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"INTEGRATION OF ADVANCED MOLECULAR ANALYSES AND MAGNETIC RESONANCE IMAGING FOR THE IDENTIFICATION OF BIOMARKERS OF DISEASE PROGRESSION IN MULTIPLE SCLEROSIS"

Direttore della Scuola: Ch.mo Prof. Gaetano Thiene
Coordinatore d'indirizzo: Ch.ma Prof.ssa Elena Pegoraro
Supervisore: Ch.ma Prof.ssa Elena Pegoraro

Dottorando: Dott. Dario Seppi

Index

Abstract

Background. In the last 15 years, it has become increasingly accepted that cerebral grey matter (GM) damage in MS is evident since early MS stages and provides the best correlate of the rate of clinical progression since early disease stages. The lack of substantial inflammatory infiltrates, complement deposition and blood brain barrier damage in MS cortical lesions led to the initial suggestion that the mechanisms underlying GM and white matter (WM) pathology substantially differ and that activated microglia are the dominant effector cell population causing GM damage. Furthermore, in the last decade, post-mortem studies have revealed that the extent of meningeal inflammation associates positively with subpial cortical demyelination and disease progression, suggesting that chronic immune activity in the subarachnoid compartment plays a key role in mediating damage in the adjacent cerebral cortex of affected patients. Factors released by immune cells circulating in the CSF and/or colonizing the subarachnoid space may diffuse across the pial membrane, inducing a gradient of glia and neuronal pathological alterations, directly and/or indirectly through activated microglia.

Aim of the study. To combine CSF molecular and protein analyses with advanced MRI imaging techniques, able to better identify cortical grey matter demyelination, in order to identify potential early biomarkers of GM pathology and disease progression with prognostic and predictive value and to obtain information on the biological and immunological mechanisms that link the inflammatory process of the GM and the progressive neurodegeneration

Methods. Two independent cohorts of MS patients have been recruited and followed by the MS Centre of Padova. The first cohort of MS patients, composed of 35 patients and 5 controls was recruited retrospectively between February 2009 and September 2011 and followed by a detailed neurological and clinical follow-up. The second cohort of 31 patients and 13 controls was collected prospectively from January 2014 to May 2015. The patients, at their disease

onset or in the very early phase of the disease, underwent a complete diagnostic work-up comprehensive of clinical evaluation, lumbar puncture and MRI evaluation inclusive of non-conventional sequences. By using the immuno-assay Bio-Plex System technique (Biorad - Bio-Plex Pro Human Chemokine panel 40-plex) we performed a protein analysis of the presence and levels of inflammatory molecules in both cohorts. In the second cohort we carried out also a gene expression analysis of the matched cellular fraction (using pre amplification real-time PCR for a panel of genes of interest).

Results. CSF protein analysis of the first cohort of MS patients reveal higher levels of proinflammatory cytokines CXCL13 (p=0,00006), CCL19 (p=0,0019), CCL1 (p=0,00018), and CCL22 (0.0009) compared to controls. Protein analysis on our second cohort pointed out an important increase of CXCL13, CXCL10, CXCL11 and CCL2 in MS population compared to controls. After stratification according to GM pathology we reported an increase of CXCL13 protein levels in the MS subgroup with higher cortical demyelination. Gene expression analysis reveals a significant increase in MS patients for CD20, CD138 and LTa compared to controls supporting the hypothesis of a key role of a B-cell response and lymphoid neogenesis in MS pathology

Conclusions. Combined CSF analysis and MRI analysis suggested that B cell immune response may play an important role in MS since the disease onset and correlates with the level of intrathecal inflammation and cortical pathology. A more detailed analysis of the CSF biomarkers suggested in the current study might provide, in addition to MRI optimization, a better indication of severity of disease process that characterized GM pathology and important tools in predicting/monitoring the evolution of the disease.

Esposizione riassuntiva in lingua italiana

Presupposti dello studio. Studi istopatologici e neuroradiologici hanno dimostrato soprattutto negli ultimi 15 anni come la sclerosi multipla, considerata classicamente una patologia elettiva della sostanza bianca, sia una patologia caratterizzata da un coinvolgimento, fin dalle prime fasi di malattia, anche della sostanza grigia corticale e profonda che ben correla con il quadro di disabilità fisica e cognitiva ampiamente descritto. Dal punto di vista istopatologico tale coinvolgimento è caratterizzato dalla presenza di lesioni corticali che si distinguono per una minor componente infiammatoria, per un'assenza di danno a carico della barriera ematoencefalica e per un minor deposito di fattori del complemento. Alla luce di tale descrizione sembra che i meccanismi che sottendono il danno a carico della sostanza grigia differiscano almeno in parte da quelli osservati a carico della sostanza bianca. In particolare è stata osservata un'associazione tra lesioni corticali e la presenza di infiltrati infiammatori meningei correlata ad una maggiore disabilità clinica. È stato quindi evidenziato come molecole pro-infiammatorie rilasciate dalle cellule infiammatorie residenti nelle meningi diffondano attraverso lo spazio subaracnoideo e agiscano, direttamente o indirettamente attivando la componente microgliale, sulla adiacente corteccia cerebrale determinando un gradiente di danno corticale.

Obiettivi. L'obiettivo di questo studio è stato quello di verificare la presenza di possibili potenziali biomarcatori di danno corticale mediante l'applicazione combinata di: a) tecniche avanzate di analisi proteica e analisi molecolare applicate allo studio del liquido cerebro spianale; b) tecniche non convenzionali di risonanza magnetica in grado di caratterizzare il danno a carico della sostanza grigia sia focale che diffuso.

Materiali e metodi. Sono state arruolate due coorti di pazienti: la prima studiata retrospettivamente era composta da 35 pazienti e 5 controlli; la seconda arruolata nello studio con un approccio di tipo longitudinale era composta da 31

pazienti e 13 controlli. Tutti i pazienti erano caratterizzati da un esordio relativamente recente e la precedente somministrazione di terapie immunomodulanti rappresentava un criterio di esclusione. Tutti i pazienti si sono sottoposti ad un iter diagnostico completo comprensivo di valutazione clinica, esami di laboratorio, esame del liquido cerebrospinale e risonanza magnetica comprensiva di sequenze non convenzionali per verificare la presenza di lesioni della sostanza grigia. Lo studio del liquido cerebrospinale prevedeva inoltre uno studio di analisti proteica mediante immuno-assay (Bio-Plex System technique Biorad - Bio-Plex Pro Human Chemokine panel 40-plex) per lo studio di 40 citochine/chemochine e uno studio di analisi di gene expression.

Risultati. L'analisi proteica del liquor nella prima coorte ha evidenziato la presenza di elevati valori di CXCL13 (p=0,00006), CCL19 (p=0,0019), CCL1 (p=0,00018), e CCL22 (0.0009) rispetto alla popolazione di controllo. L'analisi proteica della seconda coorte ha evidenziato, sempre rispetto alla popolazione di controllo, un aumento delle seguenti citochine: CXCL13, CXCL10, CXCL11 e CCL2. Dopo stratificazione in base al carico corticale abbiamo evidenziato nei pazienti con un maggior coinvolgimento della sostanza grigia un aumento dei livelli proteici di CXCL13 e una maggior espressione di CD20, CD138, CXCL13 and LTa a supporto di un ruolo della risposta infiammatoria mediata dai linfociti B.

Conclusioni. L'analisi combinata di liquor e risonanza magnetica suggerisce che la risposta immunologica mediata dai linfociti B giochi un ruolo importante nella patogenesi della sclerosi multipla e che il livello di infiammazione intratecale ben correla con la patologia corticale. I risultati del nostro studio suggeriscono quindi che l'uso di biomarker liquorali potrebbe essere di supporto nella caratterizzazione della patologia corticale nella sclerosi multipla.

1. Introduction

The first description of multiple sclerosis (MS) dates back in the 14th century, but only in the 19th century MS became a distinct disease entity when Jean-Marin Charcot defined the first correlation between the clinical feature of MS (described as "la sclérose en plaques") and the pathological changes seen in post-mortem (Compston et al., 1998). Currently MS is recognized as a chronic immune-mediated inflammatory and neurodegenerative disease of the central nervous system (CNS), and epidemiologic studies support both genetic and environmental components of susceptibility. The pathologic hallmark of chronic MS is inflammation both in the white matter (WM) and in the grey matter (GM), demyelination with partial restoration of myelin, axonal and neuronal damage, and glial scarring (Noseworthy et al., 2000, Lassmann, 2013).

1.1. Epidemiology

MS is the most common chronic inflammatory disease of the CNS and affects more than 2.3 milion people worldwide (Pathwardhan et al., 2005) with a prevalence that varies, from high levels in North America and North Europe (>30/100.000 inhabitants) to low rates in Sud America and Eastern Asia (2/100.000) (Marrie RA et al., 2004). The current prevalence of MS in Italy is estimated at 100-120/100.000 inhabitants; in the province of Padova the overall prevalence on 31 December 2009 was 139.8/100.000 inhabitants with an increased incidence rate in the last 10 years (5.5/100.00/year); other recent epidemiologic studies observed an increase of incidence in Sardinia and in the Repubblica of San Marino, suggesting changes in environmental factors more than a simply change in ascertainment (Ranzato et al., 2003, Granieri et al., 2008, Cocco et al., 2011, Puthenparampil et al., 2013).

Most people are diagnosed between the ages of 20 and 45 with a gender ratio (women/men) from 2/1 to 3/1 in Nordic Countries (Kingwell et al., 2013, Alonso et al., 2008). Previous infection with Epstein-Barr virus, smoking behaviour and vitamin D insufficiency are potential susceptibility factors for multiple sclerosis

(Marrie RA et al., 2004; Kantarci et al., 2006; Ascherio et al., 2007 a, b). Although MS is not an inherited disease, it is characterized by a strong genetic component (Di Santo et al., 2014). Indeed, the risk of developing this condition in biological relatives of MS patients increases with increasing degree of kinship (Willer et al., 2003; Kantarci et al., 2008); farther, linkage analysis studies have revealed several gene loci as risk factors including a strong association between HLA-DRB1*15:01 and MS (Hafler et al., 2007; Jersild C et al., 1973).

MS is the most disabling neurological disorder of the young adults with remarkable sanitary and social costs (Pathwardhan et al., 2005). The average annual cost of a MS patient in Italy is about € 39.000, 34.3% of which is represented by direct and 65.7% by indirect costs.

1.2. Subtypes of MS

Heterogeneity and variability characterize MS whose course may be considered as the expression of two main clinical manifestation (Confavreux et al., 2006):

- Relapse of acute neurological symptoms, that last over 24 h suggesting structural damage of the brain (D.H. Miller et al., 2012), and that end with a complete or partial recovery. It characterizes about 85% of MS disease onset.
- Progression which refers to irreversible accumulation of disability over more than six months.

In 1996 the US National Multiple Sclerosis Society (NMSS) proposed standardized definitions for the most common clinical courses of patients with MS, essential for mutual understanding between clinicians and investigators and for design of, and recruitment for, clinical trials (Lublin et al., 1996). They defined four main subtypes: relapsing-remitting, primary progressive, secondary progressive, and progressive relapsing MS.

Relapsing remitting (RR) MS: characterized by clearly defined attack with full recovery or with sequelae and residual deficit upon recovery; periods between disease relapses are characterized by a lack of disease progression

Secondary progressive (SP) MS: begins with an initial RR course, followed, in 10-15 years, by progression of variable rate that may also include occasional relapses and minor remissions. This subtype represents 40-50% of RRMS cases and 60-70% of MS cases. It is a dramatic event in the lifetime of MS patient because from this moment on prognosis is compromised and therapy is no longer effective. Indeed, once the clinical threshold of irreversible disability (score 4 on the Kurtzke Disability Status Scale (EDSS)(Kurtzke et al., 1983)) has been reached, disability proceeds irreversibly and independently (Confavreux et al., 2003 and 2006).

Primary-progressive (PP) MS (10-15% of MS cases): disease showing progression of disability from onset without plateaus or remission or with occasional plateaus and temporary minor improvements.

Relapsing-progressive (RP) MS: although this has been one of the most commonly used terms to describe an important clinical form of MS characterized by a combination of relapse and progression, there was no consensus for a definition.

In this light, disability accumulation manifests as an expression of degenerative processes that progress independently from inflammatory events.

Because imaging and biological correlates were lacking in previous phenotypes description, in 2013 Lublin and Colleagues (Lublin et al., 2014) re-evaluated definitions of clinical MS phenotypes originally described in 1996. They proposed the inclusion of two modifiers to the basic clinical courses of “relapsing” and “progressive” MS, namely, disease activity (clinical and radiological) and clinical progression in order to improve prognostication and treatment selection (Figure 1 and 2) (Lublin et al., 2014, Lassmann, 2013). Moreover they introduce new subtypes:

Clinically isolated syndrome (CIS): the first episode suggestive of MS without radiological DIS (can be a single or multiple demyelinating lesion) could be classified as CIS (Lublin et al., 2014). The symptoms should last for at least 24 h (isolated in time but not necessarily in space). Remarkably the course of MS after

CIS is variable: after 15–20 years, a third of patients have a benign course with minimal or no disability and a half will have developed secondary progressive MS with increasing disability (D.H. Miller et al 2012) while disease-modifying treatments delay the development from CIS to MS (Kennedy et al., 2013).

Radiological isolated syndrome (RIS): the evidence of a demyelinating process identified by MRI in absence of clinical relapses or neurological symptoms and clinical signs (Okuda DT et al., 2009). RIS may raise likelihood of an eventual MS diagnosis (Okuda DT et al., 2011), depending on the morphology and location of detected MRI lesions (gadolinium-enhancing lesions asymptomatic spinal cord lesions) or positive CSF findings.

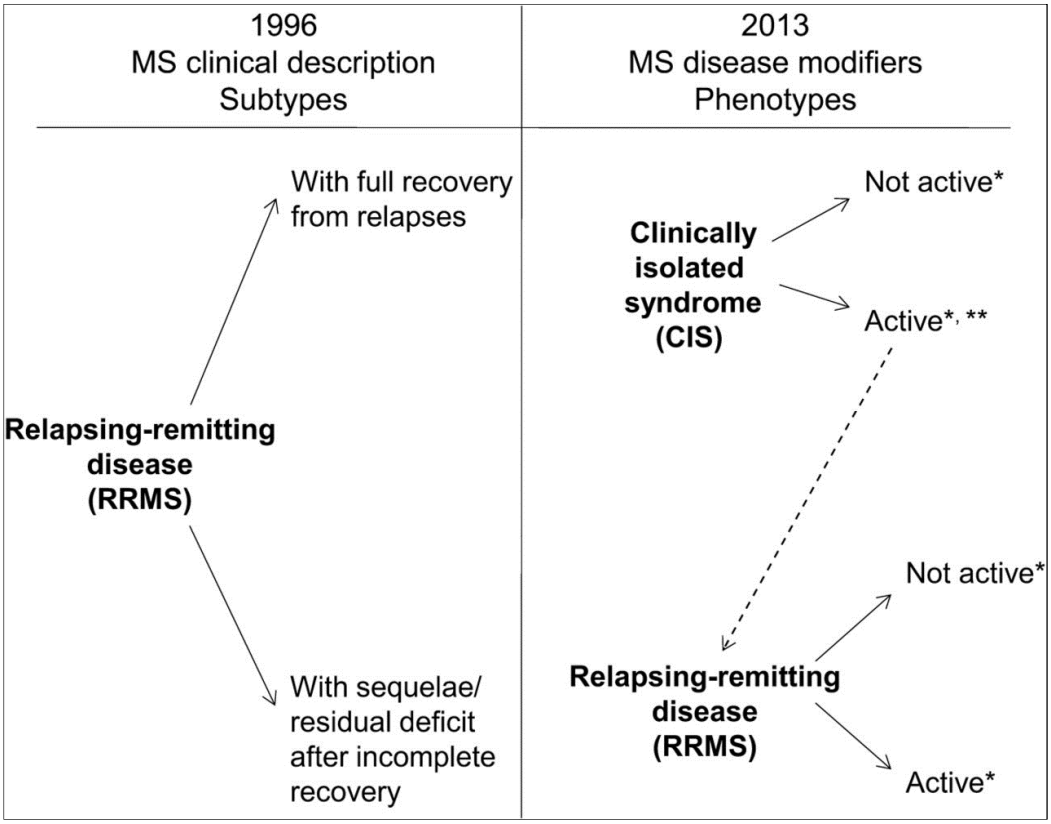

Figure 1. The 1996 vs 2013 multiple sclerosis phenotype descriptions for relapsing disease *Activity determined by clinical relapses and/or MRI activity (contrast-enhancing lesions; new or unequivocally enlarging T2 lesions assessed at least annually); if assessments are not available, activity is "indeterminate." **CIS, if subsequently clinically active and fulfilling current multiple sclerosis (MS) diagnostic criteria, becomes relapsing-remitting MS (RRMS). (Lublin et al., Neurology 2014)

