murúa et al., Annual review of pathology, 2021.*

1.2.1 Adaptive immunity in Multiple Sclerosis: role of T and B cells

1.2.1.1 T cell-mediated immunity

MS has been viewed historically as a CD4+ T cell-mediated autoimmune disease due in part to the genetic association of MS with MHC class II alleles, supported by the identification of the HLA class II haplotype HLA-DRB1∗15:01 as a major genetic risk factor for MS (Fogdell et al., 1995). The pathogenic immunological mechanisms that lead to the development of MS have been studied mostly using the animal model experimental autoimmune encephalomyelitis (EAE), induced by stimulating CD4+ T cell-mediated immunity to myelin proteins (Høglund et al., 2014; Stromnes and Goverman, 2006; Goverman, 2006), such as myelin basic protein (MBP), proteolipid

protein, and myelin oligodendrocyte glycoprotein (MOG). Self-reactive CD4+ T cells cause an acute autoimmune inflammation against myelin in the rodent CNS, with signs and symptoms that are similar to those seen in MS (Høglund et al., 2014).

The EAE is used to explore the immunological mechanisms underlying MS and the cells involved in the CNS inflammation: CD4+ T cells are activated in the periphery and then across the BBB where they are reactivated by myelin epitopes presented by dendritic cells (DCs) and by major histocompatibility complex (MHC) class II+ antigen presenting cells (APCs), mostly microglia cells (Ponomarev et al., 2005), capable of presenting antigen to CD4+ cells (Pierson et al., 2012). T cells reactivated differentiate in T helper 1 (Th1) and Th17 cells secrete proinflammatory cytokines (IFN-ɣ and IL-17, respectively), which further activate resident astrocytes and microglia, macrophage and B cells, amplifying CNS inflammation.

Proinflammatory Th1 and Th17 cells are activated in the presence of IL-12, TGF-β1, IL-23, and IL-6 or IL-21, respectively. Th1 cells activated produce IFN-γ, while the differentiation of Th17 cells is characterized by the release of IL-17, both cytokines involved in MS onset and progression (Fetcher et al., 2010). Following activation in the presence of interleukin 12 (IL-12), naive CD4+ T cells differentiate in Th1 cells, which produces interferon gamma (IFN-γ); activation in the presence of TGF-β1, IL-23, and IL-6 or IL-21 results in the differentiation of Th17 cells characterized by the production of IL-17 (Fetcher et al., 2010). Conversely, CD4+ regulatory T cells (Tregs) produce IL-10, an antiinflammatory cytokine, and limit pathogenic T cell responses. The decrease of Treg number contributes to the dysregulated autoimmune T cell response associated with MS pathology (Arellano et al., 2017). In addition to CD4+ cells, the importance in MS pathology of CD8+ cells must be considered. Although their role is not easily investigated because most preclinical models of MS are driven by CD4+ T cells, an increase in their number is detected in correspondence of the white and gray matter lesions (Arellano et al., 2017). The cytotoxic action of CD8+ T cells is mediated by their production of granzymes (GrA and GrB) and perforins and contributes to CNS damage (Arellano et al., 2017). Finally, “unconventional” T cell phenotype is represented by γδ T cells that are responsive to multiple molecular cues and can acquire the capacity to induce various cytokines, such as GM-CSF, IL-4, IL-17, IL-21, IL-22, and IFN-γ (Wo et al., 2020). Nevertheless, the

exact mechanisms responsible for γδ T cell proinflammatory functions remain poorly understood.

1.2.1.2 B cell-mediated immunity

Despite MS is considered a T cell–mediated disease, recently B cell activation has gained more attention, not only for a diagnostic reason (the presence of CSF OCBs is a hallmark of MS), but also for the development of new therapeutic approaches (e.g. Ofatumumab, anti-CD20 monoclonal antibody approved two years ago by FDA). In addition to their antibody-production function, B cells can release proinflammatory cytokines, such as TNF-α, and have important functions as antigen-presenting cells (APCs) involved in T cell activation (Bar-Or et al., 2010).

Abnormalities in the cytokine profiles of naive and memory B cells have been observed in patients with MS: activated B cells from patients with MS produce excessive amounts of the cytokines TNF, lymphotoxin-α, IL-6 and GM-CSM (Duddy et al., 2017). B cells have long been recognized as a subset of infiltrating cells in brain and spinal cord lesions in MS (Cencioni et al., 2021). Specifically, B cell accumulation is evident within the meningeal and perivascular regions (Serafini et al., 2004), resulting in extensive and active subpial grey matter demyelination, and, consequently, in a rapidly progressive clinical disease course (Cencioni et al., 2021). In SPMS forms is in fact evident an increased presence and accumulation of B cells and plasmablasts in niches similar to ectopic follicle-like structures (Serafini et al., 2004). Chemokines that are known to support the formation of such germinal centres, such as CXC-chemokine ligand 13 (CXCL13), CXCL10, lymphotoxin α, IL-6 and IL-10, are present at high levels in the CSF of patients with MS (Magliozzi et al., 2018). The degree of inflammation and the presence of follicles both associate with the severity of cortical pathology, possibly by production and subsequent diffusion of pro-inflammatory cytokines into the cortex (Magliozzi et al., 2020) In addition, B cells may contribute to disease progression in PPMS through cytokine production, specifically GM-CSF and IL-6, which can drive naïve T-cell differentiation into pro-inflammatory Th1/Th17 cells (Holloman et al., 2021).

Therefore, B cells activation contributes to a more rapid progression and aggressive disease evolution through the activation of microglia. Meningeal inflammation is in

fact strongly associated with cortical microglial activation (Bevan et al., 2018; Bevan et al., 2020) and with subpial cortical lesions (Benusa et al., 2020), which exhibit a surface-in gradient of neuronal injury and microglial activation suggests that soluble factors, released by immune cells in the meninge, diffuse across the CSF to damage the glia limitans and underlying cortex (Benusa et al., 2020).

1.*2.*2 Innate immunity in Multiple Sclerosis: role of microglia

CNS-resident microglia play a crucial role in refining synaptic networks through pruning, developmental apoptosis, positioning of neurons in the barrel cortex, and secretion of growth factors (Tremblay et al., 2011). In the homeostatic state, microglia was classically designated by a distinct morphology characterized by delicate branches, previously referred to as “resting state” (Mosser et al., 2017). Once active, microglia cells produce many factors at the base of the immune response (cytokines, free radicals and growth factors) and upregulate the MHC-II proteins of indispensable for antigen presentation to Th lymphocytes, and Toll-Like receptors Receptors (TLRs), necessary for the recognition of molecular motifs common to many pathogens (Nakagawa et al., 2014; Michell-Robinson et al., 2015). Usually, as a first defensive response, the microglia assumes the so-called “phenotype M1”, acquiring the ability to engulf foreign bodies (just like macrophages peripheral) and releasing proinflammatory factors, such as cytokines (IL-1, IL-6, TNF-α), chemokines (IL-8, MCP-1), glutamate, Reactive Oxygen Species (ROS) and Oxide Nitric (NO) (Nakagawa et al., 2014). These factors promote cell infiltration of the immune system (such as lymphocytes and macrophages) in the CNS and activate astrocytes and other surrounding microglial cells, further increasing neuroinflammation, in order to destroy the pathogen (Rivest et al., 2009). However, given that persistent inflammatory processes have a neurotoxic effect, one once the noxious stimulus is eliminated, the microglial cells assume an "M2 phenotype" e they trigger neuroreparative and neuroregenerative processes capable of suppressing inflammation, through the expression of the MRC-1 mannose receptor, which allows the removal of inflammatory factors from the circulation, the synthesis of cytokines anti-inflammatory (IL-4, IL-10 and TGF-β) and the production of growth factors (BDNF, GDNF, NGF and VEGF), thus ensuring the return to cerebral homeostasis

(Nakagawa et al., 2014). In neurodegenerative diseases, the M1 microglial phenotype assumes a value negative, as the release of inflammatory molecules aggravates neurological damage (Zhao et al., 2004; Beers et al., 2011).

When “activated” during pathological states, microglial morphology changes to resemble the typical amoeboid appearance of a macrophage (Tremblay et al., 2011). In MS microglia activation is implicated in CNS inflammation and in disease activity and pathology. Active demyelination is usually associated with a proinflammatory microglia phenotype (positive for p22phox, CD68, CD86, and Class II MHC antigens) while anti-inflammatory markers (CD206, CD163, ferritin) peak in the inactive lesion center (Guerrero and Sicotte, 2020), altrought the classification of M1/M2 or “good” or “bad” microglia fail to capture the complexity and subtly of microglial activity which changes rapidly in response to local conditions (Guerrero and Sicotte, 2020).

Microglia cells are present throughout all stages of lesion formation as a driver of inflammation, they are detectable in slowly expanding lesions linked to disease progression and they are present diffusely throughout the cortex and contribute to synaptic loss (Guerrero and Sicotte, 2020). Preactive lesion are observed in NAWM and are characterized by clustering («nodules») of activated microglia (MHC II+) in the absence of demyelination, but are not associated with blood vessels (T cell infiltrates) and with BBB disruption triggers of microglia activation (IL-10, ROS, NADH ox-2, TNF-a) might be axonal injury o oligodendrocyte stress Chronic microglial activation associated with axonal loss in turn related to demyelination (secondary to axonal damage). These lesions are observed in MS patients (67%) and are observed in the vicinity of active lesions. Most of these nodules might resolve spontaneously, while others might progress into an active lesion. In chronic active lesions, phagocytic cells “hips” (CD68 +: microglia and macrophages) surround the lesion in the presence of a closed BBB. Early chronic marker of the disease, associated with: slowly expanding lesion (WML), axonal damage (GML) and more serious neurological damage disability, faster and earlier course MS progressive (Absinta et al., 2016).

1.2.2.1 Remyelination

Following myelin and axon destruction, most lesions show signs of remyelination.

Remyelination is a regenerative process that replaces lost myelin, at least partially due to the ability of oligodendrocyte precursor cells to differentiate into myelinating oligodendrocytes although successful remyelination is complex and dependent on multiple factors as growth factors released by others glia cells as astrocytes and microglia (M2) (Tanaka and Yoshida, 2014). M2 microglia also favors this process through promote remyelination through multiple mechanisms, including the phagocytosis and proteolysis of myelin debris (Martinez et al., 2008). Remyelination of plaques is therefore possible, leading to the formation of "shadow plaques", altrought oligodendrocytes remyelinate damaged axons only partially after a relapse (Stangel et al., 2011; Marua et al., 2022). The shadow plaques are sharply demarcated areas with reduced myelin density and disproportionately thin myelin sheaths, and reflect a late phase of remyelination (Popescu et al., 2013). Most studies agree that remyelination is especially prominent at the early MS stages, whereas it is sparse after several years of disease duration. In addition, very little remyelination is found in cases of primary progressive MS (Lassmann et al., 1997). Remyelination-targeting strategies in isolation or combined with other approaches are of particular interest for the treatment of MS.

1.2.3 Intrathecal inflammation and neurodegeneration

MS was considered initially to be a demyelinating disease of CNS white matter, predominantly induced by T cell-mediated inflammation (Wekerle, 2008). However it is now known that lesions in the cortical GM can be detected from the early stages of disease, independent of white-matter pathology (Magliozzi et al., 2007). Actively demyelinating plaques in RRMS involves the movement of immune cells from the periphery into the CNS, while progressive disease involves the development of compartmentalized pathological processes within the brain mediated mainly by resident CNS cells (Correale et al., 2019). Indeed, cortical lesions are not associated with perivascular lymphocyte infiltration and BBB disruption, but with meningeal inflammation, characterized by infiltration of peripheral immune cells (T cells, B cells, and macrophages) that resembling ectopic B-cell follicular structures ([FIG.2; Hauser et al., 2020]; Howell et al., 2011) inducing microglia activation, demyelination and neurodegeneration (Popescu et al., 2013). Neuroinflammation,

mediated by microglia-the resident brain macrophage, astrocytes, neurons, T-cells and B cells infiltrates from the periphery, and inflammatory mediators released from them (such as IL-1β, IL-6, IL-8, IL-33, TNF-α, CCL2, CCL5 and GM-CSF), is crucial in the onset and the progression of neurodegenerative mechanisms. Neurodegeneration is a condition in which the neuronal structure and functions are altered, with reduced neuronal survival and increased neuronal death in the CNS (Ransohoff, 2016). The association between meningeal inflammation and cortical damage therefore suggests a strong correlation between the compartmentalization of intrathecal inflammation and a more rapid progression and aggressive evolution of the disease.

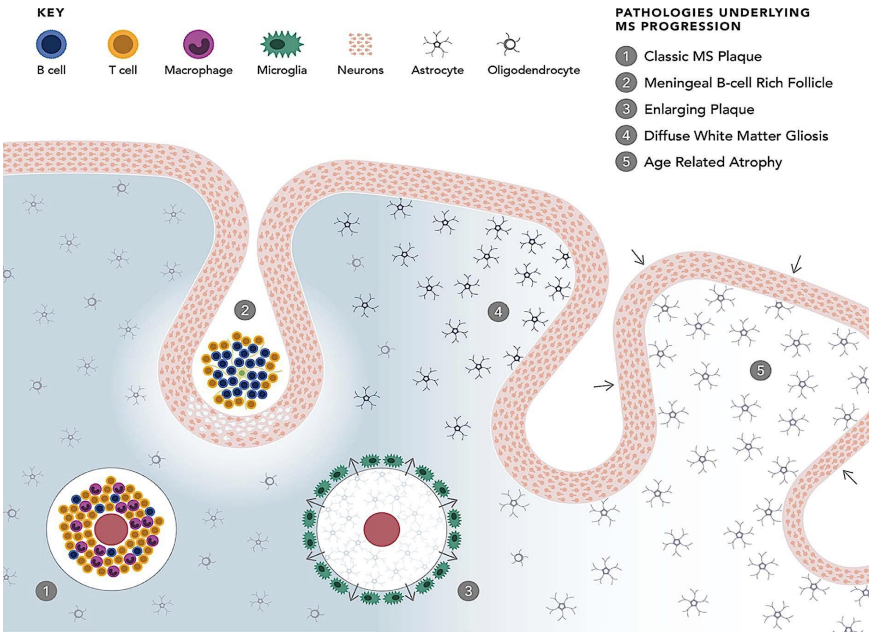

FIGURE 2. Pathologies underlying MS. (1) Focal white matter lesions are typical of RRMS, altrought are present in progressive MS diseases, and normally surround a central vein and are characterized by immune cells infiltrating from the periphery system (2) B cell rich lymphoid aggregates in the meninges, often in deep sulci, with underlying cortical demyelination and neuronal loss. (3) Slowly enlarging lesions due to gradual concentric expansion of chronic plaques, characterized by a rim of activated microglia and progressive axonal injury. (4) Widespread diffuse microglial inflammation and astrogliosis throughout the CNS white matter, associated with decreased myelin density and ongoing axonal damage. *"Treatment of Multiple Sclerosis: A Review, Hauser et al., 2020.*

The most characteristic brain tissue injury in MS is primary demyelination with partial preservation of axons (Lassmann et al., 2007).

Cortical demyelinating lesions can be subdivided into leukocortical (involve both gray and white matter at the gray matter, type I), intracortical lesions (small and perivascular demyelinated lesions confined within the cortex, type II) and subpial (extend from the pial surface to cortical layer three or four, type III; [FIG.3; Zuroff et al., 2021]).

Cortical lesions are first described in secondary progressive MS (SPMS) and PPMS but are now known to also be a feature of the very earliest stages of MS (Kutzelnigg et al., 2005; Lucchinetti et al., 2011). MRI confirmed that grey matter damage (including cortical lesions and atrophy) are already present in early disease (Wegner et al., 2006) and become more prominent during progressive MS (Amato et al., 20004). Early cortical involvement is linked with disease progression and irreversible disability, as well as cognitive deficits. Not surprisingly, the progressive forms of MS, including PPMS and SPMS, characterized by worsening of symptoms and fewer or no periods of remission, compared to RRMS cases, show extensive areas of demyelination at the level of the cerebral cortex (Kidd et al., 2019). Indeed, DMTs target peripheral infiltrators are effective in RRMS but not seem to be effective in progressive MS has forced a focus on alternative pathogenic mechanisms (Healy et al., 2022). Progressive MS disease include myelin, axonal injury and neuron loss, resulting in multifocal white matter lesions and diffuse grey matter damage in subpial and subventricular regions close to CSF and meninges (Healy et al., 2022).

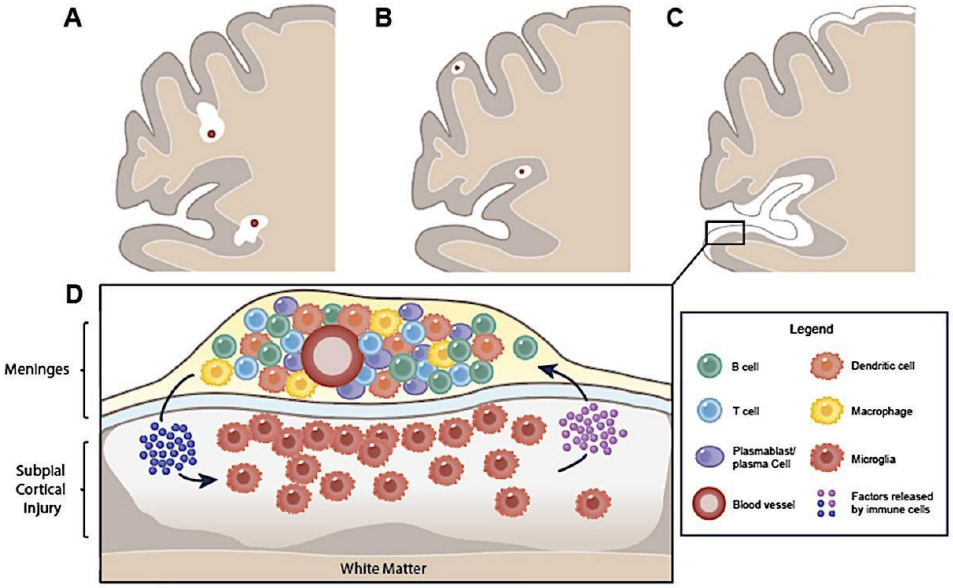

FIGURE 3. Cortical lesions in multiple sclerosis. Coronal brain sections demonstrating cortical demyelination (white) are shown in panels A-C. (A) Leukocortical (type I) and (B) intracortical (type II) lesions form around post-capillary venules (red) in the subcortical white and cortical gray matter, respectively; (C) subpial (type III) lesions extend from the superficial pial surface into the deeper layers of cortex. Type IV lesions involve all cortical layers and are not shown specifically in this schematic; (D) magnified representation of subpial cortical lesion segment demonstrating proposed inflammatory mechanism underlying subpial injury in MS. Meningeal immune cell aggregates, conteined B and T cells, are strongly associated with subpial lesions, in which a gradient of demyelination and microglial activation in the subjacent cortex is represented. Mediators released by infiltrating immune cells in the meninges as well as by glial resident cells (activated microglia and astrocytes) such as cytokines and chemokines (CXCL13, CXCL10, IL6, IL10, TNF and LTα), associated with B cells recruitment and activity, cortical pathology and rapid disease progression. *"Inflammatory mechanisms underlying cortical injury in progressive multiple sclerosis", Zuroff et al., 2021*

1.2.4 Role of Immunoglobulins in the Pathogenesis of Multiple Sclerosis

The presence of soluble clonal immunoglobulin G (IgG) also referred to as oligoclonal bands (OCBs). Intrathecal IgG detection is the only CSF biomarker used in clinical practice in MS and has been considered a central hallmark of MS, allowing an earlier and more specific diagnosis (Thompson et al., 2018). The determination of OCBs in the CSF but not in the serum, detected in >95% of patients (Link, 1978; Walsh et al., 1985) is a strong indicator of intrathecal antibody synthesis, resulting from intrathecal antigen-driven immune responses against as yet unknown target antigens (Bankoti et al., 2014). OCBs are produced by clonally expanded B cells in the CSF of MS patients (Weber et al., 2010). MS patients showed an intrathecal production of IgG, IgM, and IgA (Lolli et al., 1989); about 95% of MS patients displayed IgG OCBs (Link, 1978; Walsh et al., 1985; Villar et al., 2005), and around 40% also showed intrathecal IgM

production (Villar et al., 2005), while CSF IgA synthesis was only occasionally observed (in 13% of cases (Link and Müller, 1971; Leary et al., 2000). Therefore, IgG (predominantly IgG1), and IgM are considered the major contributors to the OCB formation in the CSF (Ziemssen and Ziemssen, 2005; Ziemssen et al., 2019). Current standard techniques for OCBs detection are isoelectric-focusing (qualitative) and nephelometry (quantitative). However, in about 5% of defined MS cases no intrathecal IgG synthesis can be detected according to standard measurements. This is due to problems of analytical sensitivity, substantial inter-laboratory variability and different indices to consider (laborious and unreliable process). Moreover, this may also be due to the presence of other IgG subgroups (IgG2, IgG3, IgG4; Fig.1; Schur, 1988), which are not usually detected with the classic method used (Fig. 4; Vidarsson et al., 2014).