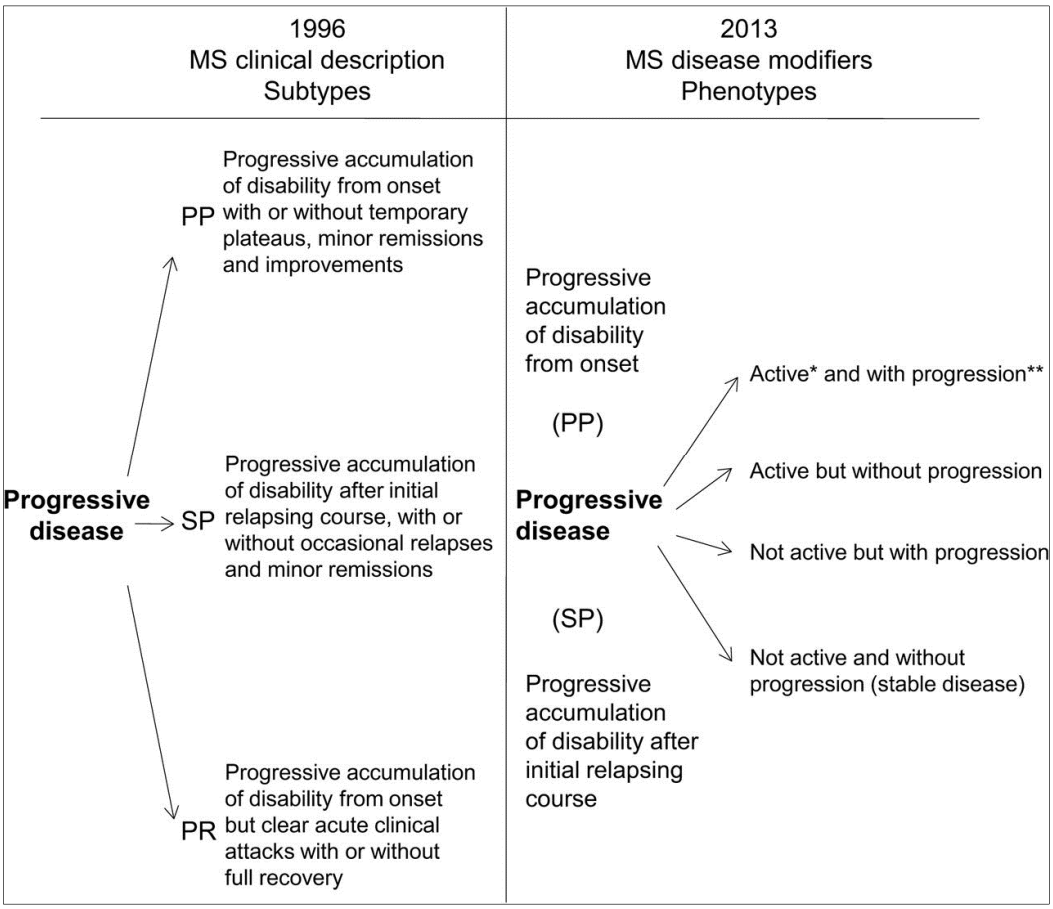

Figure 2. The 1996 vs 2013 multiple sclerosis phenotype descriptions for progressive disease. *Activity determined by clinical relapses assessed at least annually and/or MRI activity (contrast-enhancing lesions; new and unequivocally enlarging T2 lesions). **Progression measured by clinical evaluation, assessed at least annually. If assessments are not available, activity and progression are "indeterminate." MS = multiple sclerosis; PP = primary progressive; PR = progressive relapsing; SP = secondary progressive. (Lublin et al., Neurology 2014)

1.3. Diagnostic criteria

Albeit in the last twenty years the diagnostic criteria for MS have evolved significantly, at present there is no single diagnostic test. Thus, diagnosis, essentially a clinical diagnosis, is based on a detailed history, careful neurologic examination, and supportive paraclinical investigations, including MR (MRI)

imaging scans, cerebrospinal fluid (CSF) analysis, evoked potentials, and blood tests to exclude confounding diagnoses.

As the etiology of MS remains unknown and clinical manifestations result from involvement of multiple systems (motor, sensory, visual, etc.) in different period of time, the diagnosis of MS relies on the acquisition of three basic concepts:

- No better explanation: to rule out other neurological disorder that can mimics MS at onset (Table 1);
- Presence of dissemination in space (DIS): involvement of different region of the CNS demonstrated with the neurological examination or laboratory test (MRI, or evoked potential);
- Presence of dissemination in time (DIT): evidence at different time points of temporal evolution of the disease, demonstrated by a new clinical attack, by the appearance of new inflammatory lesions at MRI scan, or by continued progression for one year.

Differential diagnosis group	**Diseases**
Radiological	Tumor (glioma, isolated CNS lymphoma, tumefactive MS ADEM Balo'concentric sclerosis
Clinical	neuroboreliosis myelopathy vasculitis sarcoidosis syphilis neurocysticercosis Behcet's disease

Table 1. Differential diagnosis of MS

The first diagnostic criteria, developed by Schumacher in 1956, were based largely on the results of clinical examination (Schumacher et al., 1965). Later on in 1983, Poser and colleagues created new diagnostic criteria for clinical trials and these were supported by laboratory test (evoked potentials, CSF

examination, and MRI scan)(Poser et al., 1983). Although they have only a supportive role, the CSF study permitted the calculation of the IgG index (Link et al., 1977) and to reveal the presence of oligoclonal bands (OCBs), which represent the best evidence of the intrathecal inflammation characterizing MS from the other inflammatory neurodegenerative diseases.

In addition, the application of MRI in MS led to an increase awareness in the number of patient with white matter pathology despite lack of standard procedures (Barkhof et al., 1992).

Only in 1997-2000 Barkhof-Tintorè (Barkhof et al., 1997, Tintoré et al., 2000) defined radiological diagnostic criteria for MS; these were included in the new diagnostic criteria proposed in 2001 by the International Panel on MS Diagnosis chaired by Ian McDonald (McDonald et al., 2001). Thanks to high specificity and sensibility, MRI criteria became an alternative to clinical assessment in defining dissemination in time and space of MS. To achieve DIS criteria with MRI three out of four following conditions had to be fulfilled: a) at least one gadolinium-enhancing (Gd+) lesion must be present or at least 9 T2 hiperintensive lesions must be present if gadolinium-enhancing lesions are absent; b) at least one infratentorial lesion must be present; c) at least 1 subcortical lesion must be present; d) at least 3 periventricular lesions must be present; only one spinal cord lesion could substitute for one brain lesion. DIS was also proven in the presence of two lesions suggestive of MS in conjunction with the presence of OCBs. On the other hand, DIT could be established by the presence of at last one Gd+ lesion (not the one responsible for the clinical symptoms) in the brain in the MRI scan performed at least 3 months after the onset of first clinical symptoms, or by the presence of a new T2 hiperintensive lesion or a gadolinium-enhancing lesion in the second MRI scan performed not sooner than 3 months after the first. Besides, the diagnosis could still be achieved after a second relapse.

The revision of McDonald criteria in 2005 by Polman and colleagues reduced the time needed to prove a radiological DIT (presence of a new T2 hiperintensive lesion in the next MRI scan if the previous one was done at least 30 days after

the onset of first clinical symptoms, and not sooner than 3 months as previously described) (Polman et al., 2005). Furthermore any number of cord lesions can substitute for brain lesions and a cord lesion is also assigned the same status as an infratentorial lesion (Bot JC et al., 2004).

In 2010, the McDonald criteria (summary in Table 2) were modified to speed up the diagnosis process (Polman et al., 2011). Based on the studies conducted by the MAGNIMS (European Magnetic Imaging in MS) group, the criteria for radiological DIS were changed and stated that at least one lesion typical for MS lesion in at least two out of four locations typical for MS (periventricular, subcortical, infratentorial, and in the spinal cord) should be present in the MRI. If, at the time of the MRI scan, the patient had clinical symptoms indicating the involvement of one of the CNS lesions, this lesion should not be counted. To prove DIT MRI scan may be performed earlier (without specifying a time frame) and the coexistence of gadolinium-enhancing and non-enhancing lesions was considered sufficient. CSF testing was removed from diagnostic criteria. However because its important role in the differential diagnosis of MS and in diagnosing the primary progressive form of MS, it should be still performed.

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

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

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

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

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

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

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

Table 2. The 2010 McDonald Criteria for Diagnosis of MS (McDonald et al., 2010)

1.4. Multiple sclerosis lesions and inflammatory response

In MS, immune dysregulation, possibly triggered by genetic and environmental factors, may promote an inflammatory response against self-antigens in the CNS which causes inflammation in the brain and spinal cord and results in myelin disruption, axonal damage, neuronal loss, glia alterations (Sospedra et al., 2005) and release of new potential CNS autoantigens (as the αβ-cristallin, Bsibsi et al., 2014).

Demyelination is characterized by oligodendrocyte loss and could be followed by a variable degree of re-myelination. Axonal loss and gliosis are characterized by astrocyte proliferation, microglia/macrophage activation and glial fiber production occurring in combination with axonal/neuronal damage.

Pathological hallmark of MS is the presence in the CNS of widespread focal inflammatory demyelinating lesions which are characterized, in particular in the WM, by infiltrates composed, mostly, by activated T-lymphocytes with a dominance of MHC-I restricted CD8+ T cells (Babbe et al., 2000); infiltrating macrophages, dendritic cells, B cells and plasma cells are also present (Frischer et al., 2009); thus, this coexistence entails potential roles for both cellular and humoral immune response. Furthermore, there is an up-regulation of major histocompatibility complex class I (MHC-I) molecule expression on inflammatory cells, glial cells and neurons depending upon the severity of the disease and the activity of the lesions (Hoftberger et al., 2004). CD8+ T cells are believed to induce axonal pathology by direct injury to MHC-I antigen expressing cells such as neurons and oligodendrocytes (Batoulis et al., 2010).

Major histocompatibility complex class II (MHC-II) molecules in the brain are mainly expressed on microglia and macrophage.

In this context pro-inflammatory mediators, such as chemokines and cytokines, are also up-regulated (Holman et al., 2011) and amplify the immune response through recruitment/activation of naïve resident microglia which cooperate in mediating demyelination and neurodegeneration.

In the early stages the acute pathological changes affect mainly myelin sheaths and oligodendrocytes, however concomitant axon injury is already present and frequent in MS, even in normal appearing brain tissue (Dutta and Trapp, 2007). MRI studies show how Gd+ T2WM lesions are frequent since early phase (suggesting inflammation with blood brain barrier leakage), and how they become rare, when patients have entered the progressive phase, suggesting that the inflammatory process becomes at least in part trapped within the CNS compartment. From a clinical point of view the early phase of the disease is characterized by episodes of neurological dysfunction while the subsequent progressive phase is characterized by accumulation of disability related to pathological changes which are dominated by diffuse inflammation and extensive neurodegeneration throughout the whole brain (Kutzelnigg et al. 2005).

Clinical course, age and disease duration influence the dynamic nature of white matter MS pathology. Despite active MS plaques predominate in acute and early RRMS they are also present with a lesser degree, in the progressive MS phase and are characterized by smoldering plaques and microglial activation (Frisher et al., 2015).

Four different patterns of pathology with resulting demyelination have been also described in MS lesions (Lucchinetti et al., 2000):

Type 1 (19%): T cell-mediated where demyelination is macrophage-mediated or indirectly by macrophage toxins;

Type II (53%, the most common observed): lesions are both T cell and antibody mediated. This pattern results in demyelination via specific antibodies and complement;

Type III (26% of lesions): results from distal oligodendropathy; degenerative changes in distal processes occur and are followed by apoptosis;

Type IV (2% of lesions): related to primary (non apoptotic death) oligodendrocyte damage followed by secondary demyelination (pattern observed in a small subset of PPMS patients).

1.5. Axonal and neuronal damage in multiple sclerosis

Axon damage, a key feature of MS pathology, is an early and persistent event in MS and has a major impact on permanent neurologic deficit. It is characterized by axon swelling, axon transection, Wallerian degeneration and subsequent neurodegeneration (Dutta and Trapp, 2007). Axon damage is associated with inflammation, especially macrophage infiltration (Pascual et al., 2007). In post-mortem studies, axon loss has been identified also in the normal-appearing WM (NAWM) and cortical GM revealing a more diffuse pathology. Histopathological studies have reported the presence of discontinuous staining of axon neurofilaments in the NAWM of MS brains and the presence of terminal axonal ovoids, suggestive of transection, surrounded by macrophages and activated microglia (Trapp et al., 1998).

Inflammatory activity in MS lesion has been therefore proposed as possible immune-mediated mechanism for axon transection and damage (Ferguson et al., 1997). It has been also demonstrated that activated CD4+ and CD8+ T-cell subsets are equally neurotoxic (Giuliani et al., 2003) inducing the release, by microglia cell, of free oxygen radicals and nitric oxide (NO) which cause mitochondrial injury. CD8+ T-cell are described as possible mediators of axon transection lesions (Babbe et al., 2000) by producing direct cytotoxic factors.

Finally some studies indicate that inflammation induces aberrant glutamate homeostasis and production of nitric oxide (NO), which may cause axon damage in MS (Werner et al., 2001; Smith and Lassmann, 2002).

In the clinical setting MRI atrophy represents a reliable hallmark of neurodegeneration, and well correlates with progression of disability (Zivadinov et al., 2002). Moreover, recent investigations demonstrated that high levels of neurofilaments in CSF of MS patients strongly correlated with clinical disability suggesting that this molecules could be a good indirect indicator of axonal damage (Licke et., 1998).

1.6. Grey matter pathology

MS has been traditionally considered a disease of the WM of the CNS characterized by multifocal inflammatory lesions. However in the last 15 years it has become increasingly accepted, thanks to neuropathological and neuroimaging studies, that cerebral grey matter (GM) damage in MS is evident since early MS stages (De Stefano et al.,2003; Chard et al., 2009; Lucchinetti et al., 2011) and provides the best correlate of the rate of clinical progression (Reynolds et al., 2011; Calabrese et al., 2013) .

Grey matter damage is characterized by an extensive involvement of cortical and deep grey matter (GM) in terms of focal demyelinating lesions, “diffuse” tissue abnormalities and irreversible tissue loss (i.e., atrophy).

1.7. Grey matter lesions

Despite early reports, GM involvement has only recently received attention because of its substantial contribution to clinical disability from early disease stage. However, already in 1962, Brownell and Hughes reported that 26% of the MS lesion affected GM in or around the cortical and subcortical GM (Brownell and Hughes, 1962). Remarkable, Gilmore and coll. showed recently that the affected GM was larger than that of the WM, in particular in the spinal cord and cerebellum (Gilmore et al., 2009).

1.7.1. Classification of cortical lesions

Based on immunohistochemical detection of myelin (Peterson et al., 2001), three distinct lesion types have been described within the cortex. This classification, currently widely used, identified:

- type 1 lesions extend across both WM and GM and are often called leucocortical lesions (Figure 3.A). These lesions do not extend to the surface of the brain.
- type 2 lesions are contained within the cerebral cortex grey matter and often occur around a blood vessel (Figure 3.B)

- type 3 lesions are subpial and affect the largest cortical area (Figure 3.C)

In particular type 3 lesions are the most represented, up to 67% of the cortical demyelinated area (Bo et al., 2003; Magliozzi et al., 2007) and are often affecting several adjacent gyri. Other type 3 lesions were wedge-shaped with the base at the surface of the brain.

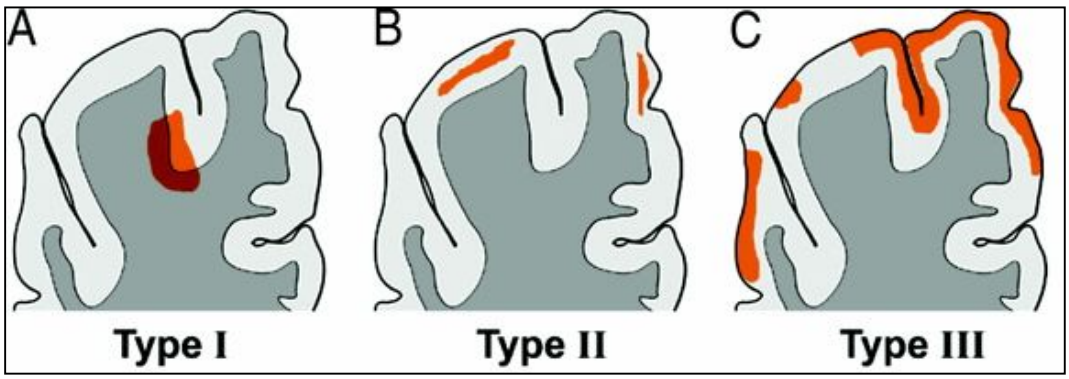

Figure 3. Cortical lesion classification. The three types of cortical lesions described by Peterson and colleagues are shown: 1.A type I (leukocortical) developing between WM and GM; 1.B type II (purely intracortical); 1.C type III (subpial) expanding from the pia mater to the internal cortical layers (Adapted from Dutta and Trapp, 2007).