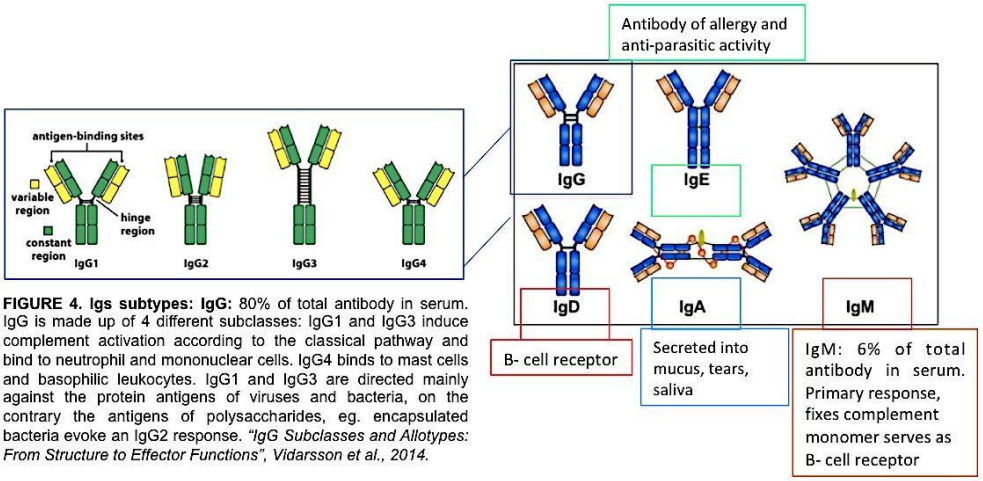

FIGURE 4. Igs subtypes: IgG: 80% of total antibody in serum. IgG is made up of 4 different subclasses: IgG1 and IgG3 induce complement activation according to the classical pathway and bind to neutrophil and mononuclear cells. IgG4 binds to mast cells and basophilic leukocytes. IgG1 and IgG3 are directed mainly against the protein antigens of viruses and bacteria, on the contrary the antigens of polysaccharides, eg. encapsulated bacteria evoke an IgG2 response. *"IgG Subclasses and Allotypes: From Structure to Effector Functions", Vidarsson et al., 2014.*

Accumulating evidence supports the pathological role of CSF immunoglobulins (Yo et al., 2020). CSF OCBs are found to be associated with greater MRI lesion load and brain atrophy, increased levels of disease activity and disability (Caroscio et al., 1986) and with a more rapid conversion from clinically isolated syndrome (CIS) to early RRMS (Avasarala et al., 2021; Bankoti et al., 2014; Yo et al., 2020), providing evidence that they may reflect more active central nervous system (CNS)-directed autoimmunity or otherwise contribute to tissue damage (Bankoti et al., 2014). In addition, the presence of co-localizing Igs and complement depositions in ongoing MS lesions and studies demonstrating that antibodies isolated from the CSF of MS patients induce axonal damage and complement-mediated demyelination when applied to

human CNS tissue ex vivo or in vitro, strongly support a key role of plasma cells and Igs, in MS pathology (Yo et al., 2020).

1.2.3.1 CSF and serum OCBs

MS CSF OCBs were not merely produced by CNS B cells, but also by peripheral B cells, which indicate that disease-relevant B cells circulate between the CNS and peripheral compartments (Bankoti et al., 2014) Recently it has been shown that serum IgG in MS was significantly elevated and there was a strong correlation between CSF IgG and CSF albumin, and also between CSF IgG and serum IgG (Beseler et al., 2017). Since CSF albumin is exclusively derived from the blood in MS, this correlation suggests that most of the CSF IgG is derived from the blood, suggesting that an ongoing exchange of immune cells between the peripheral blood and the CNS is required to maintain intrathecal B-cell stimulation and OCBs (Bankoti et al., 2014). In the brain, IgGs recognize antigens on the cell surfaces of neurons and glial cells and form immune complexes with complement factors and immune cells, inducing enhanced cytotoxicity and, consequently, demyelination and axon loss (Fig. 5; Yo et al., 2020).

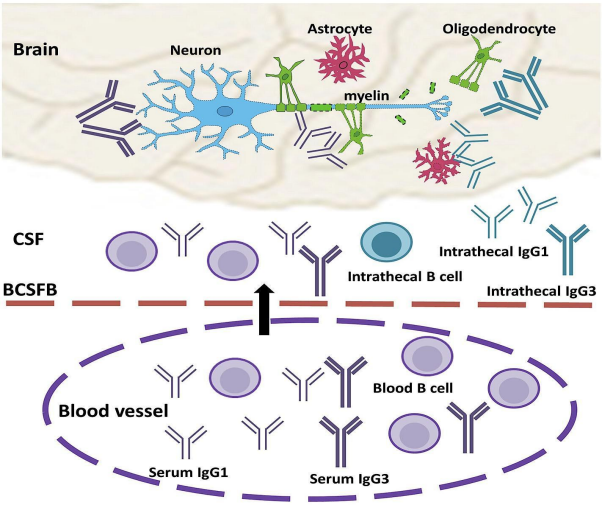

FIGURE 5. Role of serum antibodies in MS pathogenesis: circulating serum antibodies (IgG1 and IgG3, purple) and antibody-producing B cells migrate across the impaired blood-barrier (arrow), and they are present in CSF OCBs and CNS lesion together with intrathecal IgGs (IgG1 and IgG3, turquoise). In the brain, IgGs recognize antigens on the cell surfaces of neurons or/and glial cells and form immune complexes with complement factors and/or immune cells. Elevated levels of IgG1 and IgG3 induce enhanced cytotoxicity or reduced threshold to trigger injury response to CNS cells, which, in turn, result in loss of myelin sheath outside of axons. BCSFB, blood-CSF barrier.*"The Role of Antibodies in the Pathogenesis of Multiple Sclerosis", Yo et al., 2020.*

1.2.3.2 IgM OCBs

IgM are large molecules consisting of pentameter units and ten antigen-binding sites and are strong inducer of classical complement activation (Middleton et al., 2016). In the last decade, several studies have demonstrated a relevant association between intrathecally produced IgM and a more severe MS course (Villar et al., 2005, 2008; Calabrese et al., 2012). However, other studies did not find an association between IgM and a more severe MS course (Schneider et al., 2007; Stauch et al., 2011). Although CSF IgM oligoclonal bands (IgM OCB) are mainly considered a prognostic and disease activity biomarker than a diagnostic one, though not routinely used in clinical practice (Toscano et al, 2021). Indeed, intrathecal IgM synthesis is involved in demyelination and axonal injury (Piddlesden et al., 1993; Villar et al., 2005), the main source of disability in MS patients (Mead et al., 2002). In particular, IgM are the only Igs able to recognize myelin lipids, such as myelin oligodendrocyte glycoprotein, proteolipid protein, and myelin basic protein, inducing demyelination processes and also in axonal injury, the main source of disability in MS patients (Villar et al., 2005; Villar et al., 2008; Owens et al., 2009). Indeed, intrathecal IgM synthesis at diagnosis has been suggested as a prognostic marker of a more rapid and severe disease progression in MS and has been linked to higher probability of converting to SPMS in RRMS patients (Villar et al., 2002). In patients with clinically isolated syndrome (CIS), IgM OCBs detection is associated to higher risks of conversion to clinically definite MS and with a greater MRI lesion load, brain atrophy and an aggressive disease course (Villar et al., 2002). Therefore, in RRMS patients, predicted a higher probability of converting to secondary SPMS (Villar et al., 2002). Intrathecal IgM synthesis is in fact involved in demyelination and axonal injury, the main source of disability in MS patients.

1.3 Vaccination in Multiple Sclerosis

Vaccinations against infectious diseases are an important part of general health maintenance for patients with MS (Bar-Or et al., 2020). However, use of vaccines has often been problematic because of misguided concerns that they may trigger (MS onset) and exacerbate (MS relapse) the disease and/or that some DMTs may influence the immune response to immunisations and/or their safety (Reyes et al., 2020).

However, to date, no studies support a link between vaccination and causation of MS. Specifically, no association between HepB vaccination, HPV vaccination, BCG, Hepatitis B, Influenza, Measles-Mumps-Rubell and the risk of MS were found (Langer-Gould 2014; Farez, Correale 2011).

DMTs used to treat MS suppress or modulate normal immune function and may increase susceptibility to infections and may reduce vaccine effectiveness because of a decreased ability to mount an immune response (Bar-Or et al., 2020). Previous studies suggest that MS patients have an increased risk of more severe infections (Celius, 2017; Marrie et al., 2014; Montgomery et al., 2013; Smestad et al., 2009; Wijnands et al., 2017), mostly of the urinary or respiratory tract due not only to the DMT used, but also to other factors such as age, comorbidities and disability level (Jick et al., 2015; Montgomery et al., 2013; Persson et al., 2020).

Immunoglobulins (Igs) play a role in the response to infectious agents and to vaccines and their decrease is associated with a heightened risk of infections (Furst et al., 2009). Several DMTs reduce Igs levels, specifically B-cell depleting-therapy, including ocrelizumab. Ocrelizumab depletes CD20+ B cells (Genovese et al., 2008), while preserving the capacity for B-cell reconstitution and preexisting humoral immunity (Martin et al., 2006; Di Lillo et al., 2008), since the CD20 receptor is not expressed by plasma cells (Nadler et al., 1981), namely the B cells cells that produce antibodies.

1.3.1 COVID-19 pandemic and vaccination in MS

Coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has imposed an unprecedented global health emergency (Bordoni et al., 2020). Large-scale vaccination has represented the most effective measure for mitigating the COVID-19 diffusion, worldwide. However, the

vaccination and the COVID-19 itself, have raised very important concerns in patients affected with immune-mediated diseases or receiving immunosuppressant medication, like MS patients.

Among the vaccines currently available, the BNT162b2 mRNA-based vaccine has been approved and recommended by the European Medicines Agency in December 2020 in MS patients. The effect and immune response of BNT162b2 vaccine in MS patients treated with DMTs, such as cladribine, fingolimod and ocrelizumab, is debated.

Coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), whose precise pathogenesis remains mostly unclear (Bordoni et al., 2020), is the first significant pandemic of the twenty-first century. Large-scale vaccination programs represented the most effective measures for mitigating the COVID-19 severity and diffusion worldwide (Tortorella et al., 2021). Among the vaccines currently available, the BNT162b2 mRNA-based vaccine, approved by the European Medicines Agency in December 2020, was one of those recommended in MS.

The effect of this vaccine in MS patients treated with different disease-modifying therapies (DMTs) in terms of immune response is still a matter of study.

However, after vaccination, an impaired seroconversion was observed in ocrelizumab- and fingolimod-treated pwMS resulting in an absent or low presence of SARS-CoV-2 anti-spike immunoglobulin-G (IgG) in a pioneer Israeli study (Achiron et al., 2021). Subsequent studies partially confirmed an impaired seroconversion in ocrelizumab-treated patients, but revealed that most fingolimod-treated patients developed a serological response after vaccination (Agrati et al., 2021).

Contradictory results also emerged regarding the type of immunological response to the vaccine; it is well known that the BNT162b2 vaccine induces both humoral and cell-mediated immune responses against viral spike peptides (Guerrieri et al., 2022), and that pwMS showed a sustained and spike-specific CD4+ and CD8+ T cells response after vaccination (Iannetta et al., 2021) by releasing various cytokines, such as IFN-γ and TNF-α (Agrati et al., 2021). Specifically, Apostolidis and colleagues (Apostolidis et al., 2021) demonstrated a specific and robust CD3+ CD8+ T cells activation after COVID-19 vaccine in MS patients treated with ocrelizumab, while

other studies showed no difference in T-cells activity receiving immunosuppression compared to healthy people (Prendecki et al., 2021). Therefore, data on serological response and immune profile assessment in MS patients under specific treatment are still advocated.

1.4 Biomarkers Associated with Multiple Sclerosis

MS is an inflammatory-neurodegenerative disease of the central nervous system presenting with significant inter- and intraindividual heterogeneity, with regard to radiological and clinical manifestation and progression, as well as therapy response (Ziemmesse et al., 2019). Finding biomarkers able to predict the conversion from CIS to MS, to distinguish MS from other diseases and to differentiate between RRMS, SPMS and PPMS, is essential in choosing a personalized and effective pharmacological treatment from the earliest stages of the disease.

The cause of disease is not known and detecting and predicting disease progression is difficult (Paul et al., 2019) even because both imaging and clinical biomarkers are not able to predict the individual disease course. On the contrary, molecular biomarkers have so far routinely been used in clinical practice for facilitating diagnosis, prognosis and for the assessment of therapeutic response, complementing MRI and clinical characteristics.

A biomarker is defined as a characteristic that can be "objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic intervention" (Biomarkers Definitions Working Group 2001). Protein molecular biomarkers are the most common type of biomarkers used in medical diagnostics. Molecular biomarkers from blood and CSF are easily quantifiable, but sample collection, processing and storage, as well as the experimenter abilities, can influence or affect the result of the detection method. In addition, only a few molecular biomarkers have been identified as specific for MS. For these reasons, the research of more specific new biomarkers and the use of new methodologies have increased dramatically in recent years.

1.4.1 CSF biomarkers

CSF biomarkers were specifically highlighted because of their relevance to the MS process and their degree of validation (Harris et al., 2017).

Oligoclonal bands (OCBs)

The presence of OCBs in CSF supports the diagnosis of MS. Although they are not MS

specific (Dobson et al., 2013; Petzold et al., 2013), IgG OCBs occur in 90% of MS patients and remain a criterion for MS diagnosis (Thompson et al., 2018). In addition, elevated IgG index, a measure of blood-CSF barrier dysfunction, represents an additional biomarker of disease (Ziemmesse et al., 2019). On the contrary, IgM antibodies OCBs are present in the CSF of only 40% of MS patients, but their detection in CSF is predictive of conversion from CIS to clinically definite MS (Ferraro et al., 2013) and of an aggressive course of the disease (Villar et al., 2005; Ferraro et al., 2013). However, the usefulness of IgM as a predictive biomarker remains to be confirmed and thus also investigated during my PhD.

Chitinase-3-like-1

Chitinase-3-like-1 (CHI3L1, also known as YKL-40) is a secreted glycoprotein produced by glial cells (Bonneh-Barkay et al. 2010; Cantó et al., 2015). In MS, CHI3L1 is mostly expressed by reactive astrocytes and activated microglia at the rim of chronically active lesions (Cantó et al., 2015). In addition, high CHI3L1 levels in CSF were associated with an early conversion from CIS to MS (Comabella et al., 2010) and with more rapid development of disability (Martinez et al., 2015) and cognitive impairment (Quintana et al., 2018).

C-X-C motif chemokine-13

C-X-C motif chemokine-13 (CXCL13) is one of the most potent B cell chemoattractants involved in the recruitment of B cells into the CNS in MS (Ziemmesse et al., 2019). CXCL13 is expressed by B-cell follicles and contributes to the germinal center formation by recruiting activated B and follicular helper CD4+ T cells expressing its cognate receptor CXCR5 (Paul et al., 2019; Zotos et al. 2010; Crotty 2012; Victora and Nussenzweig 2012). Elevated CXCL13 levels in CSF have been found in MS patients compared to healthy controls (Kademi et al., 2011), in CIS patients converted in MS (Brett-schneider et al., 2010; Kademi et al., 2011), and during relapse in RRMS (Kademi et al., 2011).

Intrathecal inflammatory profile

A specific intrathecal inflammatory proteins profile, including proinflammatory cytokines (IFNc, TNF, IL2, and IL22) and molecules related B-cell activity and lymphoid-neogenesis (CXCL13, CXCL10, LTa, IL6, and IL10) characterizes a subgroup of MS patients with higher levels of grey matter (GM) damage at the time of diagnosis (Magliozzi et al., 2018). Similarly, increased expression of the same molecules were found in the CSF of postmortem MS cases with high levels of meningeal inflammation and GM demyelination, suggesting common pattern of intrathecal (meninges and CSF) inflammatory profile strongly correlates with increased cortical pathology and confirming an important role of CSF analysis, combined with MRI features, as a useful tool for the identification of prognostic marker for more aggressive MS (Magliozzi et al., 2018).

Neurofilaments: prognostic and predictive biomarkers

Accurate assessment of disease activity, including MRI activity, clinical relapse and disease progression, is important to determine treatment efficacy (Harris et al., 2017). Several CSF biomarkers have been shown to correlate with disease activity associated with intrathecal inflammation and neurodegeneration, such as CXCL13 (Di Sano et al., 2020) and neurofilament light protein (NFL) and (Harris et al., 2017). In MS, CSF-CXCL13 levels correlate with CSF-NFL levels (Novakova et al., 2017a), supporting an association between B cell driven inflammation and axonal injury in the pathogenesis of MS. The levels of NFL and CXCL13 in CSF are increased during relapse and are reduced after treatment with DMTs (Sellebjerg et al., 2009, Gunnarsson et al., 2011).

NFL is a neuroaxonal cytoskeletal protein that is released into the CSF, and eventually into blood, on neuronal injury (Khalil et al., 2018). Elevated levels of NFL in CSF reflect inflammatory-mediated axonal damage, primarily used in association with inflammatory disease activity in MS (Malmestrom et al., 2003). Recently, CSF NFL levels are associated not only with inflammatory outcomes, but also with neurodegenerative-mediated axonal injury and used to monitor neurodegeneration during DMTs treatment (Khalil et al., 2018).

Parvalbumin: a promising new biomarker

GABAergic interneuron populations in the human cortex expressed parvalbumin (PVALB), a calcium binding protein that protects neurons from excess intracellular calcium (DeFelipe et al., 1997; Beers et al., 2001; Dekkers et al., 2004). Loss of this protein causes the GABAergic interneurons injury and cortical hyperexcitability and has been observed in several other neurological diseases, such as MS (Cotter et al., 2002; Eyels et al., 2002). A reduction in the expression of neuronal proteins in the cortex and loss of grey matter volume occurs early in the MS disease (Chard et al., 2002). Specifically, data on postmortem MS brains have indicated a reduction in the expression of the PVALB gene, important in GABAergic neurotransmission, as well as the reduced extension of neurites in PV-expressing interneurons within normal appearing gray matter and in MS motor cortex (Dutta et al., 2006; Falco et al., 2014). Furthermore, several studies have demonstrated a significant reduction in the number of PVALB + interneurons in the motor cortex of MS patients (Dutta et al., 2006; Clements et al., 2008); however, further studies should be designed to determine if this decrease is specifically linked to MS neurodegeneration and whether it represents an initiating insult, or an effect of early neuronal dysfunction.

1.4.2 Serological biomarkers

No specific serological MS-biomarkers exist (Torkildsen et al., 2021). However, specific antibodies such as anti-aquaporin-4 (anti-AQP4) and anti-myelin oligodendrocyte glycoprotein (anti-MOG) are dosed on serum for the differential diagnosis, namely to distinguish MS from neuromyelitis optica spectrum disorder (NMOSD). Aquaporin-4 (AQP-4) is a water channel protein expressed in the CNS by astrocytes (Papadopoulos et al., 2012) and antibodies against this protein are detectable in about 75% of patients with NMOSD, but not in MS patients (Flanagan et al., 2016). MOG is a myelin protein expressed exclusively on the surface of myelin sheaths and membranes of oligodendrocytes (Delarasse et al., 2006). Anti-MOG antibodies titers are not suitable for the diagnosis or prognosis of MS, because in MS are rare, with the frequency of seropositive MS patients being highest in the pediatric patient group (Ziemmesse et al., 2019).

Furthermore, serum-NFL (sNFL) has been highlighted as a promising serum biomarker

able to quantify ongoing neuronal damage in response to the different DMTs used, to reflect acute disease activity and to predict the course of disability worsening in persons with MS. However, sNfL is not specific for MS pathology and it is not a stable measure because it increases physiologically with age and decreases with body-mass index.

1.4.3 Biomarker detection technologies

This PhD thesis is primarily focused on the evaluation of protein molecular biomarkers and their detection in MS patients. Biomarkers are present in tumor tissues, serum, CSF and other body fluids, and their detection can also be used for the follow-up of a treatment by monitoring the constant decrease in its concentration (Nimse et al., 2016). The ultimate goal of researchers in the field of biomarker detection is to develop a reliable, cost-effective, powerful detection tool for prognosis, diagnosis, and monitoring the progression of a specific disease (Nimse et al., 2016). Although several methods based on highly specific recognition biomarkers have been developed, most detection technologies are based on the principle of conventional immunoassays in which a capture antibody targets capture and a reporter antibody for assay read-out. The enzyme-linked immunosorbent assay (ELISA) system is in fact the most common method to determine the quantitative changes of cytokines/chemokines (Reiken et al., 1994). A limitation of the classic method is that only one parameter per run can be analyzed, considering that the source of samples is limited in volume and has resource issues as well (Akyüz et al., 2017). Therefore, several multiplex technologies were developed to simultaneously quantify the concentration of multiple analytes, using very small sample volumes. In this work were used two different multiplex technologies based on different platforms: the Luminex Bio-Plex® 200 System, BioRad (Luminex Corp., Texas, USA) technology and the Ella-Simple Plex (ProteinSimple, California, USA) technology.

1.4.3.1 Luminex Bio-rad Bio-plex 200 platform

The Luminex Bio-rad Bio-plex platform (X200 System, BioRad) can theoretically analyze several analytes in parallel and works on a bead-based sandwich ELISA principle (Fig. 6; Akyüz et al., 2017). However, unlike ELISA, the Luminex technology allows the simultaneous measurement of multiple analytes by using differentially color-coded beads to which cytokine-specific capture antibodies are then bound. Finally, a sandwich assay using a fluorescently labeled detection antibody is used for quantification (Akyüz et al., 2017). The Luminex kits provide ready-to-use cocktails of the respective cytokine analytes as standard for the assay (Akyüz et al., 2017).

Bio-Plex immuno-assay is therefore a qualitative and quantitative proteomics technique, used to analyze the expression of Igs and inflammatory/cytotoxic molecules in the CSF and serum samples. Specifically, this multiplex bead-based technology represents an immunological microarray: two fluorochromes and a specific antibody for a given analyte are associated with each magnetic bead characterized by a unique emission wavelength when excited by the red laser (660 nm). A specific plate, consisting of 96 wells, is used and samples, antibodies and reagents are added sequentially to each well. Firstly, several beads-antibody conjugates are mixed in each sample allowing the detection of several analytes simultaneously. Secondly, a secondary antibody conjugated with biotin is added, which binds the analyte in a different antigenic site than the primary antibody linked to the bead. At last, streptavidin (protein with high binding affinity for biotin) is added, which is in turn conjugated to a third fluorophore. Finally, the plate is read using the Bio-plex instrument, which uses the same principle as flow cytometry: the beads are aspirated by a needle and led into the reading chamber where they are passed one by one. Each ball is therefore hit by two laser beams: one that recognizes the color of the microsphere, and therefore the analyte linked to the primary antibody, going to excite the two fluorochromes that identify it; the other which instead excites the fluorochrome (with an emission wavelength of 532 nm) linked to the secondary antibody allowing the quantification of the analyte. The advantages of the Bio-Plex system are many, such as high reproducibility (it is almost completely automated technique, with very short analysis times, reading about 20.000 balls per second), high sensitivity (a small volume of sample is required for analysis, with limited

implications for patients), standard curves included in the assay that provide a correct quantification without further data processing, multiple readings on each bead set further validate the result and Bio-Plex Manager Software controls the instrument, acquires and analyzes data obtained.

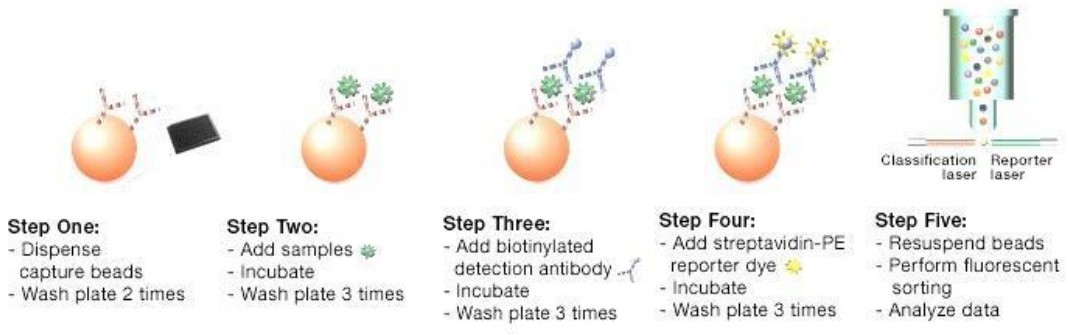

FIGURE 6. Schematic representation of a sandwich-based Bio-Plex assay workflow: *"Validation of novel multiplex technologies",* Akyüz et al., 2017.

1.4.3.2 ELLA platform

Simple Plex is a novel automated immunoassay platform, produced by ProteinSimple, used for a rapid and sensitive detection of up to four targeted protein antigens across multiple biological sources. Simple Plex consists of a multi-analysis system based on a microfluidic cartridge and an automated analyzer, the Ella instrument (Fig. 7; Aldo et al., 2016). The cartridges used by the Ella system separate the sample into four nanocapillaries. Each microfluidic channel contains two spatially separated capture antibodies, allowing the quantification of up to eight target analytes per sample. While migrating through microfluidic channels, it subsequently passes specific capture antibodies, then passes the antibody detection and is finally scanned in the glass nano-reactor where the fluorescence intensities are measured. Unlike Luminex technology, the standard values of the Ella system are provided by the company for each batch of cartridges (Aldo et al., 2016). The advantages of the Ella system are that it runs four parameters automatically in triplicates and the standard is ready-to-use, supplied by the kit, thus avoiding errors in subsequential standard dilutions. In addition, a strong correlation between the results obtained with Simple Plex and conventional immunoassays such as ELISA and Luminex was found (Aldo et al.,

2016). Simple Plex showed in fact several advantages over these traditional plate-based immunoassay approaches for multiplexing in terms of required sample volumes, high sensitivity and reproducibility: the ELLA system by ProteinSimple seems to be in fact the most reliable platform (Reiken et al., 1994). However, the disadvantage of the ELLA system is that it allows only the lowest number of analytes (four parameters) to be measured in parallel (Reiken et al., 1994).

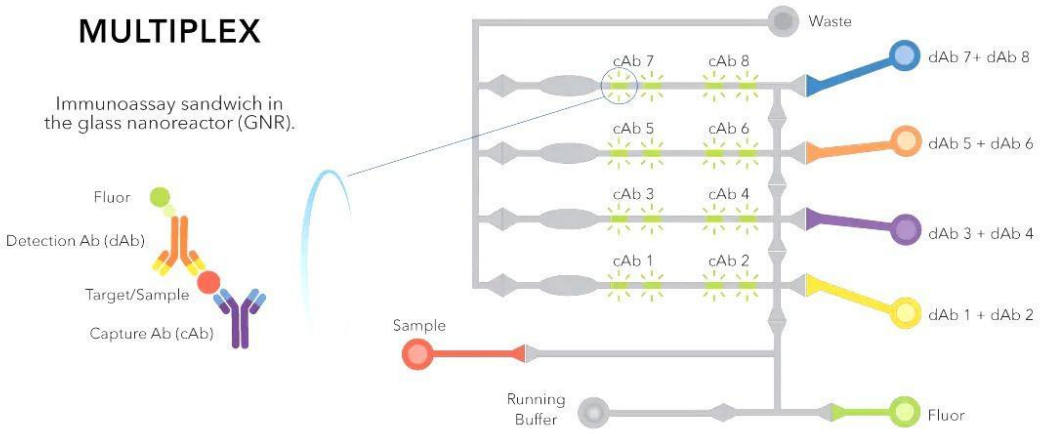

FIGURE 7. ELLA assay: ELLA splits each sample across four parallel isolated microfluidic channels. Each channel has a single-plex immunoassay for a specific analyte, therefore avoiding antibodies' cross-reactivity. Glass nanoreactors (GNRs) consist of glass capillaries that facilitate antibody-analytic interaction. This cost-effective assay allows you to maximize the data that you can extract from each sample in 90 minutes or less. Generate up to 256 reportable results from each multiplex assay (ELLA microfluidic analyzer, Protein Simple; Bio-techne, https://www.bio-techne.com/reagents/simple-plex-immunoassays).

2. AIMS OF THE THESIS

The identification of biomarkers that inform prognosis based on the degree of underlying disease activity would allow for more timely and rational individualized clinical management of MS patients. With this goal, the aim of this thesis was to identify new biomarkers associated with a high risk of disease activity.

- To explore the role and biomarkers of intrathecal inflammation and possible association with peripheral inflammation, in order to identify the pro-inflammatory factors involved in cortical damage and to better understand the mechanism at the base of progressive MS diseases. This could also help to detect possible new therapeutic targets, even for progressive MS forms;

- To investigate the relationship between intrathecal and peripheral inflammation in MS patients and clarify whether intrathecal CSF protein profiling is somehow reflected in paired serum samples;

- To define the intrathecal IgM levels production and its possible association with disease activity, considering clinical, radiological and biological parameters;

- To evaluate the role of PVALB as a new early biomarker of a severe MS disease comparing to Nf-L in MS patients;

- To study the immune response after COVID-19 mRNA vaccination in multiple sclerosis patients treated with DMTs.

3. Main studies

This thesis is based on the results of five studies, referred to as Study 1, Study 2 Study 3, Study 4 and Study 5. All studies, with the exception of the Study 2, were published in research papers.

3.1 Study 1: "The CSF protein profile of patients at risk of disease activity "

The findings described in this study were published in the article "The CSF Profile Linked to Cortical Damage Predicts Multiple Sclerosis Activity" in Annals of neurology (88(3), 562–573, doi: 10.1002/ana.25786), published in 17 May 2020, with permission from authors and with license from the journal (© 2020 American Neurological Association).

3.1.1 Introduction and rationale

During the years several studies investigate the extensive nature of cortical grey matter (GM) damage in MS (Peterson et al., 2001; Bö et al., 2003; Kutzelnigg et al., 2005, Calabrese et al., 2012), suggesting that this widespread pathology plays a major role in the accumulation of neurological disability and cognitive dysfunction (Gardner et al., 2013). Damage to the cortical grey matter in MS is characterized by leucocortical, intracortical and subpial lesions (Peterson et al., 2001; Bo et al., 2003; Kutzelnigg et al., 2005; Magliozzi et al., 2007) associated with the intrathecal inflammation (Peterson et al., 2001; Brink et al., 2005; Kutzelnigg et al., 2005; Magliozzi et al., 2010). Indeed, early stages of disease may include cortical damage correlated with considerable inflammation (Gardner et al., 2013). However, a CSF protein profile associated with a high risk of disease activity has not yet been outlined.

3.1.2 Study aim

Exploring and defining the CSF proteins profile in patients at risk of disease activity.

3.1.3 Methods

Ninety-nine treatment-naïve RRMS (female/male = 66/33; mean age = 40.4 ± 12.0 years; range 18–55; Table 1) from the MS Center of Verona University Hospital were enrolled at the time of clinical onset (T0) to participate in this longitudinal 4-year prospective study. All patients underwent baseline CSF analysis, clinical and 3T-MRI evaluation. Following the enrollment, each patient underwent a 3T-MRI and was started on one of the first-line (interferon beta-1a, glatiramer acetate, teriflunomide, and dimethyl fumarate) disease-modifying treatments, and was monitored with clinical and radiological evaluation for 4 years after the diagnosis. The neurological evaluation, including the EDSS score (Poser et al., 1983) assessment, was performed every 6 months. The number of relapses and the treatments administered were also recorded. The number and volume of white matter (WM) lesions at baseline and the number of Gad positive (Gad+), and new and enlarging WM lesions at the end of the study were assessed on FLAIR images. The number and volume of total cortical lesions (CLs) and the new CLs were assessed on DIR images (Geurts et al., 2011). Patients were classified into two groups: evidence of disease activity (EDA) or no evidence of disease activity (NEDA), considering occurence or absence of relapses, new white matter lesions, and Expanded Disability Status Scale [EDSS] change, respectively.

TABLE 1. Characteristics of Patients at Study Entry in the Whole Population, and in the Two Groups With and Without Evidence of Disease Activity

		Disease activity	
	Whole group n = 99	**EDA n = 41**	**NEDA n = 58**
Age	40.4 ± 12.0 (18–55)	34.7 ± 12.5	39.5 ± 11.3*
Gender, F:M	66:33	28:13	38:20
Disease duration, yrs	1.8 ± 2.2	1.4 ± 2.1	2.1 ± 2.3*
EDSS median, range	2.0 (0–0-6.0)	2.0 (0.0–4.0)	1.5 (0.0–6.0)
OCBs, yes/no	87/12	38/3	49/9
CLs number	4.3 ± 5.3	7.8 ± 6.0	1.8 ± 3.0***
CL volume, mm^3	230 ± 154	376 ± 76	127 ± 85***
CTh T0, mm	2.4 ± 0.4	2.3 ± 0.4	2.5 ± 0.3**
T2 WMLL T0, mm^3	960 ± 352	1000 ± 358	933 ± 350
Number of Gad+ lesions°	0.2 ± 0.6	0.3 ± 0.5	0.2 ± 0.6
Number of relapses°	1.0 ± 0.3	1.0 ± 0.4	1.0 ± 0.2
Dimethyl Fumarate	51	20	31
Teriflunomide	21	9	12
Glatiramer acetate	16	7	9
Interferon Beta1a	11	5	6

Data are reported as mean and standard deviation if not differently reported. ° = in the year before lumbar puncture. CLs = cortical lesions; CTh = cortical thickness; EDA = evidence of disease activity; EDSS = Expanded Disability Status Scale; Gad = gadolinium; LL = lesion load; NEDA = no evidence of disease activity; OCB = oligoclonal band; WM = white matter.The *p* values were obtained using Mann–Whitney *U* tests; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

CSF analysis

About 10 ml of CSF were collected from each patient and immediately centrifuged for 10 minutes at 300g, 4°C. The supernatant obtained was carefully separated from the cell pellet and frozen at -80°C until use for proteomic profiling. 18 cytokines previously identified (Magliozzi et al., 2018) to be associated with grey matter damage were were assessed using immune-assay multiplex techniques based on the Luminex technology (Bio-Plex-X200; BioRad, Hercules, CA). Specifically, customized kits from Bio-Plex Pro Human Chemokine Panel-5-Plex (CXCL13, CXCL12, CCL25, TNFα, IL6) and Bio-Plex Pro Human Inflammation Panel 1-13 plex (sTNF-R, TWEAK, APRIL, BAFF, LIGHT, IFNγ, IFNα2, IFNλ2, IL8, IL10, MMP2, Pentraxin, sCD163) were performed. To verify the reproducibility and consistency of the results, all samples were run in duplicate. The results obtained of each protein level were normalized by protein concentration (mg prot /mL) of each CSF sample and the unit of

the final result was ng/mL/mgProt. However, analyzed results were unchanged when experiments were also run using absolute concentrations of CSF protein.

Statistical Analysis

The Mann–Whitney U test was used to compare patients with different clinical and MRI characteristics both at baseline and the end of follow-up. The pairwise univariate Spearman rank correlation index was used to evaluate correlations between CSF proteins levels at diagnosis and both clinical and MRI variables at the end of follow-up.

3.1.4 Results

CSF Profiles Identified Patients with EDA

a) Forty-one patients experienced EDA and, compared to the NEDA group, had at diagnosis significant higher CSF levels of CXCL13 (27.9 ± 39.8 in EDA vs 5.4 ± 6.7 in NEDA), CXCL12 (3760 ± 3187 in EDA vs 1564 ± 954 in NEDA), IFNγ (33.8 ± 34.7 in EDA vs 7.3 ± 8.8 in NEDA), TNFα (59.8 ± 45.4 in EDA vs 19.1 ± 15.7 in NEDA), sCD163 (54,460 ± 21,801 in EDA vs 42,419 ± 22,012 in NEDA), LIGHT (0.7 × 10−3 ± 1.0 in EDA vs 0.2 × 10−3 ± 0.3 in NEDA), and APRIL (36.9 × 10−3 ± 32.4 in EDA vs 26.3 × 10−3 ± 27.6 in NEDA; adjusted $p < 0.001$ for all tests; see Table 2).

TABLE 2. Characteristics of patients at study entry in the whole population, and in the two groups with and without evidence of disease activity

	New CLs		Disease activity	
	Yes (n = 46)	**No (n = 53)**	**NEDA (n = 58)**	**EDA (n = 41)**
CXCL13	27.1 ± 37.7 [0–213] ***	4.0 ± 4.8 [0–17]	5.4 ± 6.7 [0–25]	27.9 ± 39.8 [0–213]***
CXCL12	2.9 ± 2.2 [0.1–17.9]	2.1 ± 2.6 [0.1–12.8]	1.6 ± 1.0 [0.1–5.3]	3.8 ± 3.2 [0.5–17.9]***
CCL25	1.1 ± 0.7 [0–3.0]	1.1 ± 0.8 [0–4.0]	0.9 ± 0.6 [0–3.1]	1.4 ± 0.9 [0–4.0]
TNFα	42.8 ± 41.4 [2–223]	30.0 ± 32.5 [0–133]	19.1 ± 15.7 [0–60]	59.8 ± 45.4 [6–223]***
sTNFR1	5.7 ± 4.5 [1.0–24.0]	4.3 ± 2.6 [0.3–12.1]	3.6 ± 1.7 [0.8–9.9]	6.9 ± 4.6 [0.3–24.0]***
TWEAK	2.2 ± 2.3 [0.01–10.7]	1.6 ± 1.6 [0.06–8.2]	1.6 ± 1.5 [0.1–8.2]	2.3 ± 2.5 [0.06–10.7]
APRIL	34.1 ± 32.1[0.9-125.2]	27.7 ± 27.9 [2.6-145.8]	26.3 ± 27.6[0.9-125.2]	36.9 ± 32.4 [7.9–145.8] **
BAFF	9.3 ± 6.6 [2.4–45.0]	9.5 ± 7.0 [1.5–36.6]	7.5 ± 4.3 [1.5–21.6]	12.1 ± 8.6 [2.4–45.0] ***
LIGHT	0.6 ± 1.0 [0–6]	0.3 ± 0.3 [0–2]	0.2 ± 0.3 [0–1]	0.7 ± 1.0 [0–6] ***
IFN-γ	18.3 ± 20.6 [0–78]	18.2 ± 31.1 [0–144]	7.3 ± 8.8 [0–38]	33.8 ± 34.7 [2–144] ***
IFN-α2	18.4 ± 17.4 [0–59]	18.4 ± 22.8[0.0–99.0]	10.8 ± 10.7[0.0–32.5]	29.1 ± 25.5 [0.0–99.0] ***
IFNλ2	0.4 ± 0.9 [0–5]	0.4 ± 0.8 [0–4]	0.4 ± 0.8 [0–4]	0.4 ± 0.9 [0–5]
IL-6	33.9 ± 62.6 [1–286]	19.6 ± 35.6 [1–229]	8.9 ± 8.4 [1–38]	50.7 ± 70.9 [2–286]***
IL-8	0.7 ± 0.9 [0.02–4.5]	0.5 ± 0.7 [0.02–3.1]	0.3 ± 0.2 [0.02–1.1]	1.0 ± 1.0 [0.1–4.5]***
IL-10	19.2 ± 16.9 [1–73]	23.1 ± 24.2 [0–93]	18.6 ± 19.3 [0–79]	25.0 ± 23.3 [3–93]
MMP2	2.0 ± 6.0 [0.09–36.5]	2.0 ± 5.0 [0.07–27.9]	2.3 ± 5.4 [0.07–27.9]	1.5 ± 5.6 [0.09–36.5]
Pentraxin3	0.5 ± 0.7 [0–3]	0.3 ± 0.4 [0–3]	0.3 ± 0.4 [0–3]	0.5 ± 0.8 [0–4]
sCD163	50.5 ± 18.8 [10–86]	44.7 ± 25.4 [0–126]	42.4 ± 22.0 [1–126]	54.5 ± 21.8 [0–109]**

Data are reported as: mean ± standard deviation [minimum-maximum]. Concentration of cytokines is expressed in ng/ml/mg^{Prot}. CLs = cortical lesions; CSF = cerebrospinal fluid; EDA = evidence of disease activity; MS = multiple sclerosis; NEDA = no evidence of disease activity.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

b) In addition, CSF concentrations of CXCL13, TNFα, and IFN-γ were found to have the strongest correlation with the clinical and radiological outcomes (Fig.1).