Also at early stages of the disease, the 37% of biopsy samples from MS patients revealed a clear evidence of cortical demyelination largely characterized by CL type 3.

1.7.2. Grey matter immunopathology

GM lesions are characterized by a lack of substantial inflammatory infiltrates, complement deposition and blood brain barrier damage; this aspect led to the suggestion that the mechanisms underlying GM and WM pathology differ substantially (Bo et al., 2003; Brink et al., 2005; Van Horssen et al., 2007) and that activated microglia are the dominant effector cell population causing GM damage (Peterson et al., 2001; Vercellino et al., 2007).

Despite reduced cortical inflammation, the cortical GM seems to be affected also by myelinotoxic molecules that circulate within the Virchow Robin space resulting in perivenular demyelination, and within the CSF determining subpial

and periventricular demyelination (Ransohoff et al., 2003). In the last decade, several post-mortem studies have revealed that the extent of meningeal inflammation associates positively with subpial cortical demyelination, suggesting that chronic immune activity in the subarachnoid compartment plays a key role in mediating damage in the adjacent cortical GM (Magliozzi et al., Brain 2007; Howell et al., 2011; Choi et al., 2012; Lucchinetti et al., 2011). Factors released by immune cells circulating in the CSF and/or colonizing the subarachnoid space may diffuse across the pial membrane, inducing a gradient of glia and neuronal pathological alterations, directly and/or indirectly by activating microglia (Magliozzi et al., 2010). The role of meningeal inflammation in cortical pathology is evident since the early phase of the disease (Lucchinetti et al., 2011; Calabrese et al., 2015; Gardener et al., 2013). Noteworthy, in the same study, Lucchinetti and coll. observed that active cortical demyelination and inflammation is frequent in early RR phase.

Besides, deep grey matter is different affected, suggesting that grey matter pathology in MS appears to be regionally variable and reflecting the indirect role of specific cells (i.e. interneurons).

1.7.3. Neuronal damage in cortical lesions

Histopathological studies support the hypothesis that neuron injury occurs in cortical lesion and is characterized by neurofilament protein positive swellings and consequent disruption of normal cellular transport, and by neurite transection emphasized by terminal end bulbs (Peterson et al., 2001). The correlation between neuritic transection and microglia activation suggests that dendrites and demyelinated axons are vulnerable and strictly associated to microglial activation in cortical lesions (Trapp et al., 1998; Dutta and Trapp, 2007).

In healthy conditions microglia are the resident immune cells of the CNS, exist in resting state and provide surveillance of the cerebral microenvironment. Thanks to their versatile functional state, microglia are involved in phagocytosis of

apoptotic cells, antigen presentation (APC), production of different types of free radicals, expression of cytokines and chemokines, and production of growth factors and prostanoids (Aloisi, 2001; Mittelbronn et al., 2001). Microglia contribute to neuroprotection and neurodegeneration through release of anti-inflammatory and pro-inflammatory molecules.

Besides, pro-inflammatory and cytotoxic molecules as well as cell contact-dependent mechanisms of T cell-mediated damage, may lead to microglia and/or macrophage activation and oligodendrocyte injury (Calabrese et al., 2015).

Moreover microglia seems to play an important role because it is correlated with focal loss of the glutamate transporters (EAAT1 and EAAT2); indeed alteration of glutamate reuptake found in MS cortical lesion in the presence of activated microglia could be associated with signs of neuronal and synaptic damage suggestive of excitotoxicity (Vercellino et al., 2007).

In addition to their role in immune surveillance and phagocytosis, activated microglia can play a neuroprotective role and enhance nerve repair by physically removing synaptic input with a process, known as “synaptic stripping” (Graeber et al., 1993) which promotes survival of neurons (Hardingham and Bading, 2003).

1.7.4. Cortical lesions and physical and cognitive impairment in MS

Cognitive functions are affected in approximately 45-65% of MS patients (Rao et al., 1995). MRI studies have described a significantly greater cortical/subcortical disease burden in patients with cognitive impairment (Rovaris et al.,2000). Even at the early disease stages, they confirmed the major role of cortical damage in determining cognitive disability; in this case GM is characterized by more extensive cortical volume loss in frontal temporal and parietal regions (Amato et al. 2007). Noteworthy cortical lesions seen in early MS are usually highly inflammatory and correlate with cognitive impairment (Geurts and Barkhof 2008; Lucchinetti et al., 2011).

Besides, the use of myelin immunohistochemistry and new MRI acquisition methods have permitted to better quantify the real extent of GM damage and to

reveal the relationship, present from the clinical onset, between GM pathology and physical disability and cognitive dysfunction more convincingly than WM damage (Pirko et al., 2007; Popescu et al., 2012; Lucchinetti et al., 2011; Geurts JJG et al. 2012). In a MRI study, Calabrese and coll. detected CLs, by Double Inversion Recovery (DIR), in the majority (64%) of RRMS and (73%) SPMS patients and observed that patients with higher CLs showed a higher Expanded Disease Status Scale (EDSS) score (Calabrese et al., 2007). Also MRI longitudinal studies revealed how patients with higher increase in CLs number showed a clinical worsening at follow-up (Calabrese et al., 2010; Roosendal et al., 2009).

Remarkably, the presence of CLs is associated with higher level of intrathecal synthesis of Ig (IgG and IgM), and this association correlates with highest risk of worse clinical evolution after three years (Calabrese et al. 2012).

1.8. Diffuse cortical pathology

Several neuropathological studies revealed an extensive and irreversible "neurodegenerative" process involving grey matter (Pirko et al., 2007; Howell et al 2011) characterized by GM demyelination and neuronal damage/loss which impair cortical function (Peterson et al., 2001). GM involvement differs accordingly to different disease stages. Wegner and coll. reported that also in the normal appearing cortex a diffuse reduction of cortical thickness by 10% (Wegner et al. 2006), and significant neocortical and glial degeneration.

In-vivo grey matter atrophy measures reflect combinations of demyelination, neurite transection (Peterson et al., 2001) and reduced synapse or neocortical and glial densities (Dutta et al., 2011; Wegner et al. 2006). Mitochondrial dysfunction has been proposed as one of the main mechanisms of diffuse injury, as the result of mitochondrial DNA deletions, induced by inflammation, in neurons located in layer VI and immediate subcortical white matter (WM) irrespective of lesions (Campbell et al., 2011). Currently, modern neuroimaging techniques and available software have been used to correlate measure of GM atrophy with clinical disability and cognitive impairment (Geurts et al., 2012)

Finally, the appearance of new CLs in the early phase of the diseases is one of the best correlate of the reduction of the cortical thickness (Cth), a measure of atrophy, suggesting that, at least at the beginning of the disease, the early focal cortical pathology plays a relevant role in the development of brain atrophy (Calabrese et al., 2015)

1.9.Grey matter pathology and MRI

The improvement of MRI technology, having higher sensitivity for the GM, has allowed, in the last 10-15 years, to better describe GM pathology in vivo from the early disease phase even before the appearance of WM lesions.

Numerous studies and new advanced non-conventional MRI techniques have improved the detection methods for CLs, in particular Double Inversion Recovery (DIR) improves the sensitivity of the detection of CLs (Geurts et al., 2005; Pouwels et al., 2006), in particular with 3 Tesla MR (Simon et al., 2010). Calabrese and coll. revealed in a longitudinal study of three years how an accumulation of CLs number detected with DIR correlate with disability progression (Calabrese et al., 2010).

However DIR sequences showed low sensitivity in detecting CLs, as comparative histopathological studies have demonstrated that low sensitivity depends only on the size of CLs (Seewann et al., 2011). An important improvement has been recently achieved combining DIR sequence with phase sensitive inversion recovery PSIR (Favaretto et al. , 2015). The authors describe the high ability of PSIR in disclosing and classifying CL, in particular the presence of CLs in CIS patients further pointed out the relevance of cortical pathology in MS

Besides, the availability of not-conventional MRI sequences permitted to detect, also in the early phase, cortical metabolite changes (reduced levels of N-acetyl aspartate) (Kapeller et al., 2001; Tiberio et al., 2006; Chard DT et al., 2002) and atrophy (Audoin B et al., 2006). Magnetization transfer allowed the detection of cortical abnormalities (severe axonal loss and swelling) that were related to the disability of MS patients (Fisniku et al., 2009; Fisher et al., 2007).

Noteworthy, dedicated software (as the Freesurfer software, http://surfer.nmr.mgh.harvard.edu/, or SIENAX or FSL) applied on High Resolution MRI images, allowed the assessment of global and regional GM atrophy in RRMS and SPMS patients (Davies et al., 2004, Sastre-Garriga et al., 2005). In 2003, Sailer and coll. demonstrated by means of Freesurfer, that a cortical thinning is present in the frontal and temporal lobes of MS patients (Sailer et al., 2003). Soon after, two separate studies described that the rate of atrophy progression was already present in the early phase of MS (De Stefano et al., 2003; Chard et., al 2004) suggesting once again that the neurodegeneration is a process that initiate at the disease onset.

Calabrese and coll. confirmed in a cohort of 115 MS patients that the real extend of GM atrophy (Calabrese et al., 2007) involves more cortical areas and is already present in the early MS.

It seems that diffuse GM atrophy is only partially related to the white matter damage visible by MRI (Chard et al., 2002) and the correlation between GM volume and white matter damage, revealed by diffusion tensor imaging (DTI), appears only moderate (Sbardella et al., 2013).

Finally regional analysis of GM atrophy and GM lesion maps permitted to detect specific imaging distribution both of CL distribution and regional thinning (Calabrese et al., 2010), however the correlation between focal and diffuse damage seems to be only moderate and more evident in the early phase of the disease (Calabrese et al., 2015).

All these studies suggest that in MS grey matter pathology may be a major determinant of longer-term outcomes in MS and that all together not-conventional MRI sequences represent an additional useful paraclinical tool to monitor MS evolution and for a better stratification of the patients for inclusion in clinical studies.

1.10. Meningeal inflammation and B-cell response in MS

A growing number of neuropathological studies (Prineas et al., 1979, Serafini et al, 2004; Magliozzi et al, 2007; Frischer et al., 2009) have emphasized the contribution of inflammatory process occurring in the meningeal compartment of MS patients to cortical pathology, also at early disease stages (Lucchinetti et al., 2011). Indeed, they revealed that the extent of meningeal inflammation associates positively with subpial cortical demyelination and disease progression (Magliozzi et al., 2007; Howell et al., 2011; Choi et al., 2012).

Magliozzi and coll. have shown the presence of abundant meningeal inflammation with the formation of ectopic follicle-like structures, also named tertiary lymphoid organs (TLO) in a substantial proportion of secondary progressive MS (SPMS) cases (Serafini et al., 2004; Magliozzi et al., 2007) associated with the more severe clinical features. These structures are formed by infiltrating B-cells, T-cells, dendritic cells and plasma cells, which become organized anatomically and functionally as in secondary lymphoid organs (SLO), leading to de novo formation of tertiary lymphoid organs (TLO). TLO have been previously found also in other autoimmune disorders, such as rheumatoid arthritis, lupus erythematosus systemic, suggesting that they can represent niches of chronic inflammation persisting in the target organs (Aloisi and Pujol-Borell, 2006).

In MS the presence of lymphoid follicle-like structures containing B cells and a reticulum of CXCL13+ and FDC-M1+ follicular dendritic cells within the meninges suggest that under chronic inflammatory conditions, the meningeal compartment is the site where ectopic lymphoid follicles preferentially. Such abnormal formations may contribute to pathogenic process through local amplification of autoimmune response which results compartmentalized in CNS and therefore chronic immune activity perpetuated within the subarachnoid compartment may play a key role in mediating damage in the adjacent cerebral cortex of SP and PPMS affected patients.

In particular, it may be suggested that the local persistence of inflammatory cells, such as B- and T-lymphocytes and macrophages, could be responsible for the release of inflammatory mediators (cytokines and chemokines) and proteolytic enzymes that can accumulate in the subarachnoid space and diffuse via CSF circulation. These inflammatory mediators can easily cross the pial surface and mediate directly the tissue damage of the bathed cortical GM, or indirectly by activation/alteration of the cortical microglia and astrocyte population (Calabrese et al., 2015). This is supported by the observation that high level of meningeal inflammation and cortical pathology is accompanied by increased gene expression of inflammatory cytokines, such as Tumor Necrosis Factor (TNF) and Interferon gamma (IFNg), in the meninges of post-mortem SPMS cases and elevated levels of the same proteins in the corresponding CSF (Gardner et al., 2013), compared to healthy controls.

Sellebjerg and coll. have also described an increased concentration in vivo of CXCL13 (a B-cell chemokine), which supports a role for the recruitment of B cells (Sellebjerg et al., 2009).

Molecular analysis of B-cells and plasma cells isolated from CNS lesions revealed that the observed intrathecal humoral response is antigen-driven and B-cell clonotypes persist over time in CNS compartment (Baranzini et al., 1999; Colombo et al., 2000; Owens et al., 2003; Colombo et al., 2003). However it has been not yet elucidated if antibodies, even if present from early stages (Genain et al., 1999), are only an epiphenomenon of tissue damage (Sospedra and Martin, 2005; Owens et al., 2006, Hauser, 2015). Neuropathology studies indicate that antibody-mediated demyelination is one of the predominant pathogenetic mechanisms involved in white matter lesion formation in a substantial proportion of MS patients (Lucchinetti et al., 2000).

Noteworthy the presence of elevated CNS levels of immunoglobulins (Ig) and the presence of oligoclonal IgG in the cerebrospinal fluid (CSF) are the most consistent immunological abnormalities characterizing MS compared to other inflammatory neurodegenerative diseases (Petzold et al., 2013). Such intrathecal

immunoglobulin synthesis is thought to be sustained by long-lived plasma cells recruited to or differentiating within the CNS (Prineas and Wright, 1978).

B-cells play also a role in down-regulating immune reaction and promoting repair through the release of neurotrophic factors and pleotropic cytokines (as IL10, which seems to have a protective role). Understanding how abnormal regulation of the B-cell response contributes to the pathogenesis of MS will be fundamental for the identification of specific B-cell therapies, targeting not only the antibody production but also the regulatory and antigen presenting activities of B-cells. Up to now three therapeutic monoclonal antibodies targeting CD20-positive B cells (rituximab, ocrelizumab and ofatumumab) have been investigated providing evidence for beneficial effect of B cell depleting therapies in MS. (Hauser et al., 2008; Sorensen and Blinkenberg, 2016).

1.11. CSF analysis and multiple sclerosis

CSF recent findings provided evidence for a direct correlation between the immune cells present in the CSF and CNS, especially related clonally expanded B cells (Lovato et al., 2011), which together with plasmablasts are involved in IgG secretion (Obermeier et al., 2011).

The presence of oligoclonal bands (OCBs) and the increased IgG-index (i.e. >0,7) are important hallmark of MS in the CSF of affected patients (Kabatea et al. 1948) suggesting an intrathecal chronic inflammatory process. However, given a low specificity, CSF testing alone has only a supportive role in MS diagnosis; recently Lublin and coll. emphasized that currently no biological CSF marker reliably differentiates between MS disease subtypes, and they also focalized the attention on the need to exploit the potential of CSF biological markers (Lublin et al. 2014).

Moreover, the CSF could provide important issue in order to understand the immunopathology of MS; indeed the presence of IgG suggests a humoral immunity partially responsible for the development of focal cortical damage

observed in GM. Several studies have revealed the association between the degree of meningeal inflammation together with the presence of B-cell follicles, and the gradient of neuronal and axonal loss (Magliozzi et al., 2010; Gardner et al., 2013), suggesting that the humoral cytotoxicity is a potential responsible for the cortical demyelination, even before white matter lesions have formed (Popescu et al. 2011).

Recently, the B-cell attracting chemokine C-X-C motif chemokine 13 (CXCL13) and the extracellular matrix-degrading enzyme metalloprotease-9 (MMP-9) have been proposed as possible specific CSF biomarkers (Stangel et., al 2013) which can suggest a possible connection between meningeal inflammation and cortical damage (Stangel et., al 2013). Conversely myelin basic protein, neurofilament light chain subunit and chitinase-3-like-1 (Teunissen et al., 2012; Modvig et al., 2015), have been identified as marker of tissue damage and predictor of neurological disability.

Gene-expression studies in the CSF are limited due to the small number of cells collected. These studies provided evidence of increased expression of genes involved in T-, NK-, and B-cell function in CSF cells (Brynedal et al. 2010; Jernås et al., 2013).

Recent efforts in improving diagnosis and management of MS and providing reliable biological markers have been carried out. Veroni and coll. performed a gene expression analyses in paired cerebrospinal fluid (CSF) and peripheral blood mononuclear cells (PBMC) from MS patients with a reliable improved sensitivity pre-amplification real-time reverse-transcription polymerase chain reaction (RT-PCR). They identified two factor grouping genes: factor 1 was related to a type-1 IFN response cytotoxic/Th1 T-cell activation, and inflammation; factor 2 was likely related to an inhibitory circuit involving immune regulatory cells (CD68, IL-10, MHC class II, and CD4) (Veroni et al., 2015).

Komori and coll. identified, supported by machine learning algorithms, that soluble CD27 is the best single biomarker of active intrathecal inflammation in comparison to IgG Index and OCB and it could be considered as an hallmark of a

T-cell mediated inflammation (Komori et al., 2015). CD27 molecule is selectively higher in the progressive MS subtype indicating that T-cell infiltration of CNS is represented in progressive MS, in which it may play a pathogenic role. This is in contrast with traditionally and currently accepted notions that inflammation no longer play a pathogenic role in chronic forms.

2. Hypothesis and aims

We suggest that the rate at which cortical pathology accumulates and the level of intrathecal inflammation, associated to inflammatory activity in the CSF, might provide an indication of severity of disease process and might predict/monitor the evolution of the disease and an early conversion to the progressive phase of the disease.

Therefore, the characterization of a panel of biomarkers and paraclinical tools able to identify those patients having more severe cortical damage might help the clinicians in choosing more aggressive therapeutic strategies at the earliest phase of the disease when the disease course is still modifiable.

The aim of the study is to combine CSF molecular and protein analyses with advanced MRI imaging techniques, able to better identify cortical grey matter demyelination, in order to characterize potential early biomarkers of GM pathology and disease progression with prognostic and predictive value and to obtain information on the biological and immunological mechanism that link the inflammatory process of the GM and the progressive neurodegeneration. The information obtained from these techniques is also investigated as possible indicator of clinical recovery.

It is therefore that, in this study, we have performed protein analysis and gene expression analysis of CSF samples from MS patients, in the very early phase of the disease and therapy naïve, who underwent a complete diagnostic work up comprehensive of non-conventional MRI sequences in the MS Center in Padova. Finally this combined approach may identify patients with a more severe prognosis who could benefit from an earlier and more effective long-term therapeutic strategy aimed at improving their quality of life and reducing the disease-related indirect costs.

3. Methods

3.1. Study population

First MS cohort: Thirty-five patients presenting with symptoms/signs suggestive of MS, were recruited between February 2009 and September 2011 at the MS Centre in Padova. At the time of CSF sampling none of the patients was treated with immunomodulatory or immunosuppressive therapies. Four patients were treated with high doses of steroids in the month prior to lumbar puncture. Four subjects presenting with symptoms and MRI compatible with a diagnosis of cerebrovascular disease and a woman affected by subacute combined sclerosis (in remission phase) were recruited as non-inflammatory controls (Table 3). In these subjects other neurological (inflammatory and/or degenerative) diseases were definitely excluded. The individuals were re-evaluated retrospectively according to McDonald Criteria (Polman et al., 2011): 22 were CIS and 13 RRMS (at early phase of the disease).

	n.	F/M	Age (y)	DD (m)	EDSS	EDSS 2	CIS/ RRMS	DD at LP	FU (m)	PI
MS patients	35	21/14	34.97 ±10.14	4±2 m (0-18)	1.5±0.5 (1.0-2.5)	1.5±0.5 (1.0-6.5)	22/13	4±4.5	51.2 (12-76)	0.68±0.3 (-0.92-1.12)
Control subjects	5	4/1	52±6	N/A	N/A		N/A	N/A	N/A	N/A

Table 3. Demographic and clinical data of the first MS cohort. Data are reported as mean ± standard deviation (range). For Expanded Disability Status Scale (EDSS), median and (range) are provided. Abbreviation: y year; m month; DD disease duration; FU follow up; PI Progression Index (PI) LP lumbar puncture; CIS clinically isolated syndrome; RRMS relapsing remitting multiple sclerosis

Second MS cohort (independent cohort): From January 2014 to May 2015, 31 MS patients and 13 controls (other non-inflammatory neurological diseases - ONND), at their disease onset and without any therapy, have undergone a complete diagnostic work-up, comprehensive of a lumbar puncture and a MRI (the

complete MRI protocol was set up in collaboration with the Neuroradiology Unit of the University Hospital of Padova); time interval between lumbar puncture and MRI was 18.39±10.7 days. According to McDonald Criteria (Polman et al., 2011), 12 individuals were CIS and 19 were RRMS (at early phase of the disease, disease duration less than five years)

	n.	F/M	Age at LP (y)	DD (m)	EDSS	Radiological/clinical disease activity	CIS/ RRMS
MS patients	31	18/13	36.19±9.54	17.0±28.3 (0-61)	1.52±1.0 (1.0-3.5)	11/31	12/19
Control subjects	13	11/2	42.92±10.31	N/A	N/A	N/A	N/A

Table 4. Demographic and clinical data of the second MS cohort. Data are reported as mean ± standard deviation (range). For Expanded Disability Status Scale (EDSS), median and (range) are provided. Abbreviation: y year; m month; LP lumbar puncture; DD disease duration, CIS clinically isolated syndrome; RRMS relapsing remitting multiple sclerosis.

Clinical evaluation: the degree of neurological disability has been scored using the Expanded Disability Status Scale (EDSS). Clinical and/or radiological disease activity was present in 11 out of the examined 31 patients. None of the patient was previously treated with immunomodulant or immunosuppressive, while administration of high doses of steroids in the month prior to lumbar puncture was considered an exclusion criterion. Clinical and demographical data are summarized in Table 4.

The study was approved by the local Ethics Committee (CE per la Sperimentazione, Azienda Ospedaliera – Università degli Studi di Padova). All patients gave written informed consent

3.2. MRI analysis

Morphological MRI Image Acquisition (first cohort)

Brain MRI was performed in all the patients at study entry. Images were acquired using a 1.5 T scanner (Achieva, Philips Medical Systems, Best, The Netherlands)

with 33 mT/m power gradient and a 16-channel head coil. No major scanner hardware upgrades occurred during the study, and bimonthly quality-assurance sessions assured measurement stability.

The following images were acquired for each subject:

Cerebral and spinal MRI sequences

- DIR: repetition time (TR)=15,631 ms; echo time (TE)=25 ms; inversion time (TI)=3400 ms; delay=325 ms; echo train length (ETL)=17; 50 contiguous axial slices with thickness=3 mm; matrix size=130 x 256; and field of view (FOV)=250 x 200 mm2;
- Fast fluid attenuated inversion recovery (FLAIR): TR=10,000 ms; TE=120 ms; TI=2500 ms; ETL=23; 50 contiguous axial slices with thickness=3.0 mm; matrix size=172 x 288; and FOV=250 x 200 mm2;
- Three-dimensional (3D) fast field echo (FFE): TR=25 ms; TE=4.6 ms; 120 contiguous axial slices with the off-centre positioned on zero with thickness=1.2 mm; flip angle=30°; matrix size=256 x 256; and FOV=250 x 250 mm2;
- Cervical and dorsal cord short time inversion recovery: TR= 2500 msec, TE= 60 msec, inversion time= 170 msec; 15 sagittal slices, slice thickness= 3.5mm, gap= 0 mm, matrix size= 272 x 512, filed of view= 250x250 mm2)

<u>Morphological MRI Image Acquisition (second cohort)</u>

MRI images have been be acquired with a 3 Tesla Achieva TX system (Philips healthcare, Best, The Netherlands) with a 64-channel coil. No software upgrade was performed during the study period and a weekly check scanner ensured the stability of the parameters acquired.

The following set of images has been obtained from each subject:

Cerebral MRI sequences

- Three-dimensional T1 turbo field echo (3D T1 TFE): n° slices (sagittal) 180. Duration: 5 min. Flip angle 8°. TR 8 msec. TE 4 msec. Thickness: 1 mm.

- T1 Inversion Recovery Turbo Spin Echo (T1 IR TSE) with gadolinium (gad): n° slices 34. Duration: 3 min. Flip angle 90°. TR 2000 msec. TI 800 msec. TE 20 msec. Thickness: 4 mm.
- T2 Turbo Spin Echo (T2 TSE): n° slices 34. Duration 4 min. Flip angle 90°. TR 2700 msec. TE 80 msec.
- Diffusion Weighted Imaging (DWI): n° slices 52. Duration 1.30 min. Flip angle 90°. TR 3700 msec. TE 100 msec. Thickness 4 mm.
- Apparent diffusion coefficient (ADC): n° slices 26. Duration 1.30 min. Flip angle 90°. TR 3700 msec. TE 100 msec. Thickness 4 mm.
- Three-dimensional Double Inversion Recovery (DIR 3D): n° slices (sagittal) 300. Duration 7 min. Flip angle 90°. TR 5500 msec. TE 300 msec. TI 2550 msec. Thickness 1 mm.
- Three-dimensional Fluid Attenuated Inversion Recovery (FLAIR 3D): n° slices (sagittal) 365. Duration 7 min. Flip angle: 90°. TR 4800 msec. TE 300 msec. TI 1650 msec. Thickness 1 mm.

Spinal MRI sequences

- T1_TSE: n° slices 15 (sagittal), Duration 5 min. Flip angle: 80°. TR 8 msec. TE 4 msec. Thiclness: 3 mm.
- T2_TSE: n° slices 15 (sagittal). Duration 5 min. Flip angle 90°. TR 4000 msec. TE 100 msec. Thickness 3mm.
- Short tau inversion recovery (STIR): n° slices 15 (sagittal). Duration 4 min. Flip angle 90°. TR 5000 msec. TE 60 msec. TI 210 msec. Thickness 3 mm.
- MFFE (sagittal): n° slices 15. Duration 4 min. Flip angle 28°. TR 700 msec. TE 8 msec. Thickness 3 mm

3.3. MRI image analysis

Cortical lesions (CL) and white matter (WM) lesions identification (first cohort):

All images were assessed by two experienced observers who were blinded to the patients' identity. On DIR images, particular attention was devoted to identifying artifacts, and CLs were defined as those lesions confined to the cortical ribbon

and not involving the underlying subcortical WM (Calabrese et al., 2007). However, considering the resolution of DIR images, the possibility that some CLs extend into the juxtacortical WM cannot be excluded.

The number of CLs was assessed on DIR images, and CL volumes were calculated using a semiautomatic thresholding technique based on the Fuzzy C-mean algorithm, (Pham et al., 1999 and Pham et al., 2000) included in software developed at the National Institutes of Health's Medical Images Processing, Analysis, and Visualization (http://mipav.cit.nih.gov). The same procedure was applied to FLAIR images, to identify and segment WM lesions, thus obtaining a T2 hyperintense WM lesion volume (T2WMLV) (Calabrese et al., 2010).

The number of Gd-enhancing lesions was determined on post-contrast Gd-enhanced T1-weighted images.

Global and regional cortical thickness evaluation (first cohort):

Global and regional cortical thickness (CTh - mean of right and left hemispheres) was performed on the volumetric FFE datasets by means of the longitudinal stream included in the FreeSurfer image analysis suite (release v5.1.0), available online (http://surfer.nmr.mgh.harvard.edu/) (Fischl et al., 2000). All images were accurately controlled for errors/artifacts by an experienced neurologist.

Cortical lesions (CL) and white matter (WM) lesions identification (second cohort):

On DIR images, CLs have been defined as those lesions confined to the cortical ribbon and not involving the underlying subcortical WM.

WM and GM lesion volume has been computed respectively on FLAIR and DIR images by means of a manual volume of interest (VOI) delineation of hyperintense regions. The lesion delineation has been performed by two experienced Neurologists, blinded to clinical evaluation, using the MRIcron (v. 2013) software package.

The number of Gd-enhancing lesions was determined on post-contrast Gd-enhanced T1-weighted images.

Global and regional cortical thickness evaluation (second cohort):

Cortical reconstruction and volumetric segmentation has been performed on data sets of anatomical 3D-T1 with the Freesurfer image analysis suite (release v. 5.3), which is documented and freely available for download. To avoid a possible bias in image segmentation due to the presence of WM lesions, such lesions were aligned to MNI152 standard-space (http://www.bic.mni.mcgill.ca/ServicesAtlases/ICBM152NLin6) and the T1-weighted volume (also in MNI152 standard-space) was refilled with values randomly extracted from a Gaussian distribution with the same mean and SD of normal-appearing tissue using the FSL suite (release v 5.0.6)

3.4. CSF analysis

First cohort: CSF was collected by non traumatic lumbar puncture between 8.00 and 9.00 a.m.. Routine examination on paired CSF and serum specimens included: cell count and characterization (lymphocytes/monocytes), albumin CSF/serum ratio to estimate the integrity of the blood-brain barrier, calculation of intrathecal IgG synthesis by means of quantitative formulae (IgG Index, IgG HypFunct), and demonstration of IgG oligoclonal bands (OCBs) by isoelectricfocusing and specific IgG immunofixation. After immediate centrifugation, the CSF cell pellet was separately stored at -80 °C , while the cell-free CSF supernatant was aliquoted and stored at -20°C until cytokine determination.

Second cohort: As the previous method of collecting and storing the CSF samples has shown critical steps to be improved, the methodology for CSF storing and for

RNA extraction from the cell fraction has been optimized and applied to the second independent cohort. In particular, for each individual the CSF sample have been collected by lumbar puncture and, within 30-60 min, centrifuged to separate the cellular component from cell-free supernatant. CSF supernatant has been stored at -80 °C for protein-array analysis, while the cell pellet has been separately stored at -80 °C for RNA extraction and analysis.

3.4.1. Protein analysis

On the CSF samples (first and second cohort), using the Bio-Plex System (Biorad - Bio-Plex Pro Human Chemokine panel 40-plex) in collaboration with Dr. F. Facchiano and Dr. S. Rossi at the Department of Hematology of ISS, we performed a protein immune-assay analysis to assess the presence and levels of 40 inflammatory molecules including the most important cytokines and chemokines (Table 5). The Bio-Plex multiplex system, enables the detection and quantification of multiple analytes (proteins and peptides, or nucleic acids) in a single 50 µL sample volume. In particular, Bio-Plex assays are bead-based assays that can be performed in a mixed array (multiplexed) which resembles the sandwich analysis technique: capture antibodies direct against the desired biomarker are covalently coupled to the beads; coupled beads react with the sample containing the biomarker of interest; after a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addiction of streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin serves as a fluorescent indicator, or reporter. The reader, a flow-cytometry based instrument (Luminex-based), combines two lasers, fluidics, and real-time digital signal processing to distinguish up to 100 different sets of color-coded polystyrene beads, each bearing a different assay, allowing therefore both qualitative and quantitative analysis of the analytes. Afterwards raw data were normalized according to the CSF protein concentration.

• 6Ckine / CCL21	• GCP-2 / CXCL6	• IL-4	• MCP-2 / CCL8	• MIP-3α / CCL20
• BCA-1 / CXCL13	• GM-CSF	• IL-6	• MCP-3 / CCL7	• MIP-3β / CCL19
• CTACK / CCL27	• Gro-α / CXCL1	• IL-8 / CXCL8	• MCP-4 / CCL13	• MPIF-1 / CCL23
• ENA-78 / CXCL5	• Gro-β / CXCL2	• IL-10	• MDC / CCL22	• SCYB16 / CXCL16
• Eotaxin / CCL11	• I-309 / CCL1	• IL-16	• MIF	• SDF-1α+β / CXCL12
• Eotaxin-2 / CCL24	• IFN-Ɣ	• IP-10 / CXCL10	• MIG / CXCL9	
• Eotaxin-3 / CCL26	• IL-1β	• I-TAC / CXCL11	• MIP-1α / CCL3	• TARC / CCL17
• Fractalkine / CX3CL1	• IL-2	• MCP-1 / CCL2	• MIP-1δ / CCL15	• TECK / CCL25
				• TNF-α

Table 5. Biorad - Bio-Plex Pro Human Chemokine panel 40-plex

3.4.2. Gene expression analysis

Pre-Amplification real time RT-PCR (second cohort)

Total RNA was extracted from CSF cells (median = 43987,84± 81001,69, range 1800 406000) using the AMBION RNAqueous micro kit (LifeTechnologies, Grand Island, NY), according to the manufacturer's instructions, including genomic DNA digestion. Because of the very low and highly variable RNA yield from CSF cells, the entire volume (16 µl) of RNA extracted from each CSF sample was reverse transcribed. Reverse-transcription (RT) was performed using the High Capacity Reverse Transcription kit with RNase inhibitor (LifeTechnologies). The resulting cDNA was diluted to a final volume of 50 µl and splitted into four 12,5 µl aliquots. To increase the number of targeted copies, each cDNA aliquot was amplified for the specific gene assays by pre-amplification reaction (14 cycles) using the TaqMan PreAmp Master Mix (LifeTechnologies) and pooled gene-specific primers, at the reaction conditions indicated by manufacturer. Inventoried TaqMan gene expression assays were used to study cellular genes, respectively (Table 6). Cellular gene assays were pre-amplified together with the selected house-keeping gene GAPDH. The pre-amplification product was diluted 1:5 up to 250 µl in H2O and 4 µl of this dilution were used as template for a single real-time PCR analysis. Quantitative PCR experiments were performed in triplicate with the same inventoried TaqMan assays used in the pre-amplification step (250 nM probe and 900 nM each primer), using the 7500 Real-Time PCR System (LifeTechnologies) for cellular genes. Thermocycling parameters were 50°C (2 min), 95°C (10 min), followed by 40 cycles of 95°C (15sec), 60°C (1 min) for cellular genes. The results of gene expression analysis were obtained as Ct values

(Ct = threshold cycle of PCR at which the amplified product is detected). The ΔCt is the difference in Ct values derived from the gene of interest and the reference gene GAPDH; the factor 2^-ΔCt was used to express the ratio between the gene of interest and the internal reference gene. To rule out cross-contamination of reagents and primers, all RT, pre-amplification and real-time PCR experiments included an NTC sample, containing all the components of each reaction except for the template. Considering that 12 µl of pre-amplified cDNA were analysed for each transcript and that the available volume of each pre-amplified aliquot was 250 µl, we were able to analyse in triplicate up to 20 transcripts.