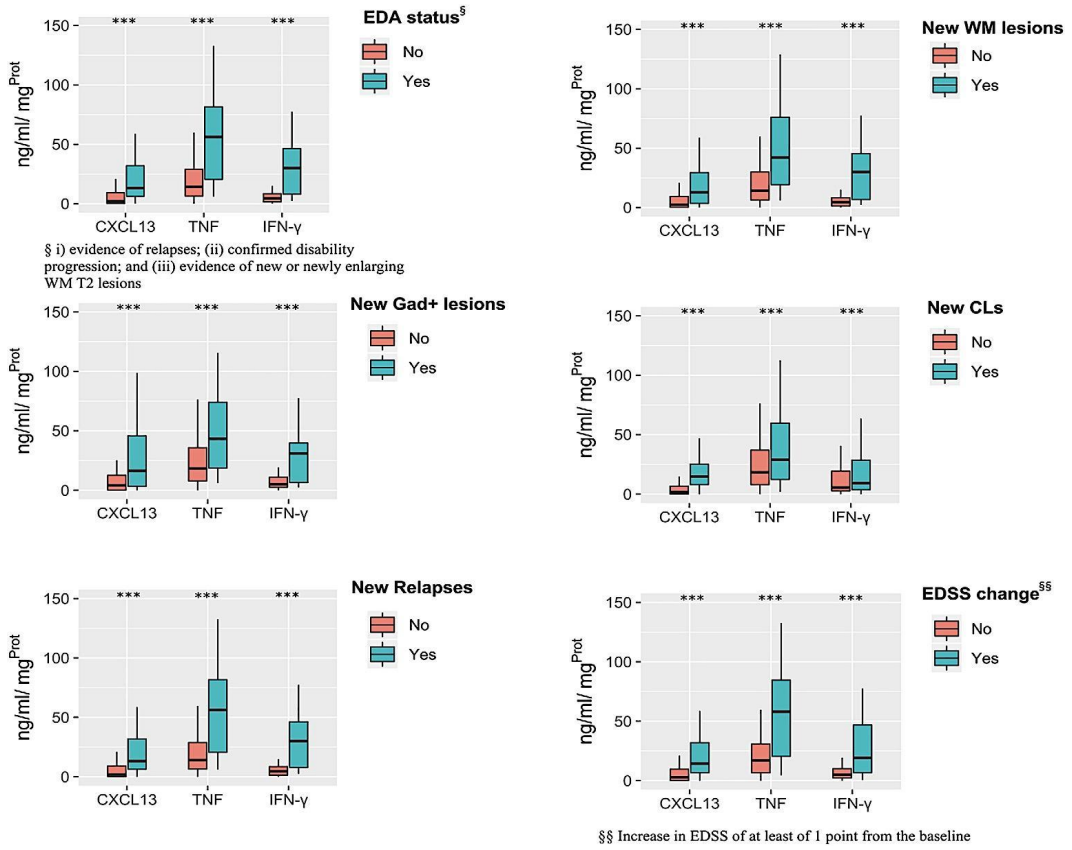

FIGURE 1: Boxplots of the CSF proteins level in correlation with clinical and MRI features at the end of study. Concentrations (ng/mL/mg^{Prot}) of CXCL13, TNF, and IFNγ are shown, among groups distinguished by the occurrence of disease activity, new WM lesions, new Gad+ lesions, new CLs, new relapses, and EDSS change. Boxplots show the medians and the two hinges. *** for p < 0.001. CLs = cortical lesions; CSF = cerebrospinal fluid; EDA = evidence of disease activity; EDSS = Expanded Disability StatusScale; MRI = magnetic resonance imaging; WM = white matter.

c) Significant univariate associations between increased cytokine level and clinical and radiological outcomes, occurring during the observation period were identified: CXCL13 correlated with EDSS worsening (rho = 0.52; adjusted p < 0.001), with the occurrence of new WM lesions (rho = 0.46; adjusted p < 0.001), and with the number of new relapses (rho = 0.45; adjusted p < 0.001); TNFα correlated with the occurrence of new WM lesions (rho = 0.50; adjusted p< 0.001), with EDSS change (rho = 0.47; adjusted p < 0.001), and with the number of new relapses (rho= 0.42; adjusted p < 0.001); IFNγ correlated with the occurrence of new WM lesions (rho = 0.57; adjusted p < 0.001), with EDSS change (rho = 0.38; adjusted p < 0.001), and

with the number of new relapses (rho = 0.38; adjusted p < 0.001; see Fig.2).

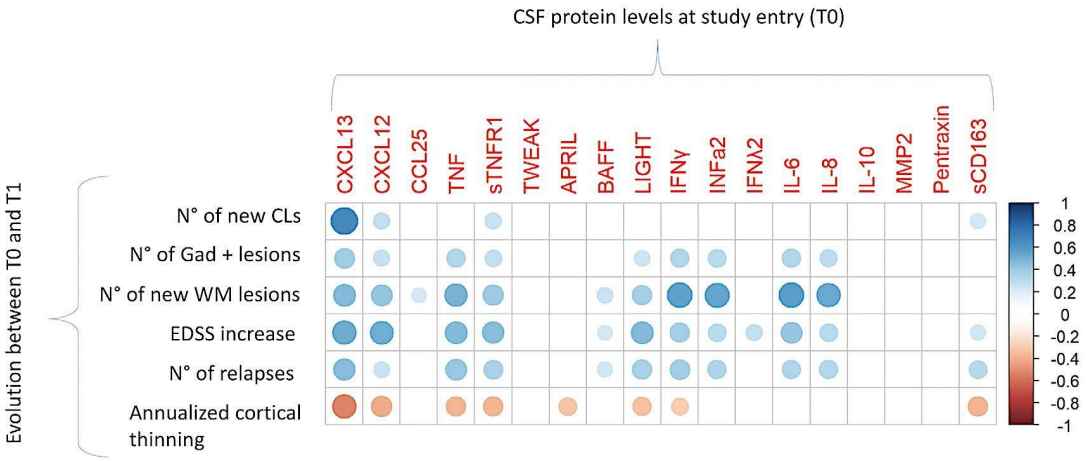

FIGURE 2: Correlation matrix showing Pairwise Univariate Spearman rank correlation index between CSF protein levels at diagnosis and both clinical and MRI variables at the end of follow-up. A FDR correction was applied. The Spearman rank is proportional to the dimension/intensity of the bubbles. CLs= cortical lesions; CSF= cerebrospinal fluid; EDSS= Expanded.Disability Status Scale; WM= white matter.

d) The same independent association between cytokines and the disease activity were confirmed by the multivariate logistic regression analysis; results were adjusted for age and type of first line treatment. ROC curve analysis was performed to assess the specificity and sensitivity of each CSF protein to discriminate between EDA and NEDA patients (Table 3). The 8 CSF proteins, which showed >70% accuracy in predicting the occurrence of disease activity (CXCL13, IFN-γ, TNFα, sTNFR, sCD163, LIGHT, CXCL12, and IFN-λ2), can be considered good biomarkers for the identification of patients at higher risk of experiencing clinical or radiological breakthrough despite being on treatment. Specifically, Forty-six patients developed one or more new CLs (mean number of CLs = 3.6 ± 2.7; range = 1–8), during the study (Table 2). This subgroup had more than fivefold increased CSF levels of CXCL13 (27.1 ± 37.7 vs 4.0 ± 4.8; adjusted p < 0.001), compared to patients without new CLs. We observed a strong positive correlation between the number of new CLs and the CXCL13 CSF level (rho = 0.67; adjusted p < 0.001; Fig.3).

TABLE 3. The Results of ROC Curve Analysis in the Testing Set was Shown

	Optimal cut-off	AUC [95% CI]	Accuracy (%)	Specificity (%)	Sensitivity (%)
LIGHT	0.16	0.78 [0.62–0.95]	80	84	72
sTNFR1	5.69	0.77 [0.60–0.93]	80	89	63
CXCL13	7.85	0.80 [0.62–0.93]	77	73	81
TNFα	37.73	0.76 [0.59–0.93]	77	79	73
CXCL12	1.91	0.80 [0.62–0.93]	77	74	82
IFN-γ	10.54	0.67 [0.49–0.85]	70	79	55
sCD163	46.07	0.67 [0.49–0.85]	70	79	55
IFNλ2	37.78	0.67 [0.49–0.85]	70	79	55
INF-α2	27.61	0.58 [0.43–0.74]	67	89	27
IL-6	11.07	0.67 [0.50–0.86]	67	63	72
BAFF	7.94	0.68 [0.50–0.87]	67	63	73
APRIL	18.786	0.61 [0.43–0.80]	63	68	54
IL-8	44.34	0.52 [0.34–0.71]	57	68	36
MMP2	449.80	0.56 [0.37–0.75]	57	57	55
CCL25	114.40	0.54 [0.35–0.73]	57	63	45
TWEAK	1.01	0.55 [0.37–0.74]	47	53	36
IL-10	17.18	0.56 [0.37–0.75]	43	42	45
Pentraxin3	0.23	0.49 [0.31–0.68]	33	15	63

For each protein, CSF was reported the optimal threshold, AUC, Accuracy, Specificity and Sensitivity, in discriminating between EDA and NEDA patients. Concentration of the cytokines is expressed ng/mL/mg^{Prot}. AUC = area under curve; CI = confidence interval; CSF = cerebrospinal fluid; EDA = evidence of disease activity; NEDA = no evidence of disease activity; ROC = receiver operating characteristic.

e) Specifically, Forty-six patients developed one or more new CLs (mean number of CLs = 3.6 ± 2.7; range = 1–8), during the study (see Table 2). This subgroup had more than fivefold increased CSF levels of CXCL13 (27.1 ± 37.7 vs 4.0 ± 4.8; adjusted $p < 0.001$), compared to patients without new CLs. We observed a strong positive correlation between the number of new CLs and the CXCL13 CSF levels (rho = 0.67; adjusted $p < 0.001$; Fig.3).

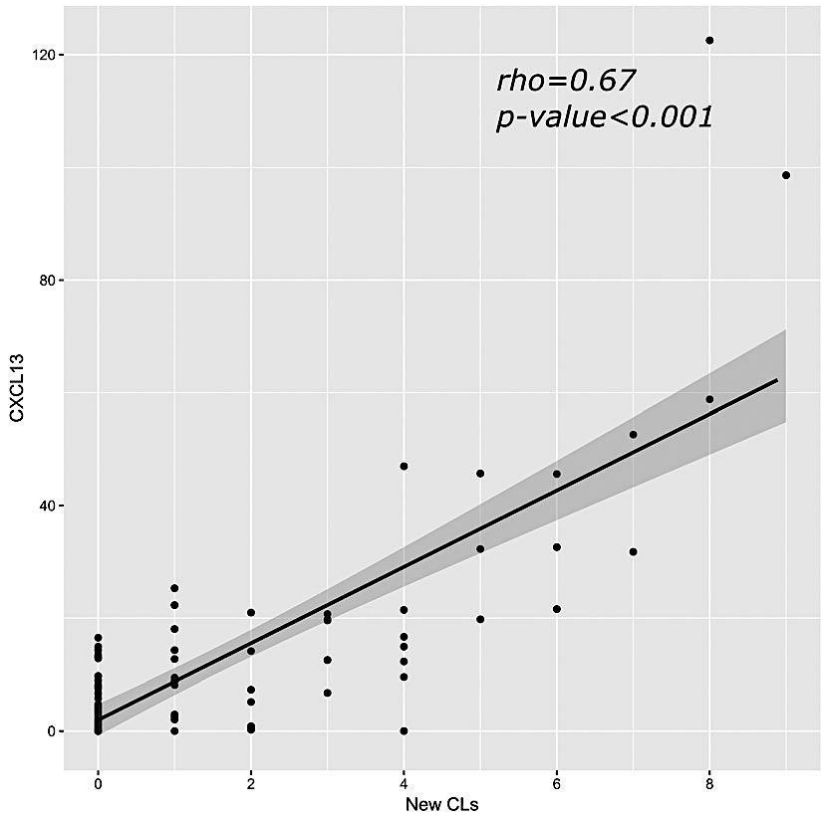

FIGURE 3: Scatter plot of relationship between the CXCL13 (ng/mL/mgProt)) and the new CLs at T1 and its corresponding correlation coefficient (rho) and p value. CLs = cortical lesions.

3.1.5 Interpretation and conclusions

Several studies have provided evidence that soluble factors, such as cytokines and chemokines, realized by B and T cells in periventricular and meningeal spaces are associated with an early cortical damage and GM pathology (Gardner et al., 2013; Lisak et al.; 2012). Among these, in our previous study 18 CSF inflammatory mediators (Magliozzi et al., 2018) were correlated with increased cortical pathology at the time of diagnosis (Magliozzi et al., 2018). In this longitudinal study we analyzed the 18 CSF inflammatory molecules previously identified (Magliozzi et al., 2018) in association with clinical and radiological parameters during 4 years of follow-up from MS diagnosis. We found significantly higher CSF levels of CXCL13, CXCL12, IFNγ, TNFα, sCD163, LIGHT and APRIL in MS patients experienced EDA compared to the NEDA group. CXCL13, CXCL12, LIGHT and APRIL are linked with B-cell intrathecal activity, while IFNγ and TNFα are the main proinflammatory cytokines

secreted by pathogenic T-cell subsets (Th and Th17). Insteads sCD163 represents a marker of activated microglia and monocyte/macrophage infiltrates in MS lesions (Stilund et al., 2015). High CSF levels of the B-cell related cytokines, CXCL13, CXCL12, LIGHT and APRIL, together with pro-inflammatory molecules, IFNγ and TNF, and the monocyte activity biomarker, sCD163, therefore represent a potential molecular signature that could be used to distinguish patients at high risk of experiencing more severe disease activity, increased GM damage, and disability progression in the early phase of the disease. Specifically, CXCL13, TNFα, and IFN-γ were found to have the strongest correlation with the clinical and radiological outcomes of disease activity, such as new WM lesions, new Gad+ lesions, new CLs, new relapses, and EDSS change. In addition, we observed a strong positive correlation between the number of new CLs and the CXCL13 CSF levels, confirming a role of this cytokine in MS pathology. CXCL13 is indeed one of the most potent B cells chemoattractants into the CNS in MS (Ziemmesse et al., 2019) that contributes to the germinal center formation in the meningeal space (Paul et al., 2019; Zotos et al. 2010; Crotty, 2012; Victora and Nussenzweig, 2012).

These results suggest that a specific CSF profile may distinguish, already at diagnosis, patients destined to have a more severe disease, activity and worsening cortical pathology over time. Since no clinical and radiological prognostic features have yet been identified (Ruggieri et al., 2018), CSF analysis may represent an important tool to early identify MS patients at high risk of disease activity, thus allowing stratifications of patients at diagnosis for optimizing therapeutic approaches.

3.2 Study 2: "Evaluating of associations between intrathecal and peripheral inflammation in MS"

3.2.1 Introduction and rationale

From our previous works (Magliozzi et al., 2018; Magliozzi et al., 2020) a specific CSF protein profile analysis was assessed in order to distinguish patients at high risk of disease activity and severe cortical damage, may allow stratifications of patients at diagnosis for optimizing therapeutic approaches. No serum protein profile and no serological predictive biomarker have been found so far in MS (Torkildsen, 2021). However, obtaining serum samples is much easier and less invasive than lumbar punctures necessary for CSF and, consequently, identifying MS-specific serum markers is a great challenge of our time.

3.2.2 Study Aim

To investigate the relationship between intrathecal and peripheral inflammation in MS patients and clarify whether intrathecal CSF protein profiling is somehow reflected in paired serum samples.

3.2.3 Methods

CSF and serum paired samples were collected from seventy naïve MS patients (female/male = 47/23; mean age = 42.4 ± 10.0 years; range 18–60) at time of diagnosis. A combination of specific proteins was evaluated by using immune-assay multiplex techniques, based on the Luminex technology (Bio-Plex X200 System). Specifically, 70 inflammatory mediators were quantified using the following kits: Bio-Plex Pro Human Chemokine Panel (40-Plex) and Bio- Plex Pro Human Inflammation Panel 1 (37-Plex). Of these 70 molecules, we focused more on those identified in our previous study (Magliozzi et al., 2018), such as: CXCL13, CXCL12, CCL25, TNFα, sTNFR1, TWEAK, APRIL, BAFF, LIGHT, INFα2, IFNλ2, IL6, IL8, IL10, MMP2, sCD163, Pentraxin-III and IFNγ. Then, we analyzed the correlation (Spearman analysis) between CSF and serum expression levels. JASP package (6 June 2022, https://jasp-stats.org, version 0.14) was used to perform the analyses. Two years

after this first study, a further 73 samples were collected, reaching a total of 143 naïve-treatment MS patients analyzed.

3.2.4 Results

CSF and Serum molecules levels and correlations

CXCL13, CXCL12, CCL25, TNFα, sTNFR1, TWEAK, APRIL, BAFF, LIGHT, INFα2, IFNλ2, IL6, IL8, IL10, MMP2, sCD163, Pentraxin-III and IFNγ, were quantify both in CSF and in serum (Table 1). Expression levels of most molecules in serum do not correlate with levels in CSF (Table 1). Indeed, preliminary results on 70 naïve MS patients demonstrated that only CCL25 (rho = 0.260, $p < 0.030$) and INFα2 (rho = 0.370, $p < 0.001$) show a significant correlation between the two sample types (Table 1, Fig. 1).

TABLE 1: Comparison between CSF and serum levels of 18 cytokines previously identified.

	Serum			CSF				
Molecules	**mean**	**±**	**sd**	**mean**	**±**	**sd**	**rho**	**p value**
CXCL13	37.876	±	47.120	10.969	±	25.831	-0.140	0.259
CXCL12	4.456.777	±	1.593.347	2.516.311	±	2.539.257	0.20	0.095
CCL25	**580.367**	**±**	**234.671**	**110.356**	**±**	**82.560**	**0.260**	**0.030***
TNFα	22.216	±	30.991	35.585	±	38.097	0.070	0.581
sTNF-R1	3.108.422	±	1.682.290	4.326.865	±	2.699.071	0.050	0.675
TWEAK	829.033	±	452.579	1.638.082	±	1.654.890	-0.040	0.772
APRIL	80.309.102	±	49.769.766	26.811.824	±	28.766.524	-0.020	0.867
BAFF	9.381.423	±	11.699.289	9.930.311	±	7.764.001	-0.130	0.293
LIGHT	9.621	±	47.587	304.584	±	436.991	-0.090	0.483
IFNγ	8.469	±	16.310	20.395	±	29.279	0.010	0.921
INFα2	**29.099**	**±**	**17.447**	**19.060**	**±**	**22.414**	**0.372**	**0.001****
IFNλ2	30.506	±	25.502	156.833	±	408.823	0.210	0.077
IL6	123.696	±	1.004.237	22.013	±	33.608	0.150	0.205
IL8	1.239.744	±	10.018.901	70.319	±	86.335	0.080	0.516
IL10	8.522	±	6.664	25.605	±	22.525	-0.180	0.124
MMP-2	41.692.435	±	18.778.401	2.228.796	±	5.569.419	0.160	0.192
Pentraxin-III	3.141.342	±	5.096.324	274.409	±	458.828	0.060	0.625
sCD163	94.372.567	±	46.209.434	44.314.965	±	21.908.453	0.100	0.390

For each protein, mean and standard deviation were reported. Concentration of cytokines is expressed in ng/ml for both serum and the CSF expression. Spearman correlation was applied. $p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

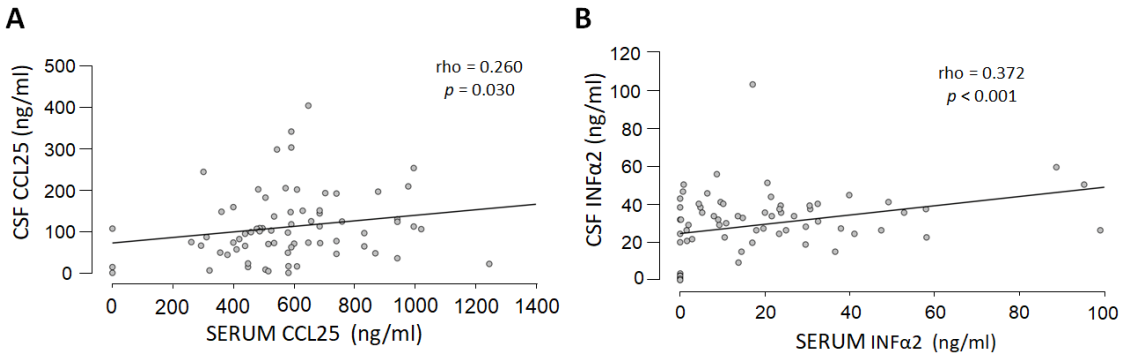

FIGURE 1: A) Correlation between CSF and serum CCL25 levels; B) Correlation between CSF and serum INFα2 levels (Spearman correlation).

a) CSF/serum association was investigated also by increasing the sample size, namely on 143 MS patients. The analyses were repeated and the only correlation found was between CSF and serum compartments for CCL25 (rho = 0.280, $p < 0.001$). However, this second sub-study is still in progress and is to be confirmed.

3.2.5 Discussion and conclusion

Although this study demonstrated small correlations between the levels of the molecules in the CSF and those of the serum, CSF/serum CCL25 levels correlations were confirmed, suggesting that this molecule could be considered as early peripheral biomarkers of an aggressive disease course, characteristic of those who have meningeal inflammation already at diagnosis. Interestingly, CCL25 is a thymus-expressed chemokine (Qiuping et al., 2004) that was found to be one-to-one combined with CCR9 (Zaballos et al., 1999). CCR9 and CCL25 are members of the CC subfamily of chemokines that are involved in a variety of inflammatory diseases and promote inflammatory responses (Wu et al., 2021). Toll-like receptor 4 (TLR4), widely distributed in Th17 cells, represents a key component in innate immunity and has been linked to CNS inflammation diseases, such as MS (Zhang et al., 2019) in that it mediates proinflammatory Th17 infiltration through CCL25/CCR9 signal during pathogenesis of experimental autoimmune encephalomyelitis (EAE; Zhang et al., 2019).

Overall this study confirms that intrathecal compartmentalized inflammation is relatively independent of peripheral immune response, and this would also explain the difficulty in targeting it by available peripheral immunosuppressive therapies. However, extending this study to a larger MS population could help to achieve a more complete analysis.

3.3 Study 3: "Role of CSF IgM levels in early MS"

The results reported in this study were described in the article "Cerebrospinal Fluid IgM Levels in Association With Inflammatory Pathways in Multiple Sclerosis Patients", published in 16 Oct 2020 on the journal Frontiers in cellular neuroscience (14, 569827. https://doi.org/10.3389/fncel.2020.569827; © 2020 Magliozzi, Mazziotti, Montibeller, Pisani, Marastoni, Tamanti, Rossi, Crescenzo and Calabrese).

3.3.1 Introduction and rationale

Several studies demonstrated a relevant association between intrathecally produced IgM and a more severe MS course (Villar et al., 2005, 2008; Calabrese et al., 2012). However, other studies did not find an association between CSF IgM levels and a more severe MS course (Schneider et al., 2007; Stauch et al., 2011). Therefore, the predictive role of CSF IgM in MS pathology remains to be elucidated.

3.3.2 Study Aim

To define the intrathecal IgM levels production and its possible association with disease activity, considering clinical, radiological and biological parameters.

3.3.3 Methods

One-hundred-three treatment-naive RRMS patients (female/male = 26/77; mean age = 38.6 ± 13.2 years; range 15–64) were recruited at the time of diagnosis and their associations with CSF inflammatory molecules, clinical and radiological measures (3T-MRI), at diagnosis and after 2 years of follow-up, were investigated. All MS patients enrolled underwent a detailed neurological evaluation (including EDSS assessment), 3-T MRI and CSF examination, at the time of diagnosis (T0). Seventy patients (female/male = 17/53; mean age = 39.5 ± 11.4 years; range 18 – 64) who did not undergo second-line therapies, in particular anti–B cell drugs, were also clinically and radiologically monitored for 2 years [24 months (T24)]. The evidence (EDA) and no evidence (NEDA) of disease activity, based on the presence of relapses, disability progression or any MRI activity (Giovannoni et al., 2015), were evaluated. Demographic, clinical, and MRI data of MS patients are reported in Table 1.

Thirty-six age- and sex-matched available patients affected by other neurological diseases (OND), were included in the study and underwent neurological evaluation and CSF examination at the time of the diagnosis included twenty-one patients with non-inflammatory neurological diseases, NIND (one idiopathic tremor, two migraine, two amyloid angiopathy, two fibromyalgia, four ischemic stroke, one spondylotic myelopathy, two amyotrophic lateral sclerosis, one olivopontocerebellar atrophy, one idiopathic spastic paraparesis, one idiopathic ataxia, one myopathy, one endocranial hypertension, two peripheral neuropathy), and 15 subjects with other inflammatory neurological diseases, OIND (one infective myelopathy, two CNS lymphoma, two intracranial abscess, one peripheral neuropathy, two Behçet disease, three neuromyelitis optica spectrum disorder, three autoimmune encephalitis, one aseptic meningitis).

MRI Acquisition Protocol and Analysis

3T MRI was performed in all MS patients, and this was repeated 2 years after diagnosis on a subset of seventy patients, including those who did not undergo second-line therapies. MRI sequences were acquired at the Radiology Unit of the University Hospital of Borgo Trento (Verona, Italy) using a Philips Achieva 3T MRI Scanner (Philips Medical Systems Best, Netherlands) as previously described (Magliozzi et al., 2018). The following image sets were acquired: a) 3D T1-weighted turbo field echo [repetition time (TR)/echo time (TE) = 8.4/3.7 ms, voxel size of 1 mm x 1 mm x 1 mm), total acquisition time of 5:51 min; b) 3D double inversion recovery (DIR) (TR/TE = 5,500/292 ms, inversion times (TI) TI1/TI2 =525/2,530 ms voxel size of 1 mm x 1 mm x 1 mm), turbo spin echo (TSE) readout with an optimal variable flip angle scheme and number of excitations (3, with total acquisition time of 10:49 min; c) 3D fluid-attenuated inversion recovery (FLAIR) (TR/TE = 5,500/292 ms, TI = 1,650 ms voxel size of1 mm x 1 mm x 1 mm), same TSE readout as the DIR sequence, number of excitations 1, with total acquisition time 5:44 min. The number of T2 hyperintense white matter lesions (WMLs) and CLs were identified on FLAIR and DIR images. The number of CLs was assessed following the recommendations for CLs scoring in patients with MS (Geurts et al., 2011). Owing to the suboptimal performance of the image-acquisition sequences on MRI in visualizing subpial lesions,

the present analysis has taken into account mainly the intracortical and leukocortical lesions.

TABLE 1: Demographic, clinical, and MRI data of MS patients.

Gender (male/female): T0, T24	26/77, 17/53
Age at diagnosis (years)	38.6 ± 13.2, 15–64
EDSS-T0	2.0 ± 2.0, 0–5
EDSS increase-T24	2.0 ± 0.0, 0–5
OCBs (positive/negative)	75/28
Albumin CSF/serum (mg/L)	4.6 ± 0.2, 2–11
WMLs number-T0	7.0 ± 2.0, 3–18
CL number-T0	2.0 ± 7.0, 0–23
New WMLs-T24	70
New CLs-T24	73
Relapses T0-T24	68
EDA/NEDA-T24	40/30

M, males; F, females; EDSS, Expanded Disability Status Scale; OCBs, oligoclonal bands; WMLs, white matter lesions; CLs, cortical lesions; EDA, evidence of disease activity; NEDA, no evidence of disease activity; T, timepoint (0 = baseline, 24 = after 2 years of follow-up). Data are shown in the following order: mean ± SD (standard deviation) and range, except for WMLs, CLs, and EDSS, for which the median ± IQR (interquartile range) are considered in place of mean and SD.

CSF examination

CSF levels of IgG, IgA and IgM were assessed by Luminex technology (Bio-Plex-X200; BioRad, Hercules, CA), using a customized Human Immunoglobulin Isotyping Panel-3-plex. Specific inflammatory molecules were selected and analyzed by Bio-Plex Pro Human Inflammation Panel 1-37-plex and by Bio-Plex Pro Human Chemokine Panel-40-plex, in order to evaluate cytokines and chemokines related to B-cell (APRIL, LIGHT, TWEAK, BAFF, CXCL12, CXCL13, CCL21, IL-10, IL-34, IL-35, and GM-CSF), T-cell (TNFα, sTNFR1, and sTNFR2, IFNγ, INFα2, IL-4, IL-8, IL-22, CCL19, CCL20, and CCL25), monocyte/macrophage (IL-1b, IL-6, CCL2,

CCL8, CX3CL1, CXCL10, CXCL11, CHI3L1, sCD163, MMP1, and MMP2), activation and recruitment.

Statistical Analysis

Mann–Whitney U test was used to test differences between MS patients and control group, as well as differences between MS patients stratified by CLs number at diagnosis (</>4, where 4 was the mean of the CL number in all the examined patients) and by the presence or not of the EDA after 2 years of followup. Analysis of variance (ANOVA) followed by post hoc pairwise comparison using the Tukey test was used to evaluate difference among immunoglobulin CSF levels in MS patients. Spearman analysis was applied to evaluate the correlation between CSF examination and demographic, clinical and MRI parameters. $p<0.05$ was considered statistically significant. GraphPad (version 5.0) and R software (version 3.5.3) were used to perform the analysis.

3.3.4 Results

Immunoglobulins assayed in the CSF of MS and control groups

Intrathecal levels of IgG, IgA, and IgM were investigated in the CSF of 103 RRMS patients and 36 controls with other neurological disorders (Table 2).

CSF IgG levels in MS patients were significantly higher compared to IgA (fold change=2.65, $p<0.001$; Fig. 1A) and IgM (fold change=2.15, $p<0.001$; Fig.1A). However, only the CSF IgM levels reached statistical significance in MS patients when compared to controls (fold change=1.46, $p=0.013$; Fig. 1B), while there was no difference in IgG (fold change=0.96, $p=0.360$; Fig. 1C) and IgA (fold change=0.27, $p=0.700$; Fig. 1D) levels between the two groups.

TABLE 2: Immunoglobulins assayed in the CSF of MS and control groups.

Immunoglobulin type	MS patients (*n* = 103)		Controls (*n* = 36)	
	Mean ± SD (ng/mL)	Concentration range (ng/mL)	Mean ± SD (ng/mL)	Concentration range (ng/mL)
IgG	3.778 ± 2.760	365–8705	3.927 ± 64.059	315–19.042
IgA	1.707 ± 3.522	0.0–7977	6.244 ± 18.929	60–82.928
IgM	1.991 ± 1.396.9	67–6114	1.367 ± 1.560	104–17.910

Ig, immunoglobulin; SD, standard deviation. IgG, IgA, and IgM were analyzed by Bio-Plex Pro-Human Isotyping Panel (3-plex) from the CSF of MS patients (n = 103) and control cases (n = 36).

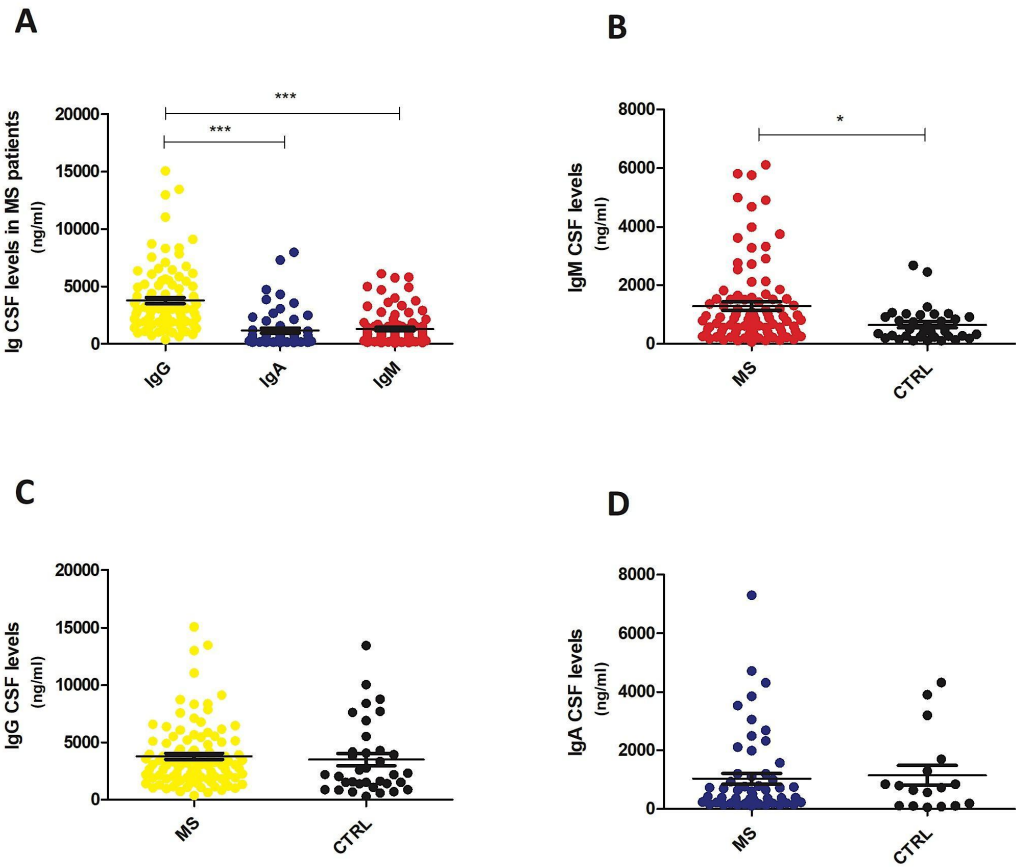

FIGURE 1: CSF expression levels of IgG, IgA, and IgM in MS and controls patients. Concentration was expressed in ng/ml. (A) IgG levels were significantly higher in MS patients (n = 103) compared to IgA and IgM levels (one-way ANOVA, p<0.001); (B) IgM was the only immunoglobulin found to be higher in MS patients (n=103) compared to controls (n = 36) (Mann–Whitney U test, p = 0.013). The difference between (C) IgG and (D) IgA expression levels was not significant (respectively, p = 0.360, p = 0.700) between MS patients and controls. *p < 0.05, ***p < 0.001.

Correlations between CSF IgM levels with demographic, clinical, and MRI data

In order to understand whether CSF IgM levels were related to peripheral Igs production, we correlated them with measurement of blood–brain barrier damage (calculated considering the CSF–to–serum albumin ratio). No correlation was found between CSF IgM levels and blood–brain barrier alteration (rho = - 0.120, p = 0.270), suggesting that IgM intrathecal levels were not related to peripheral ones;

CSF IgM levels of MS patients were also correlated with demographic, clinical, and MRI data, at the time of diagnosis and after 2 years follow-up. At T0, CSF IgM levels were correlated negatively with the age of MS patients (rho = - 0.260, p = 0.008; Fig.2A); on the contrary, no correlation was found between CSF IgM levels and EDSS and MRI data. Although there were no significant correlations between the CSF IgM levels and WMLs and CL number at diagnosis, when MS patients were stratified according to the CL load, we found a trend to increase of CSF IgM levels that were.

Almost twice higher in MS patients with CLs 4 compared to MS patients with CLs < 4 (fold change = 1.74, p = 0.072; Fig. 2B). Considering the second year of follow-up, CSF IgM levels (measured at the time of diagnosis) were significantly higher in patients with EDA compared to NEDA (fold change = 1.61, p = 0.033; Fig. 2C), and a mild correlation was found between CSF IgM levels and the presence of new WMLs (rho = 0.240, p = 0.039; Fig. 2D).

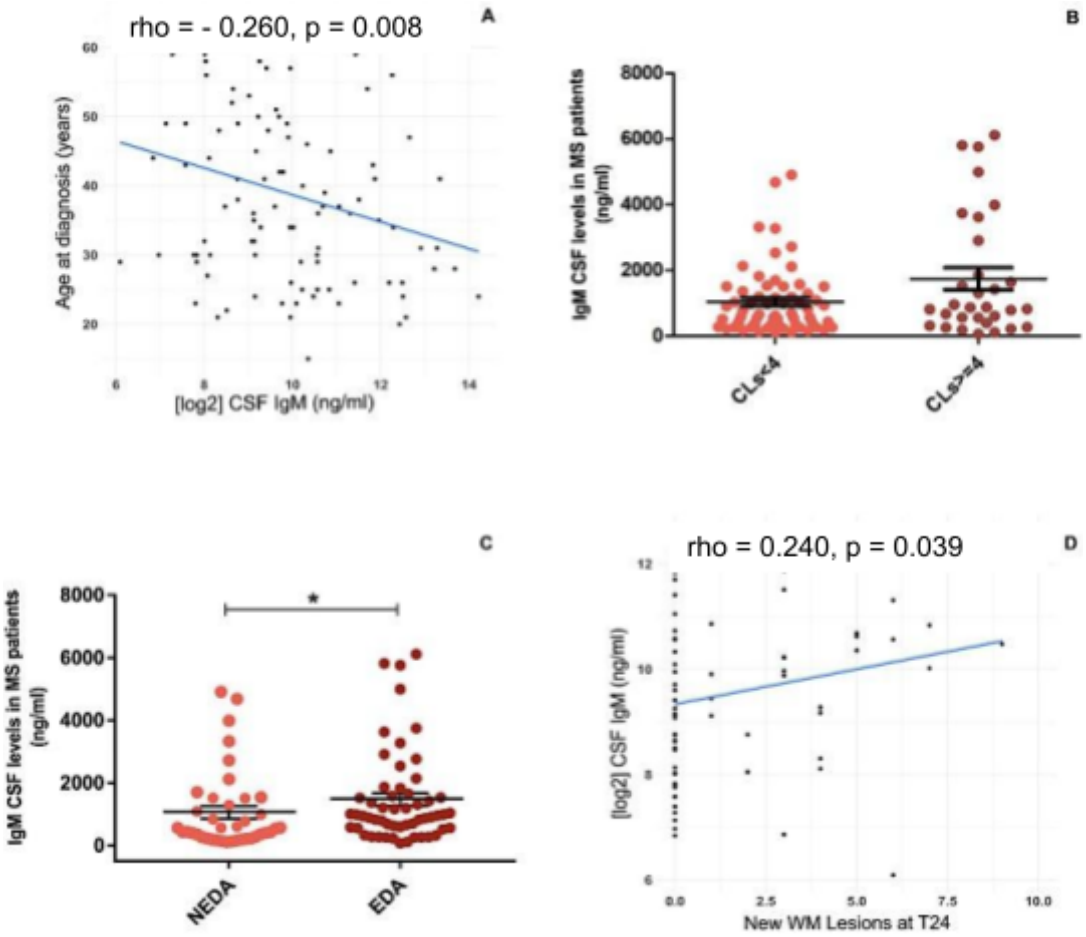

FIGURE 2: Association between CSF IgM levels in MS patients with demographic, clinical, and MRI data: (A) CSF IgM levels correlates negatively with the age at diagnosis (rho = − 0.260, p = 0.008); (B) CSF IgM levels were higher in MS patients with CLs ≥ 4 compared to MS patients with CLs < 4, at the time of diagnosis (Mann–Whitney U test, p = 0.072); (C) CSF IgM levels were higher in EDA patients compared to NEDA after 2 years of follow-up (p = 0.033); (D) CSF IgM levels correlate with the presence of new WMLs after 2 years of follow-up (rho = 0.240, p = 0.039). *p < 0.05.

Correlations between CSF IgM levels with proinflammatory biomarkers

Of all the B cell-related molecules, we found that CSF IgM levels mildly correlated with CSF IL-10 (rho = 0.220, p = 0.025; Fig. 3A), CCL21 (rho = 0.230, p = 0.023; Fig. 3B), and CXCL13 (rho = 0.200, p = 0.039; Fig. 3C). Furthermore, the CSF IgM levels are also weakly correlated with macrophage and microglia-related biomarkers such as CHI3L1 (rho = 0.190, p = 0.048; Fig. 3D), CX3CL1 (rho = 0.210, p = 0.036; Fig. 3E) and IL-12p70 (rho = 0.250, p = 0.020; Fig. 3F).

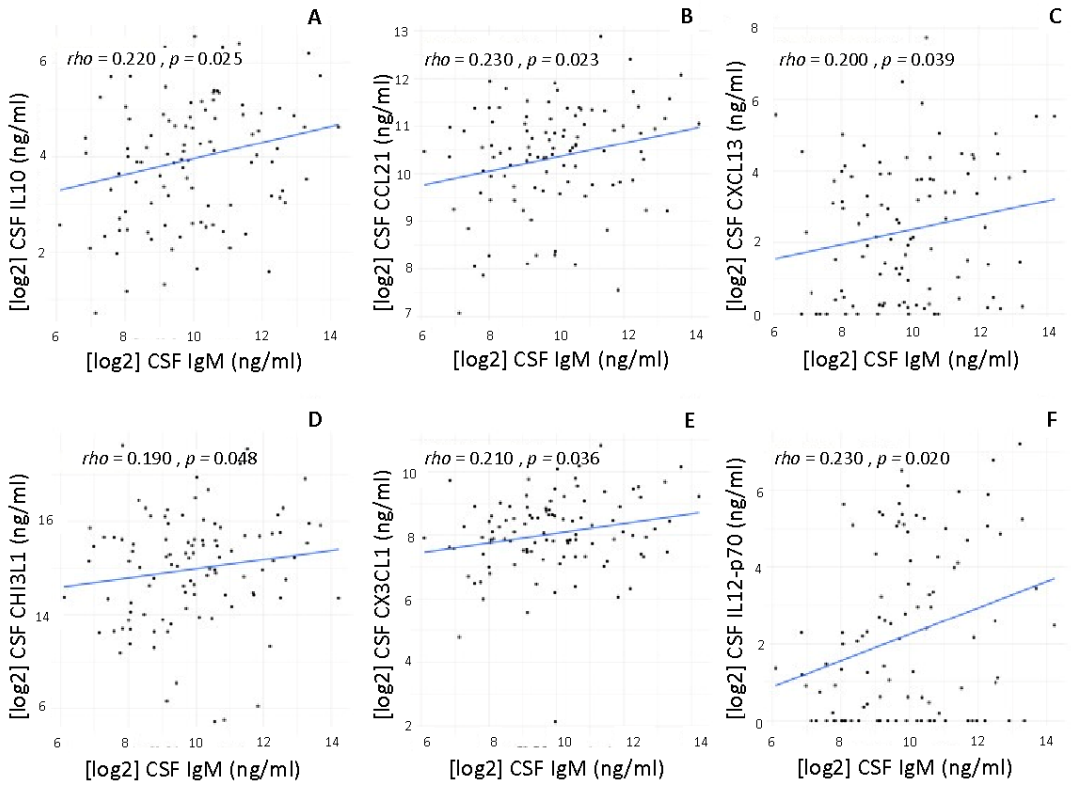

FIGURE 3: Correlations between IgM CSF levels and specific biomarkers in MS patients (n = 103). IgM CSF levels correlate with high CSF levels of (A) IL-10 (R = 0.22, p = 0.025), (B) CCL21 (R = 0.22, p = 0.023), (C) CXCL13 (R = 0.20, p = 0.039), (D) CHI3L1 (R = 0.19, p = 0.048), (E) CX3CL1 (R = 0.21, p = 0.036), (F) IL-12p70 (p = 0.020).