To check that all amplicons were amplified uniformly without bias, we performed pre-amplification uniformity experiments using non-limiting cDNA from a human non pathological pulmonary hilar lymph node (obtained from Dr. Egidio Stigliano, Institute of Pathological Anatomy, Policlinico A. Gemelli, Rome, Italy), as control for cellular genes.

GAPDH	CD8	Tbet	CD4	FoxP3
CD138	CD68	CD20	CD14	
IFN	TNF	LTa	GM-CSF	
CXCL13	IL10	IL16	CXCL10	CCL8
CCL3	CCL22			

Table 6. Gene expression panel

3.5. Statistical analyses

We analyzed demographic, clinical, MRI, CSF-protein array and gene expression data by univariate analyses for comparison between groups of patients (for continuous variables: Mann-Whitney test for non-parametric distribution or Student's t test for parametric distribution, and by Fisher's exact probability test for categorical variable).

Pathway KEGG analysis was performed in order to identify pattern of proteins/genes involved in the same biological function.

Correlation between variables was assessed by Spearman's rank correlation coefficient.

All statistical analyses were performed using GraphPad Prism 6 and R, an open source UNIX-based statistical package available at http://www.r-project.org.

4. Results

In the MS Centre of Padova two independent cohorts of patients have been recruited and followed by a detailed neurological and clinical follow-up.

The first cohort of MS patients, composed of 35 patients and 5 controls was recruited retrospectively between February 2009 and September 2011 and analysed.

The second cohort of 31 patients and 13 controls was collected prospectively from January 2014 to May 2015. The patients were at their disease onset or in the very early phase of the disease. No patient was previously treated with immunomodulant agents. Each patient underwent a complete diagnostic work-up comprehensive of clinical evaluation, lumbar puncture and MRI evaluation

Here we provide the results of combined clinical, CSF and MRI investigation.

4.1. Combined MRI / CSF analysis of the first cohort of MS patients

MRI analysis

The extent of GM and WM involvement was quantified in 35 patients. CL number and CL volume were measured on DIR images and were respectively in MS patients: CL number 5,1±6,1 (range 0-21); CL volume 453±615,3 mm^3 (range 0-2500); Global cortical thickness (Cth) measured on 3D FFE sequences was 2,38±0,15 mm (range 2,09-2,62). The observed volume of T2 WM lesions (T2WMLV) on FLAIR images was 1660,7±1922,2 mm^3 (range 0-7600). Median number of spinal cord lesion was 1,8±2,2 (range 0-9) (Table 7).

	CONTROLS (5 patients)	**CIS (22 patients)**	**RR (13 patients)**
Age at LP (yr)	51,8±5,17 (43-56)	33,09±8,83 (16-49)	38,15±11,72 (21-57)
M/F	1/4	10/12	4/9
MRI a/o clinical signs of disease activiy	n/a	11/22	5/13
Disease duration at LP (months)	n/a	2,59±4,37 (0-18)	6±4 (0-11)
Days to 1st relapse [n pts/tot]	n/a	357,42±397,71 (15-1449) [19/22]	330,44±378,86 (44-1162) [9/13]
EDSS (median±mad)	n/a	1,25±0,37 (1-2,5)	1,5±0 (1-2,5)
Follow up (months)	n/a	47,91±18,54 (13-80)	61,85±10,08 (40-76)
Progression Index at FU	n/a	0,08±0,35 (-0,92-1,12)	0,04±0,2 (-0,1-0,65)
Disease form at FU(n/tot)	n/a	3/35	32/35
Time to conversion (days)	n/a	82,16±127,97 (1-549)	n/a
Steroids in the month before LP	n/a	3/22	1/13
Shift to 2nd line therapy (n/tot)	n/a	8/22	4/13
SC.lesion number	0±0 (0-0)	1,95±2,09 (0-6)	1,69±2,43 (0-9)
CL number	0±0 (0-0)	4,59±6,34 (0-21)	5,85±5,87 (0-18)
CL volume (mm^3)	0±0 (0-0)	393,14±604,3 (0-2500)	554,23±645,04 (0-2250)
T2WMLV (mm^3)	-	1658,82±2021,43 (0-7600)	1663,85±1821,46 (200-5800)
Global.CTh (mm)	2,51±0,09 (2,43-2,61)	2,4±0,16 (2,11-2,62)	2,36±0,14 (2,09-2,59)

Table 7. Clinical and MRI data from 1st cohort of patients. Data are expressed in mean ± SD (range), except for EDSS (median±mad). Abbreviations: LP lumbar puncture, yr year, CIS clinically isolated syndrome, RR relapsing remitting, SC spinal cord, CL cortical lesion, WML white matter lesion volume, Cth cortical thickness. Progression Index = (EDSS at FU – EDSS at LP)/yr of follow up.

CSF analysis

By using the Bio-Plex System (Biorad - Bio-Plex Pro Human Chemokine panel 40-plex) we performed a protein analysis of the presence and levels of inflammatory molecules in 35 MS patients compared to 5 controls included in the first cohort of the study.

Preliminary analysis of the levels of cytokines and chemokines released in the CSF of MS cases (CIS and RRMS) and controls are shown in Table 8.

	CONTROLS (5 patients)	**CIS (22 patients)**	**RR (13 patients)**
OCBs pos	0/5	21/22	12/13
CSF Lymphocytes (number/uL)	2,52±1,85 (0,7-5,5)	10,48±14,91 (1,7-70)	10,54±10,69 (0,7-38)
Protein mg/mL	0,54±0,05 (0,47-0,61)	0,43±0,1 (0,29-0,7)	0,46±0,11 (0,32-0,69)
IgGIndex	0,46±0,06 (0,41-0,55)	0,72±0,25 (0,46-1,43)	0,91±0,62 (0,4-2,7)
X6Ckine.CCL21	648,78±226,35 (389,2-973,6)	862,93±219,32 (587,17-1247,37)	720,38±209,54 (340,41-1166,39)
BCA.1.CXCL13	0,31±0,33 (0,02-0,81)	33,04±51,72 (0,05-206,01)	34,05±36,34 (1,09-116,61)
ENA.78.CXCL5	121,27±84,9 (35,39-212,4)	154,91±81,67 (57,15-345,54)	130,37±81,36 (0-343,01)
EOTAXIN.CCL11	9,48±1,84 (7,16-11,66)	9,57±1,71 (6,84-12,86)	8,79±1,91 (5,01-11,53)
EOTAXIN.2.CCL24	21,53±2,92 (18,67-25,98)	30,11±5,74 (19,45-40,9)	28,23±7,32 (14,68-39,81)
EOTAXIN.3.CCL26	7,23±0,71 (6,64-8,43)	11,13±1,89 (7,66-14,44)	10,2±2,59 (6,19-14,41)
Fractalkine.CX3CL1	137,97±31,94 (100,66-177,61)	200,06±54,82 (125,57-289,43)	206,21±73,83 (88,97-321,81)
GCP.2.CXCL6	0,97±0,93 (0-1,89)	1,31±1,49 (0-3,77)	2,06±2,37 (0-6,83)
GM.CSF	66,09±13,75 (44,63-78,57)	100,81±18,8 (60,66-136,88)	94,87±20,54 (55,61-127,03)
Gro.alpha.CXCL1	50,51±5,02 (43,4-54,94)	68,34±13,22 (41,97-89,36)	70,72±31,02 (39,7-155)
Gro.Beta.CXCL2	3,88±2,65 (0-6,53)	9,79±3,58 (4,5-16,03)	8,14±3,58 (0-12,99)
I.309.CCL1	15,75±1,8 (13,6-18,19)	26,86±6,83 (14,48-43,25)	25,39±6,71 (14,98-34,29)
IFN.gamma	5,55±0,46 (4,86-6,09)	5,84±1,39 (3,69-8,55)	5,37±1,64 (1,66-7,95)
IL.1beta	0,42±0,07 (0,32-0,53)	0,39±0,13 (0,26-0,67)	0,44±0,27 (0,03-1,22)
IL.2	2±0,49 (1,62-2,8)	2,15±0,62 (1,22-3,33)	1,98±0,64 (0,95-2,94)
IL.4	13,15±10,62 (1,91-24,83)	7,6±5,62 (1,77-27,86)	5,96±3,77 (1,1-13,88)

IL.6	7,4±1,81 (5,36-9,07)	17,43±21,54 (4,94-97,14)	9,85±4,58 (5,23-20,57)
IL.8.CXCL8	49,44±11,04 (34,47-60,9)	86,11±39,63 (38,29-228,29)	87,74±54,9 (18,82-234,16)
IL.10	6,58±2,01 (4,24-8,81)	8,7±3,05 (4,89-16,07)	7,99±3,21 (0-13,1)
IL.16	62,78±13,13 (49,05-82,24)	47,03±20,96 (23,96-100,92)	46,07±21,15 (14,13-93,81)
IP.10.CXCL10	469,74±185,83 (281-673,72)	1181,3±1301,58 (249,21-6552,26)	982,72±684,15 (266,15-2421,68)
I.TAC.CXCL11	1,85±0,8 (1,04-2,74)	6,12±5,13 (1,61-19,64)	4,89±3,26 (1,03-11,51)
MCP.1.CCL2	791,84±280,74 (580,7-1247,24)	688,55±241,58 (278,03-1461,72)	616,51±244,64 (291,97-1197,19)
MCP.2.CCL8	11,58±3,54 (8,67-15,82)	20,19±14,81 (5,74-77,36)	14,83±7,99 (4,42-29,19)
MCP.3.CCL7	6,81±2,21 (3,62-8,85)	6,65±2,58 (2,52-11,21)	6,08±3,46 (0-11,45)
MCP.4.CCL13	1,38±0,2 (1,12-1,63)	2,29±0,62 (1,37-4,08)	2,01±0,54 (0,74-2,97)
MDC.CCL22	27,3±11,76 (14,26-46,01)	73,89±53,55 (23,18-252,78)	85,45±50,45 (16,34-204,68)
MIF	6500,63±3379,21 (3632,45-12137,56)	24219,21±15059,11 (8013,62-77459,79)	24362,44±18677,39 (3515,23-63478,97)
MIG.CXCL9	17,27±3,19 (13,87-21,41)	37,02±24 (12,61-115,34)	33,56±13,76 (11,79-51,7)
MIP.1alpha.CCL3	4,39±1,88 (2,69-7,47)	7,33±2,59 (4,79-15,89)	8±3,53 (2,83-15,39)
MIP.1delta.CCL15	534,79±174,66 (280,14-772,38)	626,78±176,36 (391,84-1077,58)	621,46±238,44 (143,29-1218,12)
MIP.3alpha.CCL20	0,75±0,11 (0,62-0,86)	0,96±0,22 (0,63-1,55)	0,98±0,37 (0,38-1,73)
MIP.3beta.CCL19	70,4±15,78 (45,81-87,88)	219,81±208,82 (70,73-1120,83)	157,5±83,12 (33,53-324,02)
MPIF.1.CCL23	8,1±3,72 (5,15-13,73)	13,36±4,17 (6,7-25,09)	11,68±6,05 (2,05-24,01)
SCYB16.CXCL16	1000,24±95,01 (933,84-1143,84)	1143,59±262,51 (640,97-1550,63)	1018,84±285,93 (676,39-1494,36)
SDF.1alpha.beta.CXCL12	4001,85±826,62 (2924,54-4891,42)	3713,79±718,11 (2480,48-5727,66)	3478,1±1251,66 (897,75-5183,56)
TARC.CCL17	0,4±0,89 (0-1,99)	2,72±2 (0-7,84)	3,9±2,77 (0-8,92)
TECK.CCL25	74,86±31,23 (43,88-117,4)	104,57±50,29 (45,65-274,35)	101,03±60,14 (0-220,55)
TNF.alpha	23,43±12,88 (9,23-38,64)	24,3±8,18 (12,63-40,63)	23,63±9,12 (7,71-38,11)

Table 8. Mean and SD of cytokines and chemokines released in the CSF of controls, CIS patients and RRMS patients. Abbreviations: OCB oligoclonal bands, CIS clinically isolated syndrome, RR relapsing remitting. Data are expressed in pg/mL

Statistic analysis has shown significantly higher CSF levels of CXCL13, CCL26, GM.CSF, CCL1, CXCL11, CCL13, CCL22 and CCL19 (and other molecules) in MS and lower levels of MIF (see Table 9) in MS patiens.

	MS patients (mean±sd)	Controls (mean±sd)	p-value
Age (years)	34,97±10,14 (16-57)	51,8±5,17 (43-56)	0,0028**
Lymphocytes (number/uL)	10,5±13,33 (0,7-70)	2,52±1,85 (0,7-5,5)	0,02562*
Protein (mg/mL)	0,44±0,1 (0,29-0,7)	0,54±0,05 (0,47-0,61)	0,02173*
IgGIndex	0,79±0,43 (0,4-2,7)	0,46±0,06 (0,41-0,55)	0,00322**
BCA.1.CXCL13	33,42±46,03 (0,05-206,01)	0,31±0,33 (0,02-0,81)	0,00006**
EOTAXIN.2.CCL24	29,41±6,33 (14,68-40,9)	21,53±2,92 (18,67-25,98)	0,00487**
EOTAXIN.3.CCL26	10,79±2,19 (6,19-14,44)	7,23±0,71 (6,64-8,43)	0,00191**
Fractalkine.CX3CL1	202,34±61,56 (88,97-321,81)	137,97±31,94 (100,66-177,61)	0,02099*
GM.CSF	98,6±19,38 (55,61-136,88)	66,09±13,75 (44,63-78,57)	0,00191**
Gro.alpha.CXCL1	69,23±21,19 (39,7-155)	50,51±5,02 (43,4-54,94)	0,01231*
Gro.Beta.CXCL2	9,18±3,62 (0-16,03)	3,88±2,65 (0-6,53)	0,00739**
I.309.CCL1	26,32±6,72 (14,48-43,25)	15,75±1,8 (13,6-18,19)	0,00018**
IL.8.CXCL8	86,72±45,1 (18,82-234,16)	49,44±11,04 (34,47-60,9)	0,00676**
IL.16	46,67±20,72 (14,13-100,92)	62,78±13,13 (49,05-82,24)	0,05262
IP.10.CXCL10	1107,54±1105 (249,21-6552,26)	469,74±185,83 (281-673,72)	0,02686*
I.TAC.CXCL11	5,66±4,52 (1,03-19,64)	1,85±0,8 (1,04-2,74)	0,00191**
MCP.4.CCL13	2,18±0,6 (0,74-4,08)	1,38±0,2 (1,12-1,63)	0,00025**
MDC.CCL22	78,18±51,98 (16,34-252,78)	27,3±11,76 (14,26-46,01)	0,00098**
MIF	24272,41±16223,27 (3515,23-77459,79)	6500,63±3379,21 (3632,45-12137,56)	0,00098**
MIG.CXCL9	35,74±20,63 (11,79-115,34)	17,27±3,19 (13,87-21,41)	0,00791**
MIP.1alpha.CCL3	7,58±2,94 (2,83-15,89)	4,39±1,88 (2,69-7,47)	0,01067*
MIP.3alpha.CCL20	0,97±0,28 (0,38-1,73)	0,75±0,11 (0,62-0,86)	0,02686*
MIP.3beta.CCL19	196,67±174,08 (33,53-1120,83)	70,4±15,78 (45,81-87,88)	0,00191**
MPIF.1.CCL23	12,74±4,94 (2,05-25,09)	8,1±3,72 (5,15-13,73)	0,04735*
TARC.CCL17	3,16±2,35 (0-8,92)	0,4±0,89 (0-1,99)	0,00673**

Table 9. Differences in CSF biomarkers between MS population and Controls. Data are expressed as mean ± SD. CSF citokines and chemokines values are expressed in pg/mL. p<0,05= *; p<0,01= **.

The MS study population was stratified in two subgroups according to low and high GM pathology [cortical lesion number cortical lesion volume/number (CL volume, cut-off 185mm3 (median value); CL volume low: n=18, CL volume high n=17); Global cortical thickness (Cth, cut-off 2,42 mm; Cth low: n=18, CL high n=17), and to T2 white matter lesion volume (T2WMLV, cut-off 1000 mm3; T2WMLV low: n=18, T2WMLV high .n=17). The two groups did not differ by sex, age at lumbar puncture, disease duration and EDSS.