3.3.5 Discussion and conclusion

We found that higher levels of IgM, but not IgG and IgA, were present already at diagnosis in the CSF of MS patients when compared with controls. This result corroborates the hypothesis that IgM production occurs from the early stages of MS. As known in MS, B cells migrate from the periphery into the meninges, CSF, and the CNS parenchyma (Levinson et al., 1983; Sandberg et al., 1986), where these cells showed a local activation and clonal expansion (Weber et al., 2010). In relation with these studies, we decided to analyze the association between the CSF IgM levels, detected at diagnosis (T0), and the clinical and MRI parameters after 2 years of follow-up [24 months (T24)]. We found that while CSF IgM levels did not correlate with any clinical/MRI parameters at time of diagnosis, they were mild correlated with the presence of new WMLs at T24 and were significantly higher in EDA patients compared to NEDA, thus suggesting a possible prognostic role of IgM levels in terms of disease activity. These results are in line with previously mentioned studies (Villar et al., 2005, 2008; Owens et al., 2009) and with studies showing an association between IgM with a severe MS course, according to both clinical and MRI outcomes (Villar et al., 2003, 2005;Ozakbas et al., 2017).

Analyzing the correlations between CSF IgM levels and the clinical and MRI parameters even at the time of diagnosis, we detected only a negative and low correlation between CSF IgM levels with and the age of patients at diagnosis, although previous studies showed that IgM levels were strongly associated with a younger age at first clinical symptoms (Tintore et al., 2008; Huss et al., 2018; Pfuhl et al., 2019). Moreover, despite that CSF IgM levels were higher in MS patients with high (CLs 4) compared to MS patients with low (CLs < 4) CL load, such a difference did not reach statistical significance. On the contrary, we detected a low negative correlation with the age of the patients. These results might be explained by the small number of examined patients and by the possibility that other underlying immunological mechanisms are involved in brain damage besides the immunoglobulin production (Lassmann, 2008). Recent studies have shown that intrathecal IgM synthesis, mainly mediated by CD5C B cells, contributes to B-cell activation and differentiation within the CNS (Villar et al., 2010). In particular, positive correlations between CSF inflammatory biomarkers, especially of humoral immunity, with MS severity support a

pathogenic role of intrathecal inflammation, particularly linked to B-cell immunity, in CNS tissue destruction in MS patients (Milstein et al., 2019), causing a more severe and rapid disease course (Magliozzi et al., 2007; Howell et al., 2011). For all these reasons, we investigated whether IgM overexpression in the CSF of MS patients at diagnosis might be associated with a specific inflammatory intrathecal milieu, by analyzing other cytokine/chemokine CSF molecules related to either B cells or other immune cell pathways. First, we observed mild correlation between CSF IgM levels with some B cell–related factors, such as CXCL13, CCL21, and IL-10. The chemokines CXCL13 and CCL21 are particularly known to regulate B-cell migration into the CNS and to favor the intrathecal accumulation of B cells (Kowarik et al., 2012). In particular, CXCL13 has recently been suggested as a prognostic marker for CIS and MS (Brettschneider et al., 2010; Ferraro et al., 2015; Magliozzi et al., 2020) and seems to play a role in the formation of ectopic lymphoid tissues within the CNS in MS (Magliozzi et al., 2007), whereas IL-10 is generally considered an immunomodulatory cytokine, but could be also released, together with IL-1, IL-6, IL-15, and TNF, by activated macrophages, which can have a key role in B-cell activation. We also found that the CSF IgM levels in MS patients weakly correlated also with molecules related to monocyte/macrophage activity and response, such as CHI3L1, IL-12p70, and CX3CL1, suggesting a mild, but significant, association between humoral and innate immune inflammatory processes. Therefore, although the correlations that we identified in the CSF at time of diagnosis between all these mediators and IgM were modest and need to be validated in a larger and independent MS population, it might be hypothesized that IgM could possibly reflect the interactions between innate and adaptive humoral immune responses, as previously suggested (Boes, 2000; Villar et al., 2010). This analysis was performed using a multiple, advanced immunoassay methodology (Bio-Plex), to obtain a simultaneous, sensitive, and reproducible evaluation of immunoglobulins and several other inflammatory mediators in the CSF of MS patients. Although further studies are needed to confirm these preliminary results, the sensitivity and reproducibility of this easily performed procedure could allow extending such a detailed CSF proteomic analysis to clinical practice.

3.4 Study 4: "Role of Parvalbumin as a new early biomarker of a severe MS disease"

The results reported in this study were described in the article "CSF parvalbumin levels reflect interneuron loss linked with cortical pathology in multiple sclerosis", published in 23 Jan 2021 on the journal Annals of Clinical and Translational Neurology (8(3):534-547. doi: 10.1002/acn3.51298; © 2021 The Authors. Annals of Clinical and Translational Neurology published by Wiley Periodicals LLC on behalf of American Neurological Association).

3.4.1 Introduction and rationale

Cortical neuronal damage in the upper cortical layers, including both dysfunction and loss, is now thought to be a major contributor to the progression of MS (Peterson et al., 2001; Klaver et al., 2015; Schirmer et al., 2019). In the last decade, several studies have confirmed an association between CSF inflammatory protein profile, meningeal inflammation and GM damage (Farina et al., 2017; Magliozzi et al., 2018). Neurofilaments are suggested to be a useful biomarker of neurodegeneration in MS (Khalil et al., 2018). Specifically, neurofilament light chains (Nf-L), a cytoskeletal component of neurons, are released into the CSF following axonal destruction, thus providing an indication of axonal damage and neuronal death. Therefore, Nf-L levels are increased in the CSF of MS patients who convert earlier to secondary progression (Salzer et al., 2010). However, Nf-L is not specific for MS: neurodegenerative diseases such as prion diseases, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, Hungtington's disease, and traumatic brain injury have all demonstrated increased levels of Nf-L (Bridel et al., 2019; Gaetani et al., 2019). This has increased the need to identify a more specific neurodegeneration marker. According to this premise, several studies investigated promising biomarkers specific for MS. Specifically, previous works showed a increase of GABA-ergic parvalbumin (PVALB)+ interneurons in the primary motor cortex of MS patients, suggesting PVALB, a calcium binding protein expressed in a subset of these inhibitory cortical interneurons, as a new biomarker of cortical damage MS (Dutta et al., 2006; Clements et al., 2008).

In addition, a recent study reported a substantial reduction in PVALB gene expression in the motor cortex of post-mortem secondary progressive MS (SPMS) brains, which reflected the degree of meningeal inflammation and cortical neurodegeneration, suggesting increase of CSF PVALB levels as a specific indicator of ongoing neurodegeneration in MS (Magliozzi et al., 2019). PVALB protein could therefore identify MS patients with a high risk of severe progression.

3.4.2 Study aim

In this work we measured CSF PVALB levels in MS cases compared to controls and evaluated their association with neuroradiological and clinical parameters at the time of diagnosis and explored the role of PVALB as a new early biomarker of a severe MS disease compared to Nf-L in MS patients.

3.4.3 Methods

One hundred and ten treatment-naive MS patients (76 females, age = 38.4 ± 2.4 years) of the MS Centre of the Verona University Hospital were enrolled at the time of diagnosis. Patients underwent a CSF withdrawal by lumbar puncture, a neurological evaluation (including EDSS assessment; median = 2, range = 0 – 4), and a 3T MRI scan. The Ethics Committee of the University of Verona approved the study and informed consent was collected from all participants (MSBioB Biological bank – A.O.U.I, Verona; Protocol number 66418, 25/11/2019).

CSF analysis

CSF levels of PVALB and Nf-L were measured using a Parvalbumin ELISA kit (MBS2022353, MyBioSource) and Human Nf-L ELISA kit (MyBioSource,), respectively PVALB and Nf-L levels were also evaluated in the CSF of thirty-two control subjects.

MRI assessment

3T- MRI was performed at the Radiology unit of the Verona University Hospital by using a Philips Achieva 3T MRI Scanner. The acquired images were analyzed to assess the lesion load both in white and grey matter: white matter lesions (WMLs)

were identified and segmented on FLAIR images, while the number of cortical lesions (CLs) was assessed on DIR images following the recent recommendations for cortical lesions scoring in patients with MS (Geurts et al., 2011). Global and regional cortical thickness (CTh) evaluation was performed on the 3D T1w scan by using the Freesurfer image analysis suite, available online(http://surfer.nmr.mgh.harvard.edu/).

Statistical analysis

Differences between groups were evaluated performing Mann–Whitney test and one-way analysis of variance (one-way ANOVA) followed by post-hoc Tukey test. Pairwise univariate Spearman rank index was used to evaluate the association between demographic, clinical, neuroradiological, neuropsychological parameters and the CSF PVALB/Nf-L. Statistical analysis were performed using GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA) and R (https://www.r-project.org/, version 3.3).

3.4.4 Results

CSF PAVLB and Nf-L levels increased in MS patients compare to controls

Significantly increased CSF PVALB levels were present in the MS patients (fold change = 3.0 ±2.1, $p = 0.002$) compared to the control cohort (Fig. 1A). Likewise, a significant increase of CSF Nf-L levels were found in the MS patients (fold change = 3.5 ±1.3, $p < 0.001$) compared to controls (Fig. 1B). A significant positive correlation was observed between CSF PVALB and Nf-L levels (rho = 0.360, $p < 0.001$) (Fig. 1C).

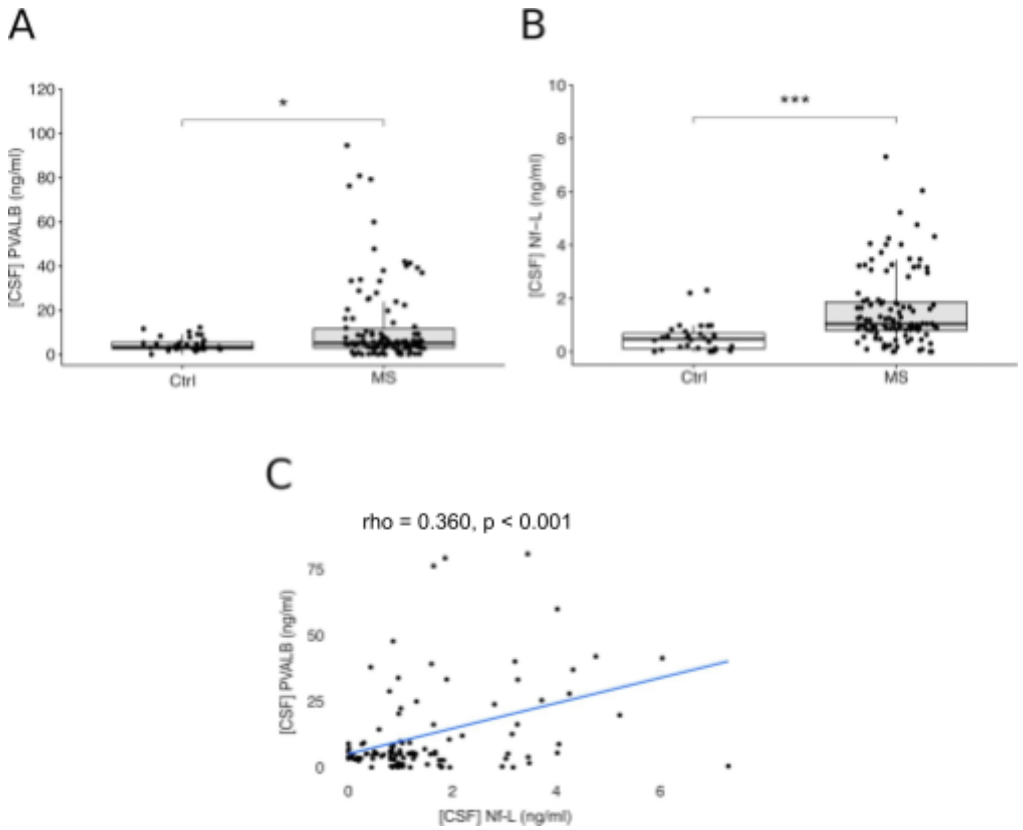

FIGURE 1: Increased CSF PVALB and Nf-L levels in MS patients compared to controls**.** (A) CSF PVALB protein concentration (ng/ml) from controls (n = 32) and MS patients (n = 110). (B) CSF Nf-L protein concentration (ng/ml) from controls (n = 32) and MS patients (n = 110). (C) Correlation between CSF PVALB and Nf-L levels in MS patients (rho = 0.036, p < 0.001, n = 110).

Increased CSF PVALB levels are associated with cortical damage

3T-MRI parameters were considered in association with CSF PVALB and Nf-L levels, such as: number of WMLs and CLs, and global and regional CTh. The global CTh (mean ± SD = 2.440 ± 0.340 mm) negatively correlated moderately with CSF PVALB levels (rho = -0.460, p < 0.001; Fig. 2A-B) and only slightly with Nf-L levels (rho = -0.230, p = 0.024) (Fig. 2A). Moreover, the number of CLs (mean ± SD = 3.88 ± 5.00) was slightly correlated with CSF PVALB levels (rho = 0.280, p = 0.006; Fig. 2C) and with Nf-L levels (R = 0.31, p= 0.003; Fig 2A), whereas WM lesion number (mean ± SD = 8.790 ± 3.990) did not correlate with CSF levels of PVALB and Nf-L (Fig. 2A).

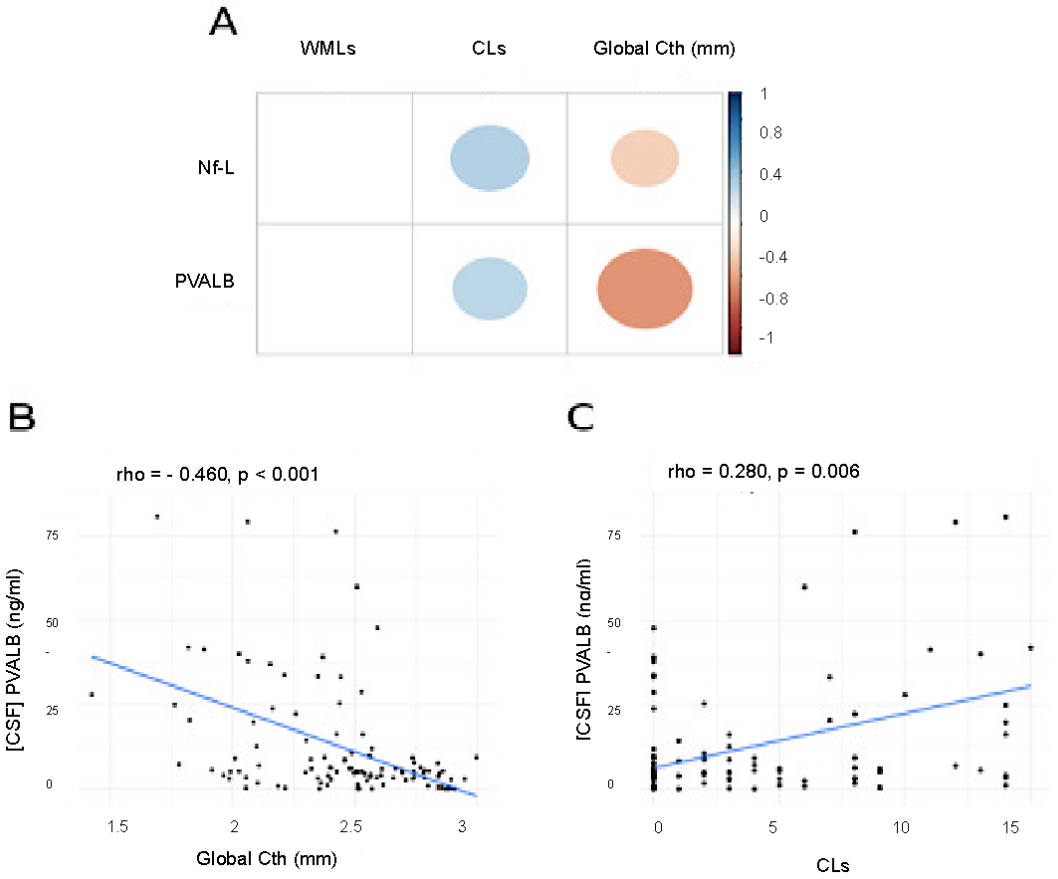

FIGURE 2: Correlations between CSF PVALB and Nf-L levels with 3T MRI outcomes in MS patients. (A) Correlation plot showing different strength of correlation (Spearman analysis) between CSF PVALB and Nf-L levels with MRI parameters. The Spearman rank index is proportional to the dimension/intensity of the bubbles. A color scale was used to determine the slope of the correlation. Blank squares indicate no significant correlation. (B) Scatter plot of relationship between CSF PVALB levels and global cortical thickness (CTh) in MS patients. (C) Scatter plot of relationship between CSF PVALB levels and number of cortical lesions (CLs) in MS patients.

The regional analysis revealed that CSF PVALB levels were significantly associated with CTh of several brain regions (Fig. 3A, Table I), such as: insula (rho =-0.550, $p < 0.001$; Fig. 3B), cingulate gyrus (rho =-0.470, $p < 0.001$; Fig. 3C), hippocampus (rho = - 0.460, $p < 0.001$; Fig. 3D), postcentral gyrus (rho = - 0.460, $p < 0.001$; Fig. 3E), parahippocampal gyrus (rho = - 0.430, $p < 0.001$; Fig. 3D), middle temporal gyrus (rho = - 0.420, $p < 0.001$; Fig. 3D), superior frontal gyrus (rho = - 0.420, $p < 0.001$; Fig. 3D). In contrast, only few significant correlations were found between Nf-L levels and regional CTh levels (Fig. 3A, Table 1).

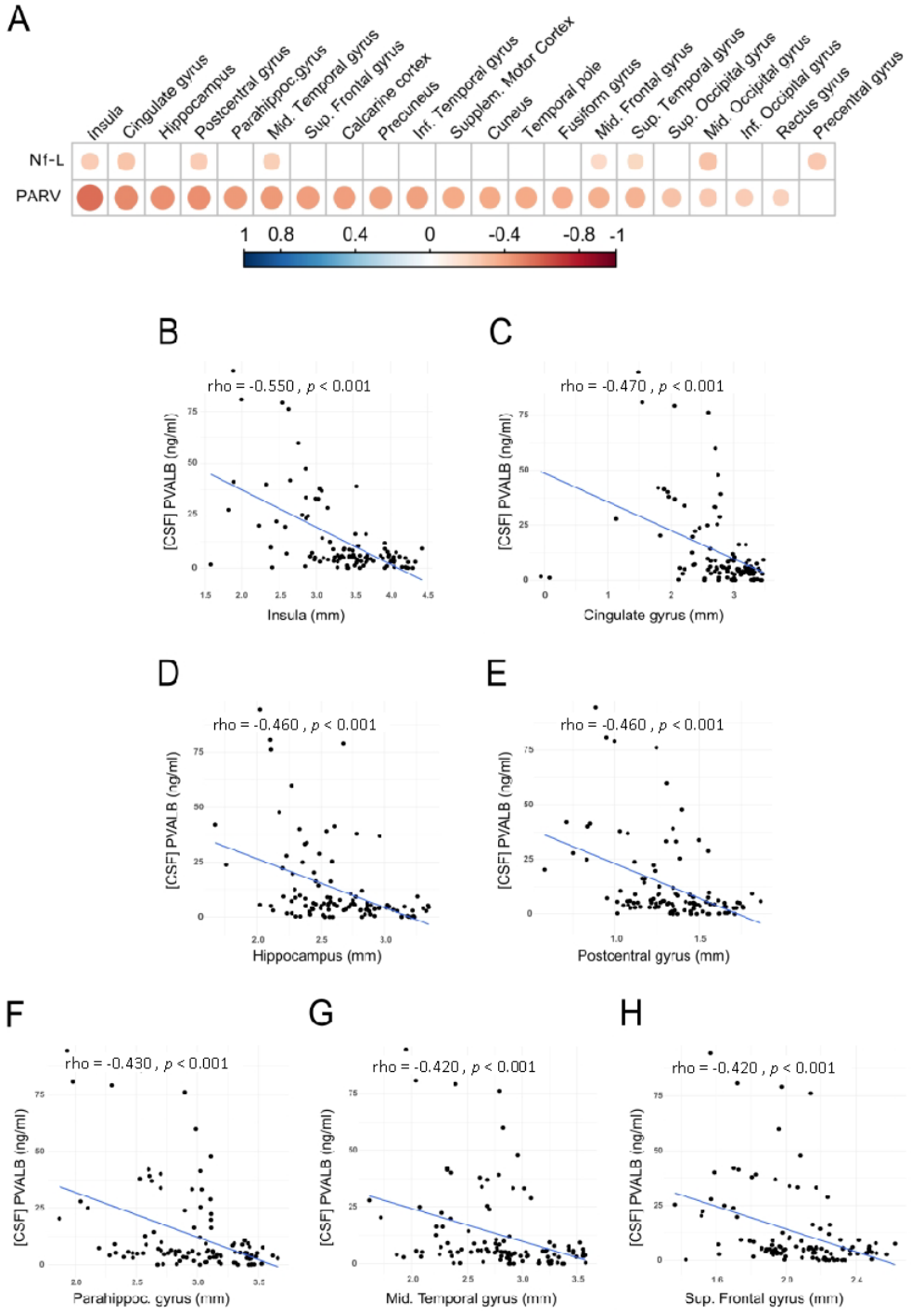

FIGURE 3: CSF PVALB levels correlations with 3T-MRI regional CTh in MS patients**.** (A) Correlation plot chart showing the correlation (Spearman analysis) between PVALB levels and Nf-L with regional cortical thicknesses. The Spearman rank index is proportional to the dimension/intensity of the bubbles. A color scale was used to determine the slope of the correlation. Blank squares indicated significant correlation. (B-H) Scatter plot of relationship between CSF PVALB levels and CTh of the most associated brain regions: (B) insula, (C) cingulate gyrus, (D) hippocampus, (E) postcentral gyrus, (F) parahippocampal gyrus, (G) middle temporal gyrus, (H) superior frontal gyrus.