MS patients with low CLs number or CL volume have significantly higher (p<0,05) CSF levels of CCL7, MIF, CXCL6 and CCL25 and a trend (p=0,05) for CXCL9 and CCL19 compared to patients with high CLs number or CL volume (Figure 4).

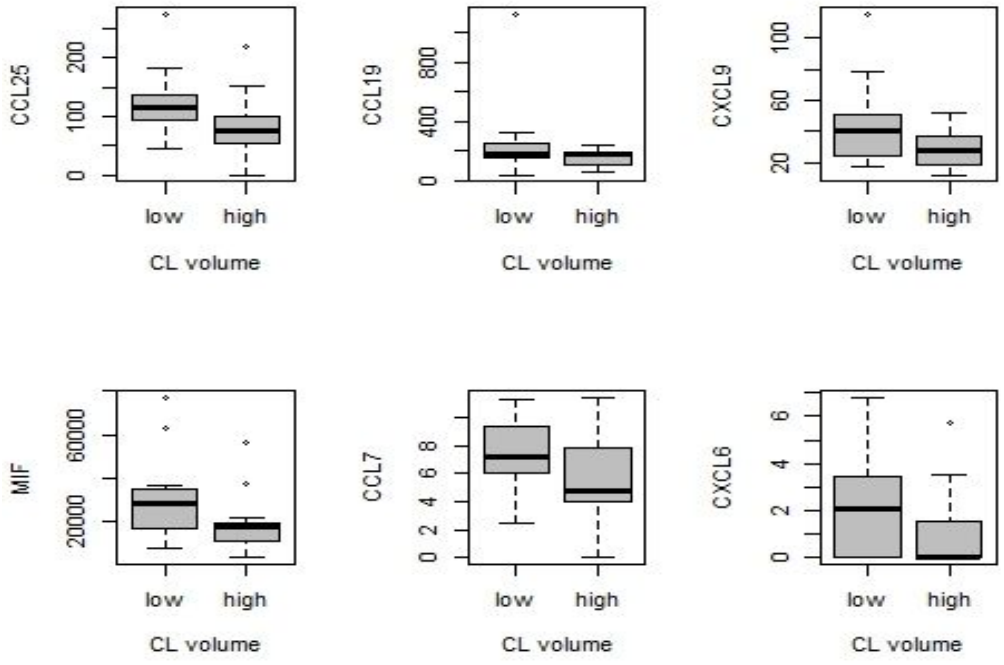

Figure 4. Boxplot representing different concentrations (pg/ml) of CCL25, MIF, CCL7 and CXCL6 (p<0.01), and CCL19, CXCL9 (p=0,05) in patients with high or low CL volume. Abbreviations: CL cortical lesion. CSF citokines and chemokines values are expressed in pg/mL

Patients with higher T2 white matter lesion volume (T2WMLV) values, indicating the load of white matter damage, have higher CSF levels of GM-CSF and lower CSF levels of CXCL9, CXCL11, CXCL13, CXCL6, CCL7, CCL17 compared to patients with lower T2WMLV (Figure 5).

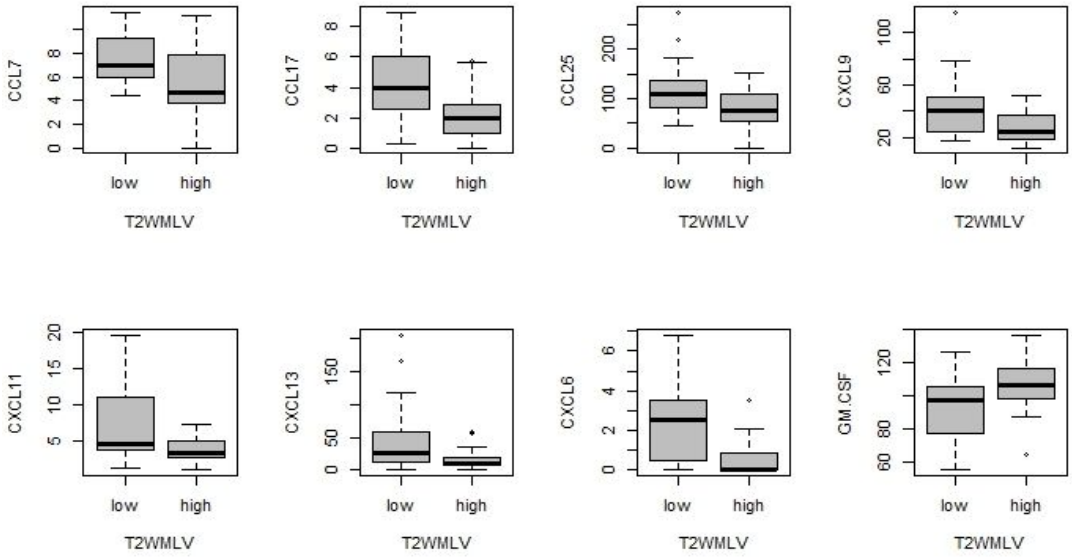

Figure 5. Patients with lower T2 white matter lesion volume (T2WMLV) express higher concentration of CCL7, CCL17, CCL25, CXCL9, CXCL11, CXCL13, CXCL6 and lower concentration of GM.CSF ($p < 0.05$) compare to patients with higher T2WMLV. CSF citokines and chemokines values are expressed in pg/mL

Patients with lower cortical thickness (Cth) values, characterizing cortical atrophy, express lower value of CCL7 and CXCL12 compared to patients with higher cortical thickness (Figure 6).

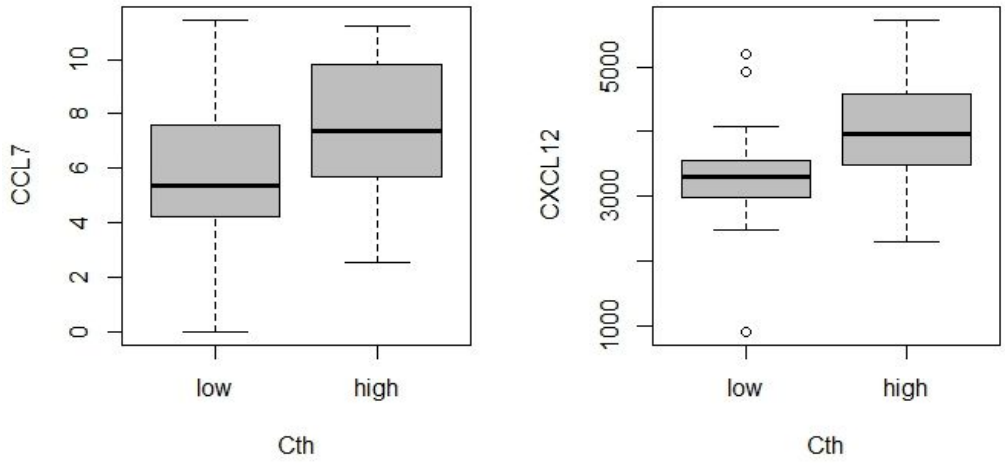

Figure 6. Patients with lower cortical cortical thickness (Cth) values express lower value of CCL7 and CXCL12 compared to patients with higher Cth. CSF citokines and chemokines values are expressed in pg/mL.

Patient with absence of disease activity, measured as presence of Gd+ MRI lesion and/or concomitant relapsing phase, show significant higher level of IL4 and a trend (p=0,05) of higher level of Interferonn gamma (IFNg) (Figure 7).

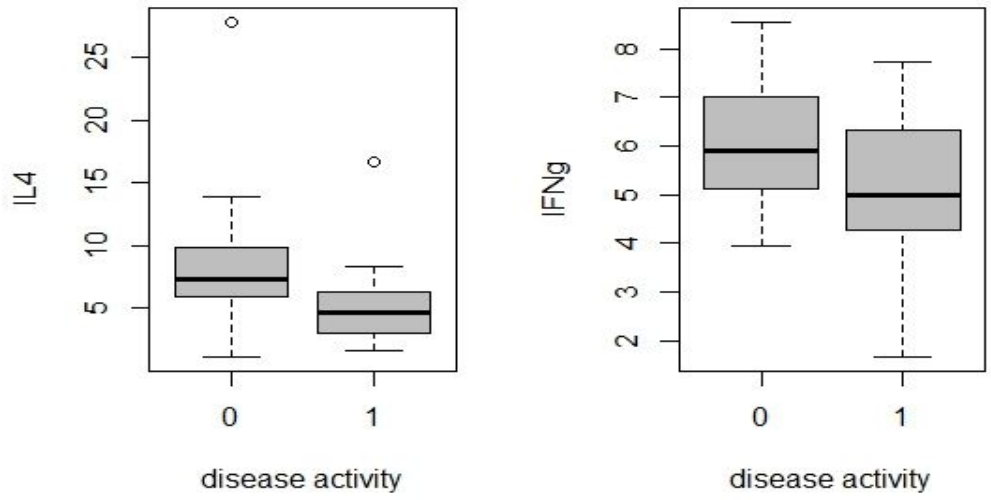

Figure 7. Disease activity ("0"= absence, "1"= presence); patient without signs of disease activity showed high level of IL4 (p<0,05)and Interferon gamma (IFNg) (p=0,05). CSF citokines and chemokines values are expressed in pg/mL

Correlation analysis. There were no significant correlation between MRI measures and other related clinical and CSF data, except for a good correlation between disease duration and time to conversion (rho di Spearmen 0.95 p<0.0001) in CIS MS population.

Correlations between CSF cytokines revealed in MS patients are summarized in Figure 8 where a correlation matrix of the protein investigated in the cohort of MS patients is displayed. Correlation coefficients are between plus 1 and minus 1 corresponding to different colors whose value are explain in the color legend. Black squares identify cluster of aggregation between protein molecules.

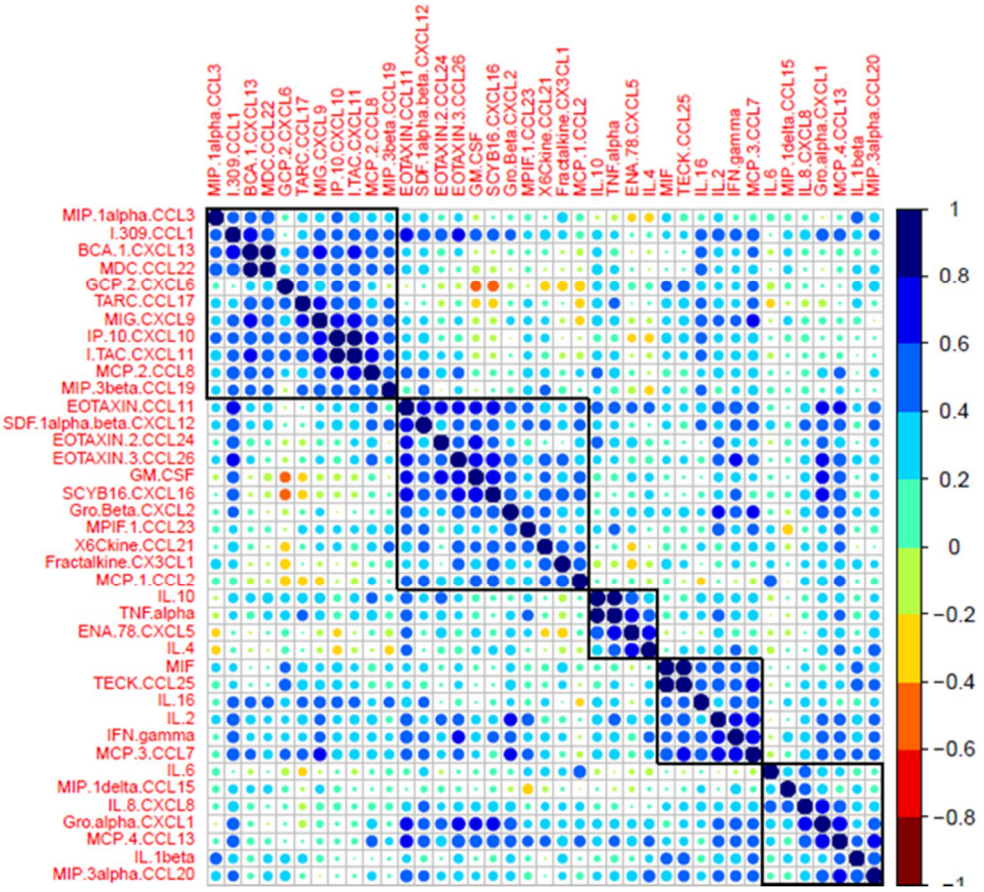

Figure 8. Correlation matrix of investigated CSF cytokines and chemokines. The results are grouped accordingly cluster analysis “ward” (black squares).

4.2. Combined MRI/CSF analysis of the second cohort of MS patients

MRI analysis

CL Number and CL Volume were measured on DIR Images and were respectively: CL Number 3,58±4,193 (range 0-16); CL Volume 103,1±233,7 mm^3; Global Cortical thickness was 2,575±0,134 mm, GMF was 0,545±0,024 (ratio). The observed volume of T2 WM Lesions (T2WMLV) on FLAIR images was 2617,7±3253,6 mm^3 (range 59,26-13530,5). Median number of spinal cord lesion was 1,48±1,95 (range 0-7). Gadolinium enhancing lesion (Gd+) number was 0,452±0,961 (range 0-5) (Table 10).

	CONTROLS (13 patients)	CIS (12 Patients)	RR (19 Patients)
Age at LP (years)	42,92±10,31 (29-60)	33,5±10,87 (22-50)	37,89±8,48 (18-50)
M/F	2/13	3/12	10/19
Disease duration at LP (months)	31,15±68,88 (0-255)	10,08±19,55 (0-69)	21,42±32,52 (0-136)
MRI a/o clinical signs of disease activity (n/tot)	0/13	2/12	9/19
EDSS (median±mad)	n/a	1,5±0,74 (0-2)	1,5±0,74 (0-3,5)
SC lesion number	0±0 (0-0)	0,92±0,9 (0-3)	1,84±2,34 (0-7)
Gd + lesion n	0±0 (0-0)	0,08±0,29 (0-1)	0,68±1,16 (0-5)
T2WMLV (mm^3)	1670,15±1706,73 (317,89-5857,54)	1988,86±3515,55 (59,26-12320,4)	3015,27±3076,54 (524,55-13530,5)
CL number	0±0 (0-0)	2,08±2,97 (0-11)	4,53±4,64 (0-16)
CL volume (mm^3)	0±0 (0-0)	41,79±97,5 (0-345,74)	141,82±284,88 (0-1260,68)
Cth (mm)	2,61±0,11 (2,43-2,82)	2,63±0,07 (2,49-2,73)	2,54±0,15 (2,27-2,77)
Cortical ratio	0,4±0,02 (0,38-0,43)	0,4±0,02 (0,36-0,43)	0,39±0,02 (0,37-0,45)
GMF	0,55±0,01 (0,53-0,57)	0,55±0,02 (0,51-0,59)	0,54±0,03 (0,51-0,61)
Altered VEP/tot	2/13	5/11	7/18

Table 10. Clinical and MRI data from 2nd cohort of patients. Data are expressed in mean ± SD (range), except for EDSS (median±mad). Abbreviations: LP lumbar puncture, n number, CIS clinically isolated syndrome, RR relapsing remitting, SC spinal cord, CL cortical lesion, WML white matter lesion volume, Cth Global cortical thickness, GMF grey matter fraction, Visual evoked potential.

CSF analysis (protein and gene expression analysis)

We performed a detailed protein analysis using the Bio-Plex System in order to detect the presence and levels of 40 inflammatory molecules expressed in the CSF of the 31 MS patients compared to the 13 controls recruited in the current study.

Preliminary analysis of cytokines and chemokines released in the CSF of MS cases (CIS and RRMS) and controls are shown in Table 11.

Gene expression data of immune related genes in CSF cells of CIS patients and RRMS patients are summarized in Table 12.