TABLE 1: Correlations between global and regional CTh with both CSF PVALB and Nf-L levels in MS patients. Spearman correlation coefficient was applied.

CTh	**PVALB**	**Nf-L**
Global CTh	−0.46 ($P < 0.001$)	−0.23 ($P = 0.024$)
Insula	−0.55 ($P < 0.001$)	−0.24 ($P = 0.012$)
Cingulate gyrus	−0.47 ($P < 0.001$)	−0.27 ($P = 0.005$)
Hippocampus	−0.46 ($P < 0.001$)	n.s.
Postcentral gyrus	−0.46 ($P < 0.001$)	−0.24 ($P = 0.010$)
Parahippocampal gyrus	−0.43 ($P < 0.001$)	n.s.
Middle temporal gyrus	−0.42 ($P < 0.001$)	−0.24 ($P = 0.014$)
Superior frontal gyrus	−0.42 ($P < 0.001$)	n.s.
Calcarine cortex	−0.42 ($P < 0.001$)	n.s.
Precuneus	−0.40 ($P < 0.001$)	n.s.
Inferior temporal gyrus	−0.40 ($P < 0.001$)	n.s.
Supplementary motor cortex	−0.39 ($P < 0.001$)	n.s.
Cuneus	−0.38 ($P < 0.001$)	n.s.
Temporal pole	−0.37 ($P < 0.001$)	n.s.
Fusiform gyrus	−0.37 ($P < 0.001$)	n.s.
Middle frontal gyrus	−0.35 ($P < 0.001$)	−0.20 ($P = 0.044$)
Superior temporal gyrus	−0.34 ($P < 0.001$)	−0.20 ($P = 0.032$)
Superior occipital gyrus	−0.29 ($P = 0.002$)	n.s.
Middle occipital gyrus	−0.27 ($P = 0.006$)	−0.28 ($P = 0.003$)
Inferior occipital gyrus	−0.25 ($P = 0.009$)	n.s.
Rectus gyrus	−0.22 ($P = 0.021$)	n.s.
Precentral gyrus	n.s.	−0.26 ($P = 0.007$)

Spearman correlation coefficients and P-values (in brackets) are listed in decrescent order of association with PVALB.

3.4.5 Discussion

CSF PVALB and Nf-L levels were found to increase in MS patients compared to controls. However, compared to Nf-L, our results show that CSF PVALB levels correlate better with global CTh and CLs number, but not with WMLs, already at the time of diagnosis, suggesting that it could represent a more specific marker of cortical neurodegeneration-related atrophy. This finding was particularly evident considering the correlation between CSF PVALB levels and cortical regions, such as insula, cingulate gyrus, hip-pocampus, postcentral gyrus, parahippocampal gyrus, middle temporal gyrus, and superior frontal gyrus, which were all previously identified by both neuropathological and MRI studies as severely affected by cortical pathology (Calabrese et al., 2015; Magliozzi et al., 2007). These cortical regions, in which a reduction of PVALB gene expression and GABA-ergic interneurons have been observed (Magliozzi et al., 2019), seem to be the most susceptible to neurodegeneration at least in the early stage of the disease. All these results suggest PVALB as a biomarker of cortical damage in early MS. Extensive cortical damage at onset is associated with florid inflammatory clinical activity and predisposes to a rapid occurrence of the progressive phase (Scalfari et al., 2018), consequently, PVALB might represent an early, new MS-specific biomarker of cortical neurodegeneration.

3.5 Study 5: Study on the immune response after COVID-19 mRNA vaccination in multiple sclerosis patients treated with DMTs

The results reported in this study were described in the article "Immune Response after COVID-19 mRNA Vaccination in Multiple Sclerosis Patients Treated with DMTs", published on 24 Nov 2022 in the journal Biomedicines (10, 3034. https://doi.org/10.3390/biomedicines10123034;

3.5.1 Introduction and rationale

Coronavirus disease 2019 (COVID-19) outbreak, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is considered the first significant pandemic of the twenty-first century, causing global mortality of over 6 million deaths and important public health consequences. Pathogenetic mechanisms underlying COVID-19 have not been completely clarified (Bordoni et al., 2019), and the large-scale vaccination programs represented the most effective measures for mitigating the COVID-19 severity and diffusion worldwide (Tortorella et al., 2021). COVID-19 vaccination is recommended in all persons with MS (pwMS), including those treated with disease-modifying therapies (DMTs) (Reyes et al., 2021; Wu et al., 2022) (70% of pwMS) (Kalincik et al., 2017). DMTs are immunosuppressive or immunomodulatory drugs, and their impact on the immune response to the COVID-19 vaccine in pwMS is still controversial.

Among the vaccines currently available, the BNT162b2 mRNA-based vaccine was approved by the European Medicines Agency in December 2020 as one of those recommended for persons with MS (pwMS) (https://www.ema.europa.eu/en/news/ema-recommends-first-covid-19-vaccine-authorisation-eu). BNT162b2 vaccine induces both humoral and cell-mediated immune responses against viral spike peptides (Agrati et al., 2021). Impaired seroconversion after BNT162b2 vaccination was observed in ocrelizumab- and fingolimod-treated pwMS, whereas pwMS treated with cladribine developed a specific humoral response (Achiron et al., 2021, *a*; Achiron et al., 2021, *b*). Subsequent studies partially

confirmed a blunted seroconversion in ocrelizumab-treated patients but revealed that most fingolimod-treated patients developed a serological response after mRNA COVID-19 vaccination (Tortorella et al., 2021; Guerrieri et al., 2022). Furthermore, despite the absent or low presence of anti-spike immunoglobulin (Ig)-G (Achiron et al., 2021, *a*), ocrelizumab-treated pwMS showed a preserved cell-mediated immune response after BNT162b2 vaccine compared to healthy people [11], characterized by an increased release of inflammatory mediators, such as granzyme B (GrB), interferon (IFN)-γ and tumor necrosis factor (TNF)-α (Brill et al., 2021). On the contrary, other studies demonstrated a more robust cell-mediated immune activation mainly driven by T cells after the COVID-19 vaccine in pwMS treated with ocrelizumab compared with healthy controls (Apostolidis et al., 2021).

Further research on the immune response to BNT162b2 mRNA-based vaccine in DMTs-treated pwMS might be helpful in elucidating the basis of these contradictory findings.

3.5.2 Study aim

In this longitudinal study, we evaluated the impact of DMTs on the immune response to COVID-19 vaccines in pwMS treated with cladribine (c-pwMS), fingolimod (f-pwMS) and ocrelizumab (o-pwMS) who completed the first BNT162b2 vaccination cycle. The immune response was assessed considering seroconversion vaccine-related, anti-spike immunoglobulin-G (IgG) titers, inflammatory mediators release and immunophenotype profile. Specifically, the serum levels of GrB and cytokines (IFN-γ and TNF-α) were measured with a highly reproducible and ultrasensitive assay to identify new potential vaccine-related biomarkers and obtain a more rapid evaluation of vaccine efficacy, compatible with the routine monitoring of DMTs-treated pwMS.

3.5.3 Materials and Methods

Study Protocol Approvals and Patient Consents

The Ethics Committee of the Verona University Hospital approved the study (2413 CESC) and informed consent was obtained from all participants.

Patients Cohort

Ninety-eight pwMS (94 relapsing-remitting [RRMS], 4 primary-progressive [PPMS], mean age 45.0 ± 10.0 years, 64 female), followed at the MS Center of the Verona University Hospital (Italy), were enrolled from January 2021 to September 2021 and then followed over time in this 15 months longitudinal prospective study, in order to collect data relative to a possible breakthrough SARS-CoV-2 infection. Demographic, clinical and biological data of enrolled patients were reported in Table 1. Inclusion criteria for the enrollment of patients with MS were: (a) diagnosis of MS according to the most recent diagnostic criteria (Thompson et al., 2018), (b) ongoing treatment with cladribine, ocrelizumab or fingolimod for at least 12 months before the study entry, (c) the willingness to undergo a two-approved dose (given 21 days apart) the primary BNT162b2 COVID-19 vaccination cycle. PwMS undergoing cladribine and ocrelizumab treatments, the timing of the first vaccine dose was scheduled at least three months after the last DMT administration, according to the recommendation of both the Italian and European Academy of Neurology for COVID-19 vaccination.

Study protocol, Samples Collection and Analysis

The enrolled patients underwent a first blood sample at least 1 month prior the first BTN162b2 vaccination and a second blood sample 20-30 days after the fulfillment of the BTN162b2 vaccination cycle, in order to assess T cells-associated cytokines production and immunophenotyping of blood lymphocytes pre- and post- vaccination. Concomitantly with the second blood sample collected, SARS-CoV-2 IgG test was performed.

All blood samples were collected according to the Consensus Guidelines for Blood Biobanking (Teunissen et al., 2011). Once collected, the blood samples were centrifuged, and the serum obtained was stored at -80°C until use in the "MSBioB" biobank (Ethics Committee Protocol n° 66418). The health status of patients was monitored at the time of collection samples to exclude symptoms associated with infections or MS relapse.

Anti-spike IgG titers

The level of SARS-CoV-2 IgG antibodies against the spike-protein was measured in all the 98 pwMS in a centralized laboratory with an automated serologic enzyme immunoassay (EIA) test (Abbott, Chicago, Illinois, USA). Anti-spike IgG values were expressed as Arbitrary Units (AU)/mL and values ≥ 1.1 were considered positive.

Inflammatory mediators' release

The cellular response was evaluated considering the expression of several inflammatory mediators, such as GrB, IFN-γ and TNF-α, in a subset of treated pwMS (n=43, 15 c-pwMS, 11 f-pwMS, 17 o-pwMS). Inflammatory mediators' levels were measured by using the ultrasensitive Ella-Simple Plex technology (ELLA microfluidic analyzer, Bio-techne, Protein simple, San Josè, California, USA), an automated enzyme-linked immunosorbent assay-ELISA. Differences between cytokines serum levels before and after vaccination were specifically considered in o-pwMS, dividing them into two groups: non-seroconverted and seroconverted patients.

Immunophenotyping of blood lymphocytes

No sufficient lymphocytes immunophenotype data were available for f-pwMS, therefore the absolute counts of lymphocytes subsets (CD19+ B cells, CD3+, CD4+ and CD8+ T cells) was investigated only in c-pwMS and o-pwMS (total: n=63). Lymphocyte subsets were assessed by flow cytometry analysis (Navios, version 1.3, Beckman Coulter, Brea, California, Usa).

Statistical Analysis

Analysis of covariance (ANCOVA) was performed on identified variables associated with the seroconversion COVID-19 vaccine-related. ANCOVA, with age as a covariate, followed by post-hoc comparison Tukey test was used to evaluate differences in anti-spike IgG seroconversion and titers, Inflammatory mediators' levels and lymphocytes counts among pwMS treated with different DMTs. Student's paired t-test was used to assess inflammatory mediators' levels, and lymphocytes count in pairwise samples of each DMTs group before and after vaccination. Pearson correlation analyses were applied to evaluate the association between anti-spike IgG

titers and lymphocyte count. JASP package (https://jasp-stats.org, version 0.14) was used to perform the analyses.

3.5.4 Results

Anti-spike IgG seroconversion

The age effect on seroconversion was significantly ($p < 0.001$; Table 1). O-pwMS show a rate of seroconversion significantly lower (n = 20/54, 37%, $p < 0.001$) compared to c-pwMS (n = 25/29, 86%) and f-pwMS (n = 12/15, 80%; Table 1). No differences were found between c-pwMS and f-pwMS ($p = 0.166$, data not reported).

TABLE 1: Demographic, clinical and seroconversion COVID-19 vaccine-related data

	Cladribine (n=29)	**Fingolimod (n=15)**	**Ocrelizumab (n=54)**	***p***
Gender (F:M)	20:9	12:3	22:32	*p=0.107* [a]
Age, years (mean ± SD)	41 ± 12	40 ± 10	51 ± 10	***p=0.023*** [a]
EDSS score [median (range)]	1.5 (0-7.5)	1.0 (0-4)	6.0 (0-7.5)	*p=0.525* [a]
DMTs (n)	29	15	54	***p<0.001*** [a]
Disease duration, years (mean ± SD)	7.1 ± 7.0	10.5 ± 6.4	10.7 ± 6.3	*p=0.571* [a]
Treatment duration, years (mean ± SD)	1.4 ± 0.6	2.6 ± 2.0	2.2 ± 0.1	*p=0.739* [a]
Seroconversion vaccine-related (n; %)	25; 86%	12; 80%	20; 37%	***p<0.001***[b]
COVID-19 infection post-vaccine (n; %)	0; 0%	0; 0%	1; 1.85% [c]	

Spike-specific IgG antibody production was measured by EIA test (cut-off ≥ 1.1 AU/mL), from the blood sample of pwMS treated with ocrelizumab (n=54), cladribine (n=29) and fingolimod (n=15). DMTs, disease-modifying therapies; EDSS, expanded disability status scale. Anti-spike IgG production was measured by EIA test (cut-off ≥ 1.1 AU/mL) from the blood sample of pwMS treated with ocrelizumab (n=54), cladribine (n=29) and fingolimod (n=15).[a]Statistical analysis was performed using ANCOVA. [b]Differences among the DMTs groups on seroconversion: here was reported ANCOVA, with age as a covariate, result. [c]O-pwMS, who developed COVID-19 following the vaccine, was non-seroconverted

Anti-spike IgG titers

Among the pwMS who showed anti-spike seroconversion (n = 57/98, 58%), o-pwMS demonstrated on average a significant lower level of anti-spike IgG compared to c-pwMS ($p < 0.001$) and f-pwMS ($p = 0.003$; Table 2; Fig.1).

TABLE 2: Anti-spike IgG titers

DMTs	Seroconversion (n; %)	Spike-specific IgG antibody titers (mean± SD)	Range (min-max)
Cladribine	25/29 (86%)	5.607 ± 3.002 AU/mL	0.100 - 9.500
Fingolimod	12/15 (80%)	4.087 ± 2.832 AU/mL	0.300 - 9.300
Ocrelizumab	20/54 (37%)	1.441 ± 2.320 AU/mL	0.000 - 9.900

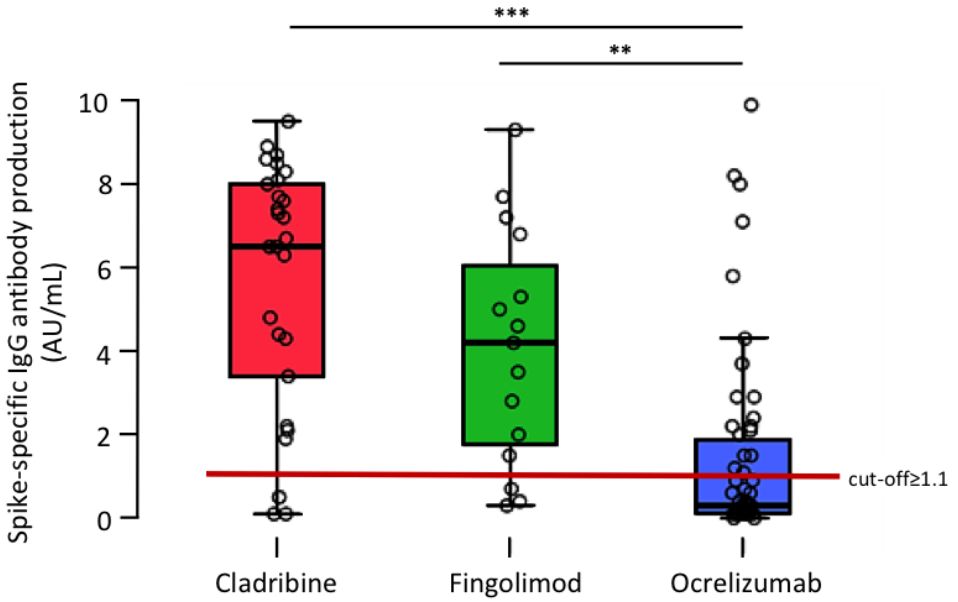

FIGURE 1: Anti-spike IgG titers**.** Anti-spike IgG titers, obtained by EIA test (cut-off ≥ 1.1 AU/mL), was significantly lower in seroconverted and o-pwMS (n=20/54, 37%) compared to and c-pwMS (n=25/29, 86%; p < 0.001) and f-pwMS (n = 12/15, 80%; p = 0.003, ANCOVA followed by post-hoc pairwise comparison using the Tukey test).

Inflammatory mediators levels pre-and post-vaccination

No significant differences of T cells-associated cytokines were observed between pre- and post-vaccination in c-pwMS and f-pwMS (Table 3), while in o-pwMS on a significant increase in GrB serum levels was found (p = 0.021; Fig.2, A).

TABLE 3: Inflammatory mediators levels pre-and post-vaccination: differences in each IS-DMT group

Cladribine-treated pwMS		***p***
GrB_pre-vaccination (pg/ml)	GrB_post-vaccination (pg/ml)	
9.876 ± 5.022	9.309 ± 3.448	0.642
IFN-γ_pre-vaccination (pg/ml)	IFN-γ_post-vaccination (pg/ml)	
0.634 ± 0.544	0.580 ± 0.447	0.984
TNF-α _pre-vaccination (pg/ml)	TNF-α_post-vaccination (pg/ml)	
9.133 ± 2.990	9.006 ± 2.050	1.000
Fingolimod-treated pwMS		***p***
GrB_pre-vaccination (pg/ml)	GrB_post-vaccination (pg/ml)	
9.587 ± 6.914	9.849 ± 4.728	0.847
IFN-γ_pre-vaccination (pg/ml)	IFN-γ_post-vaccination (pg/ml)	
0.551 ± 0.480	0.778 ± 0.411	0.178
TNF-α _pre-vaccination (pg/ml)	TNF-α_post-vaccination (pg/ml)	
12.513 ± 4.964	11.051 ± 2.980	0.653
Ocrelizumab-treated pwMS		***p***
GrB_pre--vaccination (pg/ml)	GrB_post-vaccination (pg/ml)	
10.555 ± 3.264	13.115 ± 4.407	0.021
IFN-γ_pre-vaccination (pg/ml)	IFN-γ_post-vaccination (pg/ml)	
0.986 ± 1.008	0.981 ± 0.865	0.985
TNF-α _pre-vaccination (pg/ml)	TNF-α_post-vaccination (pg/ml)	
10.904 ± 2.258	12.036 ± 4.368	0.311

No significant differences were found in serum GrB, IFN-γ, TNF-α levels (pg/ml) between pre-and post-vaccination in each DMTs group (repeated measures ANCOVA).

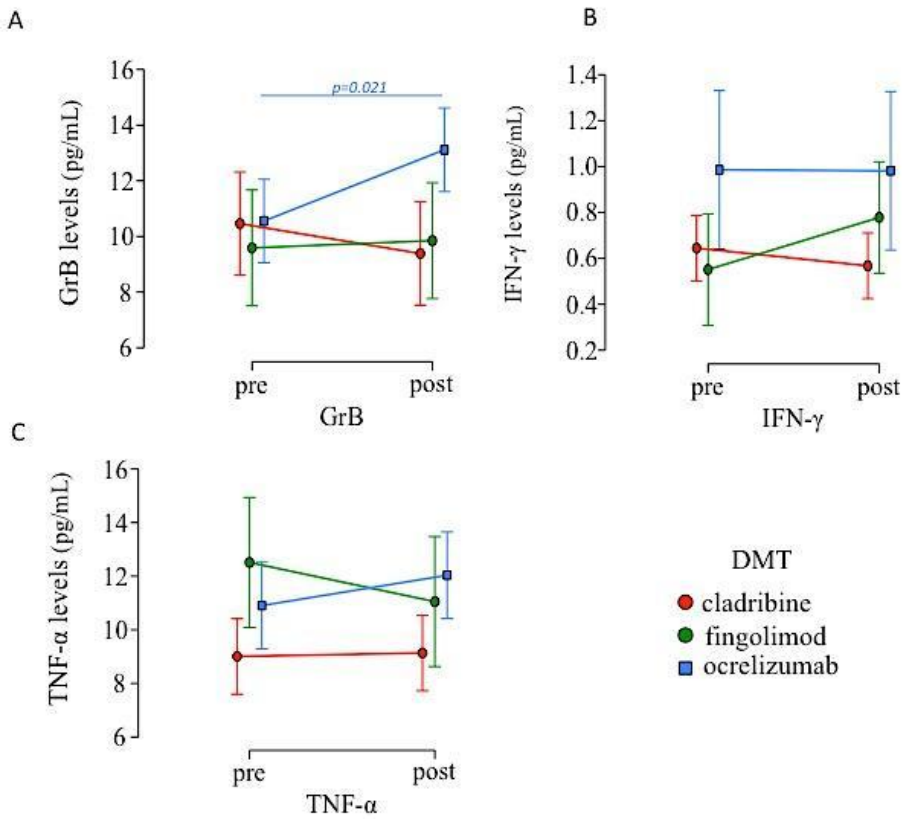

Figure 2: T cells-associated cytokines levels pre-and post-vaccination. (A) A significant increase of GrB serum levels was observed only in o-pwMS after vaccination (mean ± SD: 13.115 ± 4.407) compared to before (10.555 ± 3.264; p = 0.021, Student's paired t-test), while no differences were found of (B) IFN-γ and (C) TNF-α serum levels pre- and post-vaccination in each DMT.