	CONTROLS (13 patients)	CIS (12 Patients)	RR (19 Patients)
OCB positive	0/13	7/12	16/19
IgG index	0,47±0,06 (0,4-0,61)	0,68±0,33 (0,32-1,48)	0,86±0,63 (0,38-3,2)
Protein mg/mL	0,29±0,1 (0,14-0,49)	0,31±0,07 (0,17-0,41)	0,32±0,13 (0,19-0,65)
CSF - cell number/uL	2,17±1,3 (0,3-5,5)	7,94±11,64 (1-43)	8,78±12,55 (1,7-58)
6Ckine/CCL21	2597,27±1508,56 (915,03-6475,57)	2981,17±1339 (1663,87-6234)	3346,97±1993,81 (1363,6-9482,36)
BCA-1/CXCL13	0,71±0,59 (0-1,7)	17,47±41,7 (0-148,15)	29,2±31,01 (0,68-122,48)

ENA-78/CXCL5	228,54±131,51 (64,12-501,69)	295,17±176,64 (150,71-756,41)	352,99±176,91 (108,52-704,4)
EOTAXIN/CCL11	27,59±12,47 (13,27-52,94)	31,18±13,69 (21,68-71,71)	37,46±20,52 (14,45-107,11)
EOTAXIN-2/CCL24	92,8±61,07 (23,67-217,64)	88,5±33,17 (51,96-174,76)	109,47±84,01 (0-414,32)
EOTAXIN-3/CCL26	29,61±19,15 (8,93-81,5)	27,94±11,88 (10,3-54,24)	33,05±23,38 (3,43-117,57)
Fractalkine/CX3CL1	360,53±115,3 (207,48-586)	367,47±116,25 (227,44-587,18)	436,03±307,15 (78,98-1325,61)
GM-CSF	143,98±83,32 (0-327,5)	133,71±41,62 (89,32-239,94)	151,38±108,3 (58,92-541,07)
Gro-alpha/CXCL1	124,09±58,01 (67,38-265,29)	118,4±31,34 (90,59-196,53)	143,81±93,43 (56,27-482,68)
Gro-Beta/CXCL2	20,83±17,02 (0-50,64)	19,27±26,82 (0-95,61)	25,47±14,92 (0-59,11)
I-309/CCL1	70,07±40,9 (20,17-158,36)	81,84±24,48 (55,59-133,35)	100,67±55,8 (48,55-301,93)
IFN-gamma	14,23±6,34 (6,83-31,21)	13,73±4,96 (7,93-24,52)	15,7±11,85 (4,02-61,21)
IL-1beta	0,55±0,22 (0,31-0,96)	0,43±0,31 (0-1,21)	0,49±0,49 (0-2,43)
IL-2	4,06±1,35 (2,05-6,43)	3,53±1,67 (1,48-8,09)	3,97±2,73 (1,22-14,36)
IL-4	19,38±17,74 (3,53-65,13)	25,59±24,54 (5,43-95,65)	27,03±17,55 (4,88-59,67)
IL-6	13,1±4,24 (8,88-21,79)	13,86±12,56 (6,58-52,67)	24,78±28,74 (2,65-109,32)
IL-8/CXCL8	75,24±29,73 (31,89-139,07)	75,94±25,63 (49,15-129,35)	88,4±39,33 (38,11-177,39)
IL-10	11,71±3,59 (7,87-19,63)	10,88±3 (7,68-17,94)	13,67±7,7 (4,43-41,71)
IL-16	123,45±47,91 (70,62-245,29)	142,17±70,6 (51,15-264,97)	158,65±91,18 (33,2-443,96)
IP-10/CXCL10	744,61±347,78 (393,8-1592,19)	1031,91±441,57 (409,44-1865,88)	1484,82±757,1 (599,07-3219,53)
I-TAC/CXCL11	4,61±1,99 (1,69-8,5)	7,42±4,1 (3,32-17,47)	10,5±6,32 (3,61-25,73)
MCP-1/CCL2	1113,91±367,1 (524,55-1703,14)	685,89±281,45 (251,73-1186,95)	1016,72±1024,04 (170,04-4698,18)
MCP-2/CCL8	18,15±4,27 (12,73-29,29)	21,01±7,68 (9,26-32,05)	38±27,81 (11,2-131,71)
MCP-3/CCL7	15,03±6,09 (6,7-31,04)	17,35±19,78 (0-73,12)	18,24±15,07 (0-75,86)
MCP-4/CCL13	4,18±2,23 (1,74-9)	4,75±1,25 (3,08-6,91)	5,5±3,17 (2,05-16,96)
MDC/CCL22	40,94±16,34 (13,59-76,07)	58,68±45,07 (20,44-188,46)	80,67±38,05 (35,65-155,15)
MIF	22430,55±18582,96 (2912,63-74856,36)	21159,44±11464,92 (4790,8-39936,53)	21808,52±23959,29 (5451,74-115563,93)
MIG/CXCL9	45,58±26,17 (20,02-117,73)	56,29±29,09 (23,19-116,71)	81,37±58,07 (21,51-262,71)
MIP-1alpha/CCL3	5,75±1,81 (2,66-9,48)	7±3,07 (3,63-13,23)	9,08±4,81 (3,14-22,29)
MIP-1delta/CCL15	783,3±354,35 (398,9-1592,77)	560,08±191,79 (360,59-921,05)	672,08±549,01 (215,94-2807,5)
MIP-3alpha/CCL20	2,16±1,07 (1,07-4,71)	2,36±1,02 (1,19-4,58)	2,7±1,53 (0,69-8)
MIP-3beta/CCL19	155,91±53,99 (91,6-275,79)	200,44±83,35 (93,9-338,95)	245,9±188,28 (68-939,11)
MPIF-1/CCL23	14,6±5,58 (7,88-26,25)	14,56±4,85 (6,48-21,27)	16,77±11,02 (5,4-53)
SCYB16/CXCL16	2068,01±775,76 (1002,08-3666,19)	1774,93±387,88 (1331,03-2499)	2220,68±1538,21 (621,51-7667,75)
SDF-1alpha+beta/CXCL12	6226,59±1742,04 (4130,88-10697,64)	5654,05±1488,43 (3484,19-8187,41)	6819,24±5931,34 (1772,1-29784,75)
TARC/CCL17	0,88±1,4 (0-3,59)	2,34±5,83 (0-20,21)	2,57±3,56 (0-12,29)
TECK/CCL25	351,06±248,24 (79,5-952,21)	385,1±161,98 (211,39-779,82)	421,2±309,66 (151,63-1636,32)
TNF-alpha	42,41±41,8 (17,1-173)	46,32±30,92 (13,97-120,12)	54,6±32,18 (19,06-119,86)

Table 11. Mean and SD of cytokines and chemokines released in the CSF of control patients, CIS patients and RRMS patients. Abbreviations: OCB oligoclonal bands, CIS clinically isolated syndrome, RR relapsing remitting. CSF cytokines and chemokines values are expressed in pg/mL.

	CONTROLS (13 patients)	CIS (12 Patients)	RR (19 Patients)
CCL22	0,06±0,06 (0-0,18)	0,04±0,03 (0-0,11)	0,03±0,03 (0-0,09)
CCL3	18,76±18,36 (0,57-47,5)	30,87±43 (0,53-135,02)	33,24±30,48 (1,52-96)
CCL8	0,11±0,08 (0-0,27)	0,03±0,03 (0-0,07)	0,07±0,08 (0-0,21)
CD138	0±0 (0-0)	0±0 (0-0)	0,01±0,01 (0-0,03)
CD14	22,84±20,89 (4,23-69,36)	28,48±38,24 (4,5-114,09)	26,99±21,97 (2,55-67,6)
CD20	0,01±0,01 (0-0,04)	0,11±0,23 (0-0,77)	0,22±0,4 (0-1,38)
CD4	5,15±4,97 (0,06-14,06)	2,55±2,36 (0,45-8,73)	2,01±1,28 (0,06-4,69)
CD68	3,45±2,21 (0,78-8,87)	2,3±2,09 (0,9-6,93)	2,08±1,73 (0,13-6,41)
CD8	0,35±0,35 (0-1)	0,8±1,15 (0,06-3,99)	1,05±1,23 (0-3,96)
CXCL10	0,01±0,02 (0-0,06)	0,01±0,01 (0-0,02)	0,01±0,01 (0-0,02)
CXCL13	0±0,01 (0-0,02)	0,04±0,12 (0-0,39)	0±0,01 (0-0,03)
FoxP3	0,04±0,07 (0-0,26)	0,07±0,14 (0-0,49)	0,2±0,17 (0-0,55)
GAPDH	1±0 (1-1)	1±0 (1-1)	1±0 (1-1)
GM-CSF	0±0 (0-0)	0±0,01 (0-0,02)	0±0,01 (0-0,02)
IFN	0±0 (0-0,02)	0,01±0,02 (0-0,06)	0,01±0,01 (0-0,03)
IL10	0,27±0,28 (0-0,87)	0,07±0,04 (0-0,15)	0,13±0,11 (0-0,32)
IL16	0,41±0,27 (0,09-1,01)	0,82±0,74 (0,24-2,84)	1,03±0,98 (0,04-2,96)
LTa	0±0,01 (0-0,05)	0,01±0,01 (0-0,05)	0,03±0,03 (0-0,11)
Tbet	0,03±0,04 (0-0,14)	0,11±0,13 (0-0,4)	0,16±0,17 (0-0,51)
TNF	0,48±0,47 (0-1,44)	0,13±0,15 (0,01-0,51)	0,25±0,28 (0-0,87)

Table 12. Expression of immune related genes in CSF cells CIS patients and RRMS patients. Gene expression values are presented as -2^ΔCt relative to GAPDH and are expressed as median ± SD.

Statistic analysis by using Mann Whitney signed rank test shows high significantly different levels (p<0,01) of different CSF protein as CXCL13, CXCL10, CXCL11, and CCL22, and high significantly different levels (p<0.01) in gene-expression of Lymphotoxin-alpha (LTa) and T-box transcription factor TBX21 (Tbet), and significant different (p<0,05) levels in gene-expression of in CCL8, CD138, CD20, CD68, CXCL13, FoxP3 and IFNg in MS patients compared to controls patients. Results are summarized in Table 13.

	Control	SM patients	p-value
IgG index	0,47±0,06 (0,4-0,61)	0,79±0,54 (0,32-3,2)	0,0009**
CSF cell number/uL	2,17±1,3 (0,3-5,5)	8,46±12,02 (1-58)	0,0018**
BCA-1/CXCL13	0,71±0,59 (0-1,7)	24,66±35,33 (0-148,15)	0,00002**
IP-10/CXCL10	744,61±347,78 (393,8-1592,19)	1309,5±682,42 (409,44-3219,53)	0,0005**
I-TAC/CXCL11	4,61±1,99 (1,69-8,5)	9,31±5,7 (3,32-25,73)	0,0011**
MCP-1/CCL2	1113,91±367,1 (524,55-1703,14)	888,66±827,69 (170,04-4698,18)	0,0192*
MCP-2/CCL8	18,15±4,27 (12,73-29,29)	31,42±23,59 (9,26-131,71)	0,0104*
MDC/CCL22	40,94±16,34 (13,59-76,07)	72,16±41,62 (20,44-188,46)	0,0066**
MIG/CXCL9	45,58±26,17 (20,02-117,73)	71,66±49,88 (21,51-262,71)	0,0598*
MIP-1delta/CCL15	783,3±354,35 (398,9-1592,77)	628,73±444,3 (215,94-2807,5)	0,0438*
CCL8	0,11±0,08 (0-0,27)	0,05±0,06 (0-0,21)	0,0285*
CD138	0±0 (0-0)	0,01±0,01 (0-0,03)	0,0485*
CD20	0,01±0,01 (0-0,04)	0,17±0,33 (0-1,38)	0,0185*
CD68	3,45±2,21 (0,78-8,87)	2,18±1,87 (0,13-6,93)	0,039*
CXCL13	0±0,01 (0-0,02)	0,02±0,08 (0-0,39)	0,0178*
FoxP3	0,04±0,07 (0-0,26)	0,14±0,17 (0-0,55)	0,044*
IFN	0±0 (0-0,02)	0,01±0,01 (0-0,06)	0,0124*
LTa	0±0,01 (0-0,05)	0,02±0,03 (0-0,11)	0,0023**
Tbet	0,03±0,04 (0-0,14)	0,14±0,15 (0-0,51)	0,0085**

Table 13. Differences in CSF protein (first box) and gene expression (second box) between MS population and controls. CSF cytokines and chemokines values are expressed in pg/mL Gene expression values are presented as -2^ΔCt relative to GAPDH. The relative amount expressed as median ± SD. Comparison was made by Mann Whitney signed rank test.p<0,05= *; p<0,01= **.

By stratifying the study population in two subgroups according to high and low cortical lesion volume (CL number, cut-off: median value; CL volume low: n=16, CL volume High n=15) and Cth (Cth, cut-off: median value ; Cth low: n=16, CL High n=17) and T2 white matter lesion volume (T2WMLV, cut-off: median value; T2WMLV low: n=16, T2WMLV High n=15) it was found that MS patients with

higher CL volume are characterized by higher level of CSF cell number, Gd+ lesions and T2WMLV compared to patients with lower CL volume. They have significantly ($p<0.05$) higher CSF protein levels of CXCL13 and higher values of gene expression of CD138 and GM-CSF and a trend ($p=0.05$) for CD20 compared to patients with lower CL volume(see Figure 9)

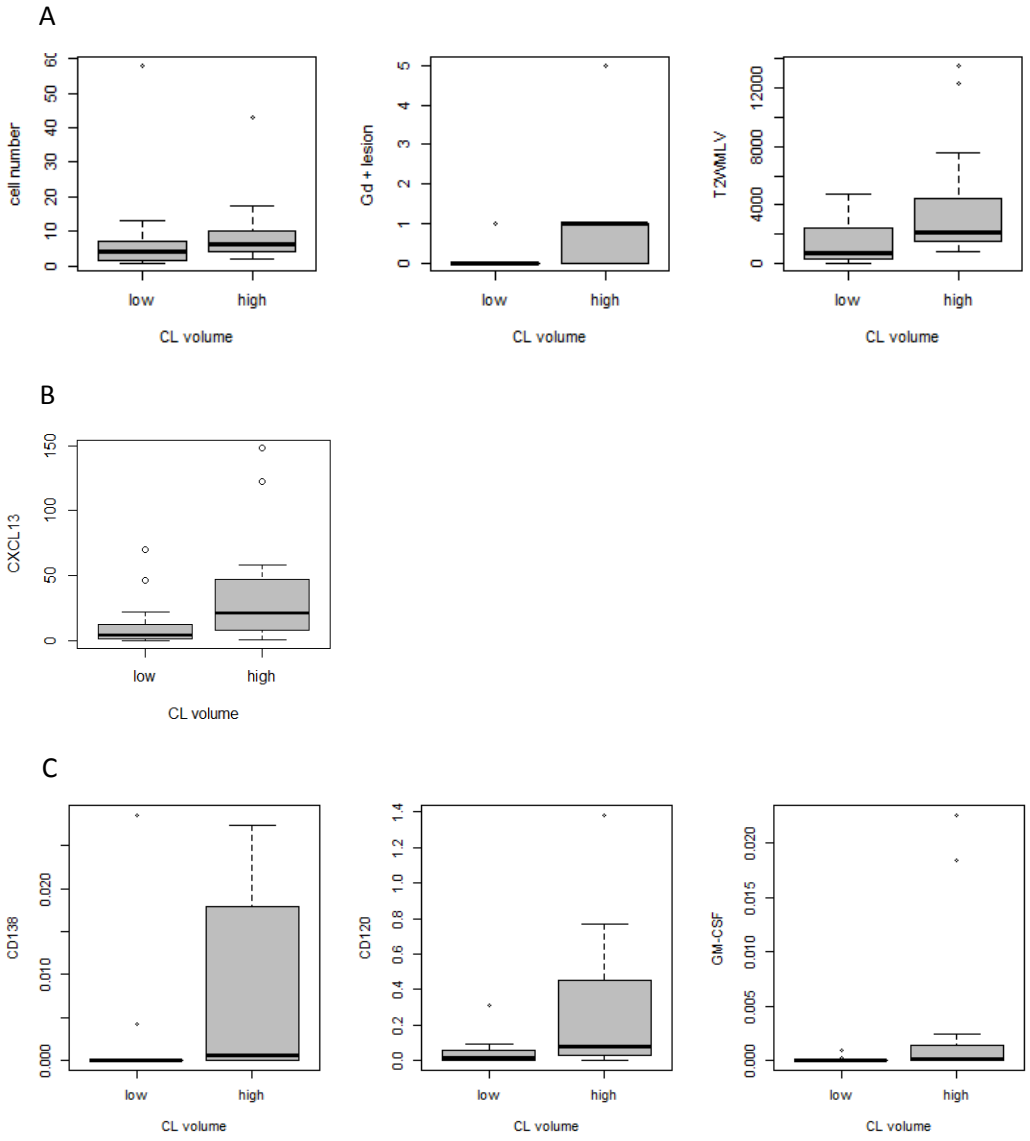

Figure 9. Patients with higher CL volume express higher value of A) CSF cell number, Gadolinium enhancing lesion (Gd+) number, and T2WMLV volume (mm^3) B) CXCL13 protein values C) values of gene expression of CD138 and GMCSF and a trend for CD20 compared to patients with lower degree of CL volume. CSF cytokines and chemokines values are expressed in pg/mL. Gene expression values are presented as -2^ΔCt relative to GAPDH and are expressed as median ± SD.