Considering non-seroconverted o-pwMS (n = 34), GrB serum levels were significantly increased after vaccination (p = 0.008; Fig.3, A,) while no difference was observed in seroconverted o-pwMS. No differences were found in TNF-α (although a trend to increase was evident) and IFN-γ serum levels pre- and post-vaccination (Table 4).

TABLE 4: Inflammatory mediators levels pre-and post-vaccination: differences between seroconverted (1) and non-seroconverted (0) ocrelizumab-treated pwMS patients.

Descriptive	GrB_pre (pg/ml)		GrB _post (pg/ml)	
	0	**1**	**0**	**1**
Mean	10.069	11.720	13.813	11.440
SD	2.914	4.106	4.645	3.658
Minimum	6.040	5.800	6.240	7.700
Maximum	17.300	15.800	24.900	17.200
	IFN-γ_pre (pg/ml)		**IFN-γ _post (pg/ml)**	
	0	**1**	**0**	**1**
Mean	0.972	0.986	0.723	1.442
SD	1.143	0.246	0.533	1.205
Minimum	0.270	0.650	0.270	0.560
Maximum	4.650	1.320	2.260	3.120
	TNF-α _pre (pg/ml)		**TNF-α _post (pg/ml)**	
	0	**1**	**0**	**1**
Mean	10.340	12.637	12.702	11.027
SD	2.263	4.521	4.829	3.035
Minimum	6.560	8.250	6.490	6.900
Maximum	13.900	20.100	27.000	15.300

Among the 43 patients tested with ELLA, o-pwMS non-seroconverted (1; n=13) showed GrB serum levels increase after vaccination (mean ± SD: 13.813 ± 4.645 pg/ml) compared to before (10.069 ± 2.914 pg/ml), while no difference was observed in seroconverted o-pwMS. No differences were found in TNF-α (although a mild trend to increase was evident) and IFN-γ serum levels pre- and post-vaccination (Student's paired t-test).

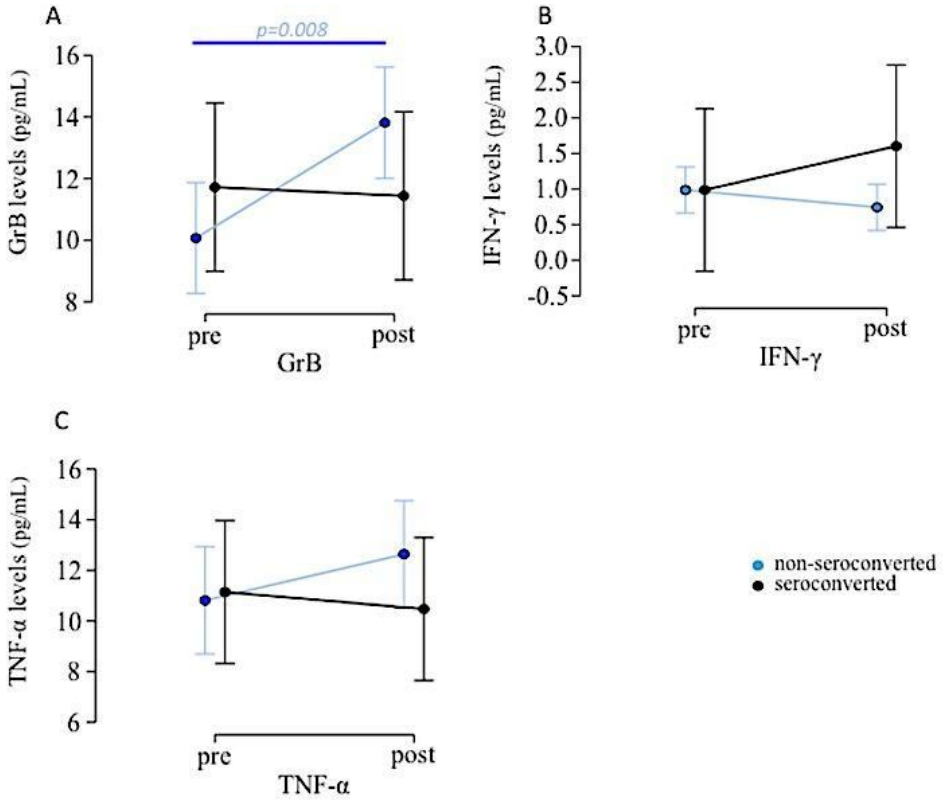

FIGURE 3: Inflammatory mediators' levels pre-and post-vaccination in o-pwMS. (A) Among the 43 patients tested with ELLA, o-pwMS non-seroconverted (n = 13) showed GrB serum levels significantly increase after vaccination (mean ± SD: 13.813 ± 4.645 pg/ml) compared to before vaccination (10.069 ± 2.914 pg/ml; p = *0.008*). No differences were found in (B) IFN-γ and (C) TNF-α (although a mild trend towards increasing was evident, p = *0.196*) and IFN-γ serum levels pre- and post-vaccination (Student's paired t-test).

Lymphocytes immunophenotype pre-and post-vaccination and their association with anti-spike IgG production

Comparing lymphocytes immunophenotype in o-pwMS and c-pwMS, CD19+ B and CD3+ T cell subsets count did not change after vaccination, in both DMT groups (Table 5). However, o-pwMS showed a trend of CD4+ T cells to increase after vaccination. Specifically considering non-seroconverted o-pwMS, a significant increase of CD4+ cells count was found after vaccination, which was not observed in seroconverted o-pwMS, although even in these an increase of CD4+ T cells was present (Fig. 4, p = 0.040).

TABLE 5: Lymphocytes immunophenotype pre-and post-vaccination

Lymphocytes/ul	Pre- vaccination	Post-vaccination	p
	mean ± SD (cells count/ul)	mean ± SD (cells count/ul)	
CD19+ B cell			
Cladribine	81.616 ± 77.455	93.874 ± 75.597	1.000
Ocrelizumab	7.401 ± 17.658	7.573 ± 16.432	1.000
CD3+ T cell			
Cladribine	811.905 ±496.021	768.905 ± 421546	0.998
Ocrelizumab	1.217.126 ± 527.237	1.305.160 ± 593.981	0.759
CD4+ T cell			
Cladribine	475.524 ± 284.689	464.619 ± 320.932	1.000
Ocrelizumab	837.833 ± 394.981	910.143 ± 451.069	0.436
CD8+ T cell			
Cladribine	299.905 ± 241.424	286.333 ± 207.881	1.000
Ocrelizumab	379.293 ± 225.432	395.017 ± 256.120	0.898

No differences were found considering lymphocytes count pre-vaccination and post-vaccination both in c-pwMS and o-pwMS (Student's t-test). However, o-pwMS showed a trend to increase of CD4+ T cells. No sufficient lymphocytes immunophenotype data were available of fingolimod-treated patients pre-vaccination.

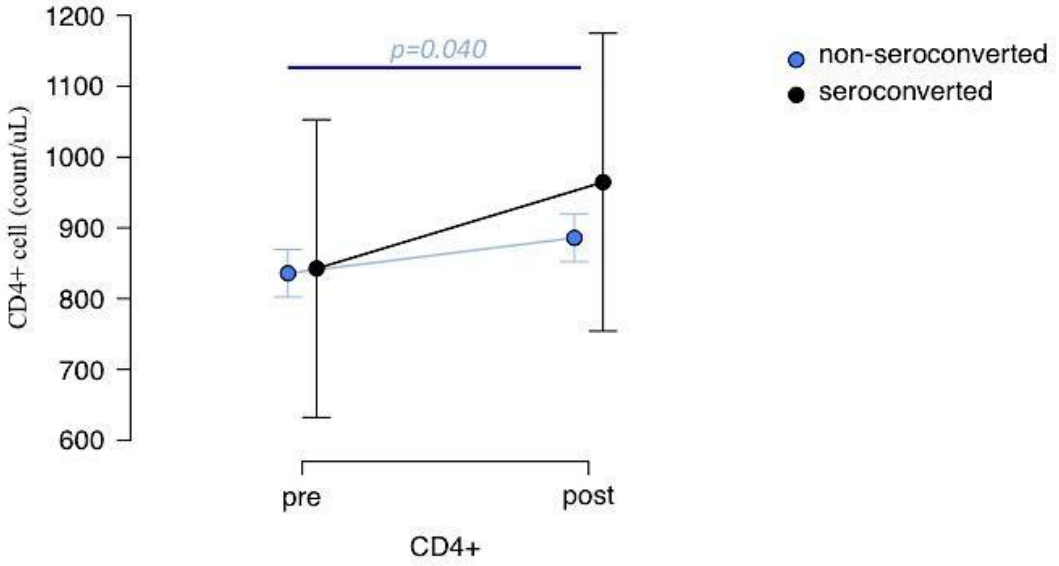

FIGURE 4: CD4+ T cells count pre-and post-vaccination in o-pwMS. Non-seroconverted o-pwMS blood analyzed through flow cytometry (n = 34), showed a significant increase of CD4+ cells after vaccination (mean ± SD: 885.793 ± 352.271 cells count/ul) compared to before (mean ± SD: 835.759 ± 321.184 cells count/ul, p = 0.040; Student's t-test). No differences were found between CD4+ T cells count pre- (842.462 ± 540.486 cells count/ul) and post-vaccination (964.462 ± 633.230 cells count/ul) in seroconverted o-pwMS (n = 13).

Therefore, only in non-seroconverted o-pwMS a significant negative correlation was found between CD4+ T cells count post-vaccination and anti-spike IgG production (r = -0.438, p = 0.014; Fig.5).

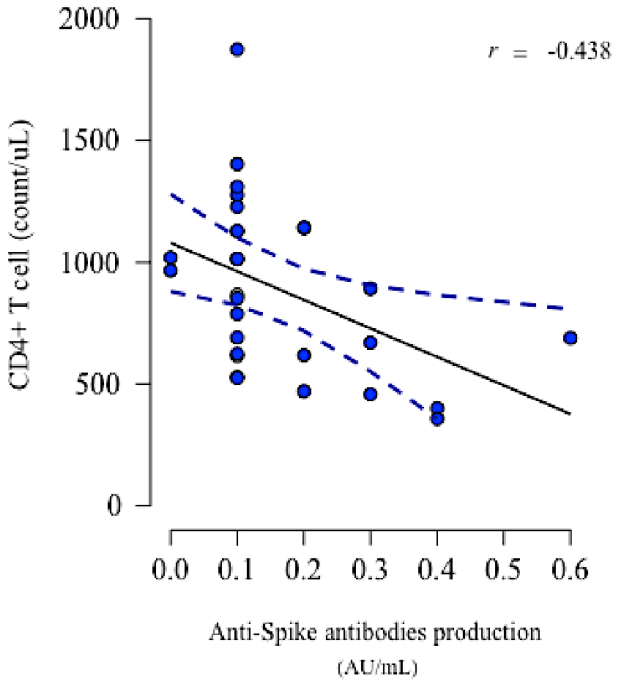

FIGURE 5: CD4+ T cells and their association with anti-spike IgG production. Negative correlation was found between anti-spike IgG production and CD4+ T cells count in non-seroconverted o-pwMS (r=-0.438, p=0.014, Pearson correlation).

3.5.5 Discussion and conclusion

The ongoing COVID-19 represents one of the deadliest pandemics in history (Schulte et al., 2021). The COVID-19 mRNA-based vaccines continue to be effective against severe disease, activating a coordinated induction of both humoral- and cell-mediated immune responses (Agrati et al., 2021). Specifically, the Pfizer-BioNTech COVID-19 (BNT162b2) vaccine, containing mRNA vector encoding the prefusion spike-glycoprotein of SARS-CoV-2 (Corey et al., 2020), has been demonstrated safe in pwMS and no increased risk of MS relapse activity has been observed following vaccination (Dreyer-Alster et al., 2022). However, investigating vaccine efficacy is extremely important in pwMS treated with DMTs that could make vaccination ineffective, exposing them to a higher risk of infection and severe symptoms.

In the present study, we evaluated BNT162b2-mRNA vaccine response in terms of antibody production, inflammatory mediators release and lymphocytes immunophenotype assessment in DMTs-treated pwMS under three different MS treatments: cladribine, fingolimod and ocrelizumab.

In line with previous results, we found a fully detectable humoral response in c-pwMS and in most f-pwMS (Tortorella et al., 2021; Guerrieri et al., 2022). As expected by B-cell depleting therapy (Frampton, 2017; Bar-Or et al., 2020), we found that most o-pwMS patients did not develop the antibody response to mRNA-based vaccines (Tortorella et al., 2021; Guerrieri et al., 2022). In addition, o-pwMS who developed antibodies had significantly lower antibody titers compared to c-pwMS and f-pwMS, as shown in previous works Achiron et al., 2021, *a*; Frampton, 2017). Furthermore, treatment duration and disease duration were not associated with spike-specific antibody production for each DMT considered.

However, despite the absence of antibodies, we observed that only one case of COVID-19 infection in the o-pwMS group, suggesting a limited increased risk of COVID-19 infection compared to the other DMTs examined (cladribine and fingolimod) in line with previous observations (Tortorella et al., 2021; Gadani et al., 2021; Iannetta et al., 2021).

Identification of molecular mediators' induction following COVID-19 vaccination is therefore important to evaluate vaccine response in pwMS treated with different DMTs, especially in those who do not develop an antibody response (Guerrera et al., 2021). Furthermore, the use of a straightforward and reproducible assay for molecular detection could represent a new tool for routine monitoring of vaccinated pwMS**.**

For all these reasons, in addition to the humoral immune response, we also explored through the ELLA platform the cellular immune response to the BNT162b2-mRNA vaccine considering the serum levels of specific inflammatory mediators, mainly released by spike-specific activated T cells, such as GrB, IFN-γ and TNF-α (Krzysiek et al., 2013; Taoufik et al., 2021). These molecular mediators have a pivotal role in the development and regulation of cellular and humoral immunity to vaccination, including the BNT162b2-mRNA vaccine (Agrati et al., 2021; Apostolidis et al., 2021; Krzysiek et al., 2013; Taoufik et al., 2021). Specifically, in our study, we found that GrB serum levels in o-pwMS (but not in the other DMTs groups) were increased after

vaccination and such increase was also more relevant in those patients who failed to generate anti-spike IgG. In the same patients, furthermore, an increasing trend in TNF-α serum levels was observed.

These results, in line with previous works (Achiron et al., 2021, *b*) suggest that in o-pwMS, who underwent the B-cell depleting therapy, a cell-mediated response, presumably associable with the activation of CD4+ and CD8+ T cells, has occurred.

In order to confirm this hypothesis, we also assessed the immunophenotypic lymphocyte profile in c-pwMS and o-pwMS, considering CD3+ T cell subsets. We found that non-seroconverted o-pwMS showed a significant increase in CD4+ T cell count post-vaccination compared to pre-vaccination.

Anti-spike IgG production and lymphocytes immunophenotype were correlated both in c-pwMS and in o-pwMS. We found a strong negative correlation between anti-spike IgG production and CD4+ cell count after vaccinations only in non-seroconverted o-pwMS, supporting previous findings and confirming a CD4+ T cell-mediated response.

This study is not free from limitations. The low number of patients recruited and the lack of a control group, and of some data (e.g. lymphocytes immunophenotype in f-pwMS) represent limitations in our study. However, the extension of the data to a larger cohort of patients will confirm and clarify the different immunological setup in non-seroconverted pwMS.

Furthermore, the study lacks a functional assay that demonstrates the direct production of inflammatory mediators by T lymphocytes including ELISpot assay or flow cytometry (intracellular cytokine staining analysis). Nevertheless, although the ELLA simple-plex assay used does not represent the gold standard for a functional analysis of the expression of T-cells related molecular mediators; it is highly sensitive and reproducible, making our results robust and indicative of the inflammatory levels of these patients after vaccination.

Overall, this study confirms differences in anti-spike IgG production among different DMTs and provides evidence of cell-mediated immunity preservation after BNT162b2 vaccination in o-pwMS, specifically in those patients lacking anti-spike IgG

antibodies. Such hypothesis seems confirmed by the observation of a low rate (and the mild evolution) of COVID-19 infection in o-pwMS.

Finally, the evaluation of specific molecular mediators, through innovative and immediate technology, could represent a helpful tool for routine monitoring of vaccination response in vaccinated DMTs-treated pwMS.

4. Conclusions

Early cortical gray matter damage was related to a more severe and rapid disease course in terms of disability progression and cognitive impairment. Progressive MS forms, characterized by a worsening of neurological function and by an accumulation of disability over time, show extensive areas of demyelination at the level of the cerebral cortex. DMTs, that target constituents of the peripheral immune system, are ineffective in progressive MS, suggesting an alternative pathogenic mechanisms underly this MS pathology due to intrathecal inflammation, rather than to perivascular infiltrates. Specifically, in this work we found an increase of cytokines and chemokines released into the CSF levels by meningeal infiltrates and resident glial cells activated. Specifically, high CSF levels of the B cell-related cytokines (CXCL13, CXCL12, LIGHT and APRIL) Th1-proinflammatory molecules (IFNγ and TNF) and of the monocyte/microglia activity biomarker (sCD163) were found. CXCL13, TNFα, and IFN-γ were found to have the strongest correlation with the clinical and radiological outcomes of disease activity, such as new WM lesions, new Gad+ lesions, new CLs, new relapses, and EDSS change. In addition, we observed a strong positive correlation between the number of new CLs and the CXCL13 CSF levels, confirming a role of this cytokine in MS pathology. The CSF profile identified could represents a promising biomarker in MS, detectable in those patients with high risk of experiencing more severe disease activity, increased GM damage, and disability progression in the early phase of the disease.

We continued the work with the analysis of the same protein profile found in the CSF in the previous study, to investigate the relationship between intrathecal and peripheral inflammation in MS patients and clarify whether intrathecal CSF protein profiling is somehow reflected in paired serum samples, but we found only one correlations, confirming a clear separation between intrathecal and peripheral inflammation. Overall this study confirms that intrathecal compartmentalized inflammation is relatively independent of peripheral immune response, and this would also explain the difficulty in targeting it by available peripheral immunosuppressive therapies.

In addition to CSF cytokines and chemokines levels, we measured CSF IgM levels in MS patients compared to patients with other neurological diseases. IgMs are the

immunoglobulins mostly expressed in the CSF of naive MS patients compared to other neurological conditions at the time of diagnosis. The association between increased CSF IgM levels and molecules related to both B-cells immunity (IL-10) and recruitment (CXCL13 and CCL21) and to macrophage/microglia activity (IL-12p70, CX3CL1, and CHI3L1) suggests possible correlation between humoral and innate intrathecal immunity in early disease stage. Furthermore, the association of IgM levels with WMLs and MS clinical and MRI activity after 2 years supports the idea of key role of IgM in the disease course.

Moreover, we continued with the study of prognostic biomarkers considering the levels of parvalbumin and neurofilament in MS cases compared to controls and evaluated their association with neuroradiological and clinical parameters at the time of diagnosis. Specifically we explored the role of PVALB as a new early biomarker of a severe MS disease. We found that CSF PVALB and Nf-L levels were found to increase in MS patients compared to controls. However, compared to Nf-L, our results show that CSF PVALB levels correlate better with global CTh and CLs number, but not with WMLs, already at the time of diagnosis, suggesting that it could represent a more specific marker of cortical neurodegeneration-related atrophy. This finding was particularly evident considering the correlation between CSF PVALB levels and cortical regions previously identified as severely affected by cortical pathology at least in the early stage of the disease. All these results suggest PVALB as a specific biomarker of cortical damage in early MS.

Finally, given the recent COVID-19 pandemic, a specific study on the antibody and cell-mediated response by MS patients treated with different immunosuppressive therapies (cladribine, fingolimod and ocrelizumab) was included in this thesis. The study demonstrated a different vaccine effect in patients treated with different treatments: patients receiving ocrelizumab showed an impaired antibody production and, on the contrary, a preserved cell-mediated response, associated with an inflammatory mediator's production, despite the absence of antibody titers.

The identification and validation of prognostic biomarkers that underlying disease activity would allow for more timely and individualized clinical management of MS patients. We hope that this study will aid in improving the management of MS patients, with the aim of reducing, halting, or one day even reversing the disability associated with multiple sclerosis. Extending this study to a larger MS population could help to achieve a more complete analysis and long-term follow-up of the MS cohort is necessary to confirm the results.

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