MS patients with higher T2WMLV (mm^3) are characterized by higher number of CL volume (mm^3), higher level of IgG Index and CXCL11 protein in the examined CSF (see Figure 10)

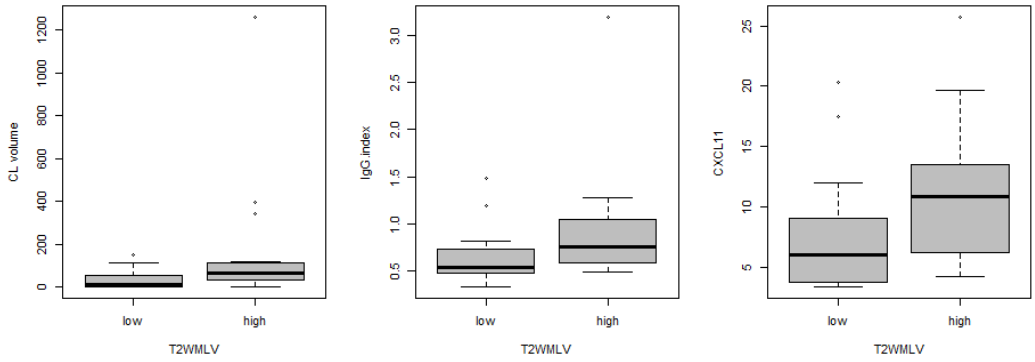

Figure 10. Patients with higher value of T2WMLV present higher value of cortical lesion (CL) volume(mm^3), higher level of IgG Index and higher level of CXCL11 compared to patients with lower value of T2WMLV. CXCL11 values are expressed in pg/mL

Patients with lower Cth values have lower CSF protein levels of CCL21, CCL24, CXC3CL1, CXCL1, CCL2, CCL13 and CXCL16 compared to patients with higher values of Cth (Figure 11).

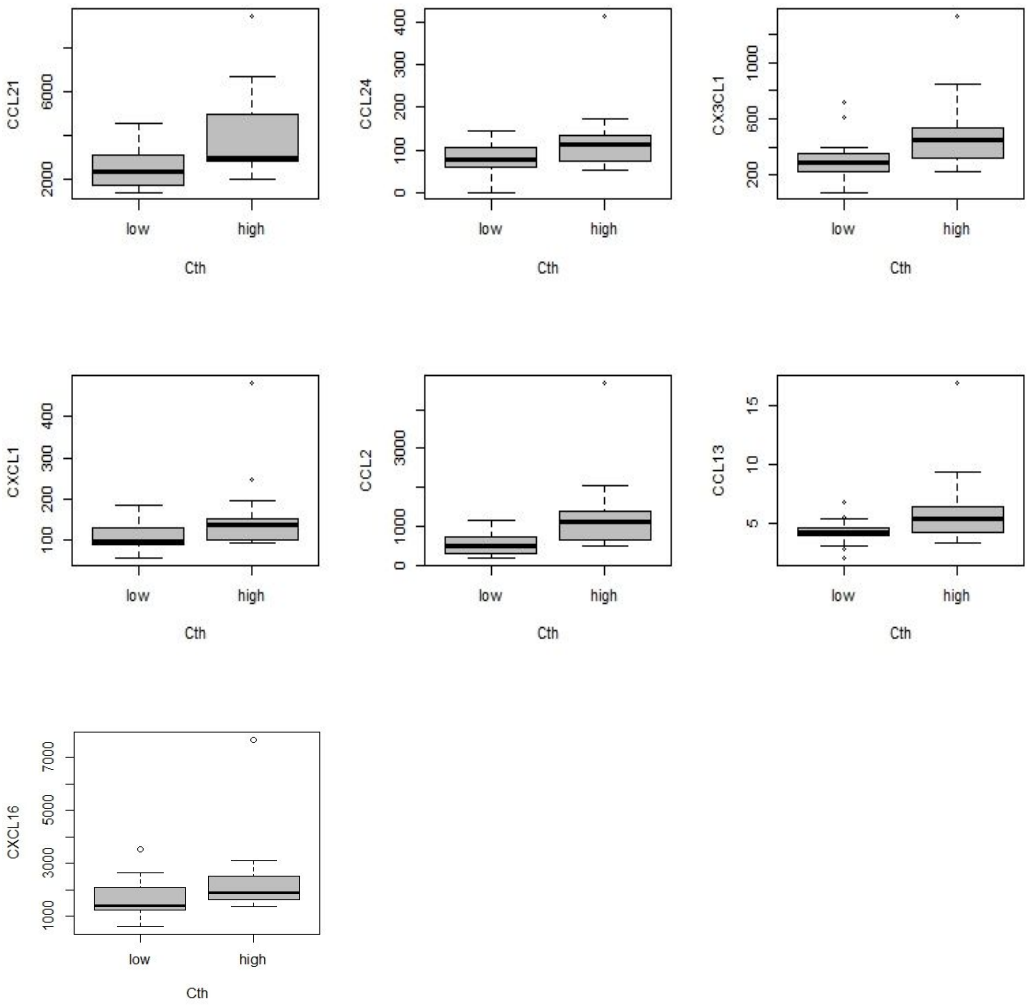

Figure 11. Patients with lower Global cortical thickness (Cth) present lower value of CCL21, CCL24, CXC3CL1, CXCL1, CCL2, CCL13 and CXCL16 compared to patients with higher values of Cth. CSF cytokines and chemokines values are expressed in pg/mL .

Patient with clinical and MRI evidence of disease activity have significantly higher levels of CLs number, higher CSF levels of IgG Index, CXCL13, CXCL10, CXCL11, CCL8, CCL22, CCL3 compared to patients without any clinical and radiological evidence of disease activity (Figure 12).

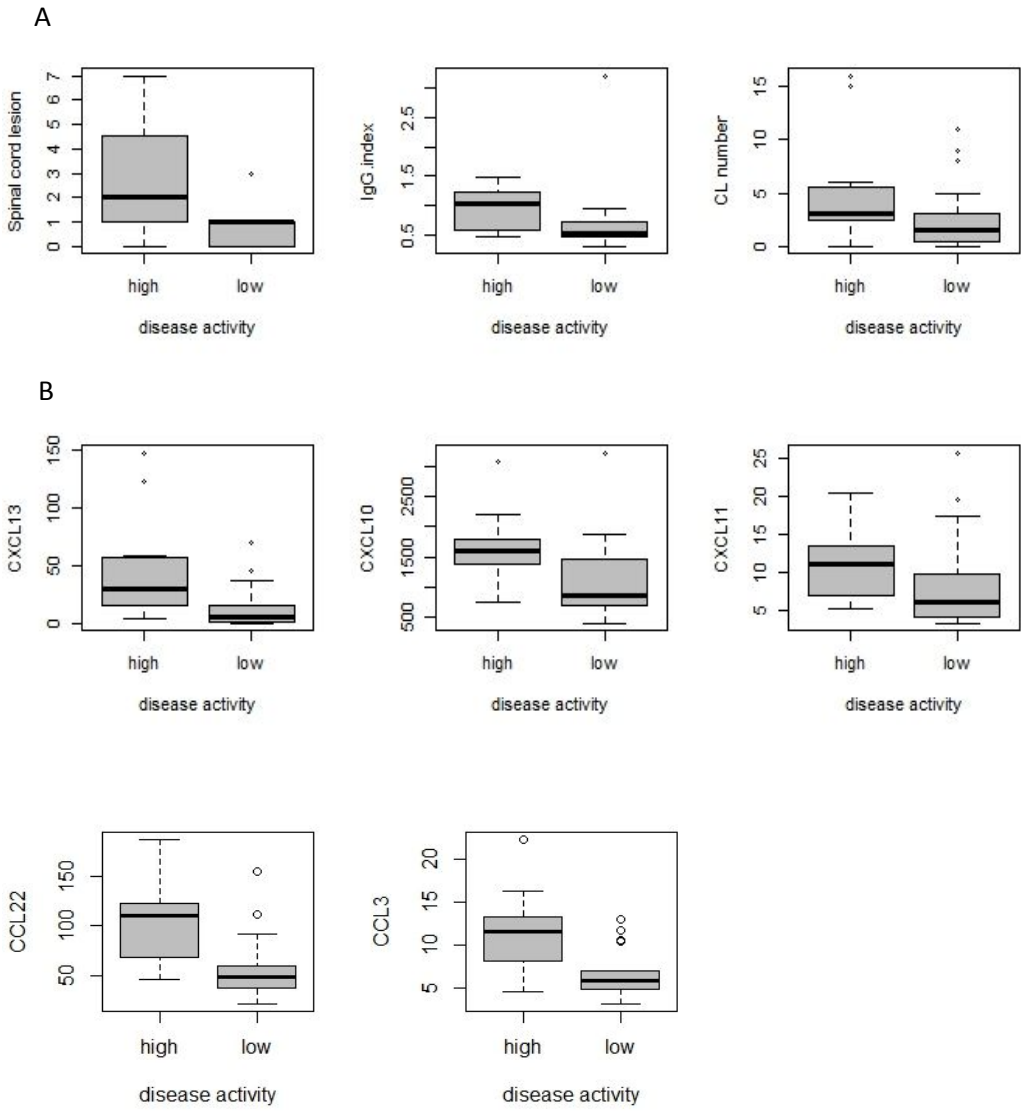

Figure 12. Patients with higher disease activity show higher value of A) spinal cord lesion number and CL number and higher level of IgG Index B) higher protein level of CXCL10, CXCL13, CXCL11, CCL 22 and CCL3. CSF cytokines and chemokines values are expressed in pg/mL.

5. Discussion

Our study shows that cortical pathology, as revealed by advanced MRI methodology, positively correlate with CSF bio-markers of intrathecal inflammation, in particular associated with B-cell response since early disease stage.

This finding strongly corroborate the hypothesis of a key role of meningeal inflammation in cortical pathology, as previously suggested by several neuropathological studies on post-mortem and bioptic MS tissues (Magliozzi et al., 2007, 2010; Howell et al., 2011; Lucchinetti et al., 2011).

Soluble factors secreting from meningeal inflammatory aggregates, populated by infiltrating B-cells, plama cells, CD4+ and CD8+ positive cells, have been suggested to promote a gradient of demyelination and neuronal injury in the cortical grey matter bathed by the CSF flow (Magliozzi et al., 2010; Gadner et al., 2013). The extent of meningeal inflammation indeed associates positively with cortical demyelination and disease progression, suggesting that chronic immune activity in the subarachnoid compartment plays a key role also from the early disease stage (Lucchinetti et al., 2011).

Furthermore, elevated IgG index and the presence of OCBs, which represent an extremely useful CSF marker of intrathecal chronic humoral immunity in MS may be hypothesised to be in somehow linked to the presence of compartmentalized intrathecal B-cell response, possibly within ectopic B-cell follicles structures settled in the subarachnoid space.

Concomitant MRI studies in the last decade have emphasized the relevance of cortical pathology, which occur at the earliest phase of MS, on clinical and physical disability (Calabrese et al., 2010).

For this reason in attempt to investigate how cortical pathology in vivo could be related to intrathecal inflammation, possibly related to the presence of meningeal infiltrates, we performed a combined analysis by using non-conventional MRI sequences and molecular CSF analysis. In particular we carried

out a protein analysis of 40 cytokines and chemokines in the CSF and a gene expression analysis of the matched cellular fraction.

We firstly investigated the contribution of MRI data and CSF molecular analysis characterizing MS patients from control.

Hereafter MRI parameters of GM damage were adopted to stratify patients having more severe cortical pathology and to find possible inflammatory mediators linked to higher or lower degree of cortical lesion load.

In the first cohort we observed that MS patients were characterized with higher rate of cortical atrophy than controls. Under the investigated molecules, the significantly overexpressed in the CSF of MS patients compared to controls were CXCL13 (p=0,00006), CCL19 (p=0,0019), CCL1 (p=0,00018), and CCL22 (0.0009), suggesting that the CSF of MS patients represent an inflammatory milieu with potential key role in the pathogenesis of the disease.

In line with previous studies we revealed an increase of CXCL13 a potent B-cell attracting chemokine and a CSF promising biomarker indicative of the humoral immune response in CNS. In the current study, this chemokine, although it is not disease specific, because is related to several other chronic inflammatory diseases, is increased in active patients, is present in early MS and finally is associated with higher risk to conversion to definite MS. Furthermore it has been proposed as a potential biomarker to assess therapeutic effects on intrathecal inflammation of B-cell depleting therapies as rituximab (Piccio et al., 2010). It was recently demonstrated that CXCL13 is not essential in mounting effective peripheral humoral responses, but specifically promotes CNS accumulation of differentiated B cells (Phares et al., 2016), suggesting that overexpressed protein levels in the CSF of MS patients may have an important role in the accumulation of B cells in the CNS of affected patients since the disease onset as well as in the perpetuation of the humoral response during the chronic stage.

Indeed CXCL13, together with CCL19, is reduced after rituximab treatment, an anti CD20 therapy that has been reported to have also some efficacy on disease progression (Hauser 2008). CCL19 bind to receptor CCR7 and its transcripts were

found in both active and inactive MS lesions. Elevated levels were already detected in the CSF of MS patients and were correlated with higher level of IgG production (Krumbolz et al., 2007). CCL19 may also play both a physiological immunosurveillance and pathological role.

Regarding CCL1 and CCL22 these chemokines are involved in Th2 response, Th2 cell migration, and Treg migration. However their role have been less investigated yet in multiple sclerosis. Galimberti and coll. attribute to CCL22, which is secreted by dendritic cells and macrophages, a role, in females, in the development of a lesser severe type of MS through recruitment of Th2 cells (Galimberti et al., 2009). However CCL22 promotes, through the receptor CCR4 the recruitment of circulating memory T cells (Th0) which, depending on the CNS milieu, have the ability to differentiate into Th1 and Th2 and acquire either a pro- or anti-inflammatory phenotype.

In order to better understand the role of these cytokines we stratify our first cohort according to GM and WM damage and we surprisingly observed an increment of pro-inflammatory chemokines in patients with lower GM and WM damage without recognising common immunological pathway. Furthermore stratifying according to disease activity we disclosed only a modest decrease of cytokine IL4. It has to be mentioned that not proper procedures of CSF storing have been followed during the recruitment of this first cohort of patients. Therefore, further analysis of the results of our first retrospective cohort has shown some critical steps and results has to be interpreted with caution. Methodologies has been also implemented and criteria of selection have become more stringent for our second prospective cohort. The interpretation of the data of the first cohort has been taken in consideration only by high rate of significance. Moreover follow up analysis has not been showed for lack of significance and because the small sample size and the variety of therapies adopted from MS patients has not allowed to interpret our data correctly.

On the second cohort preliminary protein analysis pointed out an important increase of CXCL13 in MS population compared to controls. Moreover we found

a relevant increase of CXCL10, CXCL11 and CCL22. CXCL10 and CXCL11 are responsible for Th1 response and for Th1, CD8, Natural Killer (NK) cells trafficking. In particular, IFNg induced CXCL10 together with CXCR3 (its receptor) and has a crucial role in the accumulation of T cells in the CNS of MS patients and positively correlate with cell count (Sørensen et al., 2002). Once again we revealed an increase of CCL22 chemokine. Its moderate correlation with levels of CXCL10, and CCL8 and a higher correlation with gene expression of FoxP3 suggest a potential role in T cell trafficking regulatory role in particular during disease activity. Also CXCL10 is more expressed during activation phases of MS. We can speculate that BBB disruption in active phase of the disease may alter the subtle balance of cell trafficking.

On the second cohort data analysis was also improved introducing a relative simple procedure in order to perform large gene expression analysis, thus allowing a better understanding of immunopathological mechanisms in MS. Based on the experience of a previous work which explored the reliability of pre-amplification real-time reverse-transcription polymerase chain reaction (PreAmp RT-PCR) to analyze expression of different genes (Veroni et al., 2015), we explore the expression of 20 immune related genes together with protein array analysis.

The most interesting data, after stratification according to GM pathology, revealed once again a significant increase of CXCL13 protein. Noteworthy gene expression analysis in MS patients also reveals a significant difference in MS patients for CD20, CD138 and LTa supporting the role of a B-cell response and lymphoid neogenesis in MS pathology. All these molecules and cells play a relevant role in fact in the organization and activity of secondary and tertiary lymphoid organs (Aloisi, 2001).

However patients with higher level of CL volume are not characterized by higher expression of CXCL13. This combined analysis may indicate that the source of this cytokine should not be sought in CSF cells but probably mostly in the meninges resident inflammatory cells. These data confirm the role of CXCL13 in

B-cell trafficking, but more surprisingly the impact on GM pathology expressed as CL volume.

Interestingly, when stratifying for Cth we observed (with higher statistically significance, p= 0,001) higher level of CCL2 related to higher value of Cth which may suggest a possible protective role of this cytokine, thus suggesting that cortical atrophy may be influenced by altered balance of pro and anti - inflammatory cytokines. In a precedent study Sørensen and coll. observed that CCL2 negatively correlates with measures of inflammation (Sørensen et al., 2001). However this results are in contrast with those conducted recently on animal model where lack of CCL2 and CCL3 correlate with less severe demyelination (Janssen et al., 2015).

In conclusion, combined CSF analysis and MRI analysis suggest that B cell immune response plays an important role in MS since the disease onset and correlates with the level of intrathecal inflammation . A more detailed analysis of the CSF biomarkers suggested in the current study might provide, in addition to MRI optimization, a better indication of severity of disease process that characterized GM pathology and important tools in predicting/monitoring the evolution of the disease in order to choose the most adequate and aggressive therapeutic strategies in those patients at higher risk to develop clinical and cognitive disability in shorter time. Finally, anti B-cell depleting strategies seems to be a promising option in slowing down GM damage.

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