Toscano *et al*. *Neuroimmunol Neuroinflammation* 2021;8:14-41
DOI: 10.20517/2347-8659.2020.12

Neuroimmunology and Neuroinflammation

Review

Open Access

CSF biomarkers in multiple sclerosis: beyond neuroinflammation

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How to cite this article: Toscano S, Patti F. CSF biomarkers in multiple sclerosis: beyond neuroinflammation. *Neuroimmunol Neuroinflammation* 2021;8:14-41. http://dx.doi.org/10.20517/2347-8659.2020.12

Received: 31 Jan 2020 **First Decision**: 20 May 2020 **Revised**: 10 Jun 2020
Accepted: 28 Jun 2020 **Fisrt online:** 21 Aug 2020 **Published:** 21 Mar 2021

Academic Editor: Roberta Magliozzi **Copy Editor**: Cai-Hong Wang **Production Editor**: Jing Yu

Abstract

For many years, quantifiable biomarkers in neurological diseases have represented a hot topic. In multiple sclerosis (MS), cerebrospinal fluid biomarkers have played a diagnostic role since the introduction of Poser's criteria in 1983, with IgG oligoclonal bands playing a supporting role in an epoch prior to magnetic resonance imaging and a complementary one after the introduction of McDonald criteria in 2001. Nowadays, that supporting role has turned into a main one in substituting for dissemination in time and defining the diagnosis of MS in patients with a first clinical event, according to the 2017 revised McDonald criteria. Possibly kappa free light chains, N-CAM, chitinase 3-like protein 1 and IgM oligoclonal bands, not yet implemented in clinical practice, could similarly gain importance in the near future. Furthermore, the increasing knowledge of molecular mechanisms leading to chronic inflammation has enhanced interest in looking for biomarkers of disease activity, better defining the MS phenotype and patients with highly active disease. Accordingly, myelin proteins, intermediate filaments, metalloproteinases and other molecules involved in the inflammatory cascade, are currently under investigation. Finally, it has long been known that axonal loss occurs from the early phases, leading to a progressive neurological deterioration. Since established criteria to assess treatment failure and transition to progressive forms are still lacking, both treatment response and prognostic biomarkers would be useful to predict MS course, and neurofilaments seem to have this potential. The purpose of this review article was to illustrate biomarkers that have been already validated or require further validation after proving to be useful in exploratory studies and potentially could prove useful in clinical practice in the coming years.

Keywords: Multiple sclerosis, biomarkers, cerebrospinal fluid, neurofilaments, oligoclonal bands, disease activity

INTRODUCTION

In the framework of neurological diseases, the need for objective and measurable indicators of the underlying pathological processes is more and more pressing. Therefore, the search for biological markers, or biomarkers, is a continuously expanding field, and a large number of molecules have been explored so far; but only a few have been validated, and even fewer are currently used in clinical practice[1].

Indeed, the assessment of the clinical validity and utility of a biomarker requires a multistage process, which has been proposed as a five-phase procedure going from preclinical exploratory studies (phase 1) to clinical assay development (phase 2), retrospective studies (phase 3), prospective diagnostic accuracy studies (phase 4), and disease burden reduction studies (phase 5)[2]. Moreover, the level of evidence relies on the number of supporting studies and patients included while exploring the independence of the results in independent cohorts[3].

With effective therapeutic strategies in neurodegenerative diseases still lacking, biomarkers would allow us to define the start time of degeneration and make an early diagnosis, to monitor the disease course and predict the prognosis, but also to identify potential therapeutic targets[4].

In the context of inflammatory diseases, biomarkers would be useful to specifically define the involved actors of the immune response and potential therapeutic targets, and also to better understand the etiopathogenesis and to monitor disease activity and treatment response[5].

Multiple sclerosis (MS) is a challenging disease, since it is now clear that both inflammatory and degenerative components occur since early phases[6]. Although the introduction of the first approved medications for progressive MS (PMS) is a recent achievement[7,8], therapeutic options are actually limited in progressive phases. The majority of currently available disease-modifying drugs (DMDs) target inflammation, and therapeutic efforts are mainly focused on early phases of the disease, with the purpose of influencing long-term evolution[9]. It is for this reason that the concept of "no evidence of disease activity" (NEDA) has been introduced as the main therapeutic goal to achieve in patients with MS. Extensively used in clinical trials to define and compare the efficacy of DMDs, the concept of NEDA is still evolving, integrating an increasing number of measures able to define the absence of disease activity. Even though the achievement of this goal is the main one in a clinical real-life setting while monitoring patients under treatment as well, long-term studies are needed to provide evidence of its utility in clinical practice[10]. Currently, NEDA-3 is mainly used in clinical trials and considered the aim of therapeutic strategies in clinical practice, consisting in the absence of relapses, magnetic resonance imaging (MRI) activity and sustained disability worsening during follow-up[11]. Indeed, the increasingly prominent role of MRI and recent advances in technology have led to the inclusion of the measurement of brain volume loss in NEDA (NEDA-4), which may further evolve with the inclusion of biomarkers (NEDA-5)[12]. In this respect, the role of neurofilament light chain (NF-L) as a marker of disease activity, correlating with long-term prognosis seems to be promising[11]. The availability of biological markers reflecting such a disease heterogeneity would definitely help us to better understand its complexity and would be an instrument of unquestionable value.

According to the functional classification provided by the FDA-NIH Biomarker Working Group, such molecules can be categorized in susceptibility, diagnostic, monitoring, prognostic, safety and response biomarkers[13] [Table 1].

Susceptibility biomarkers would be useful to detect among asymptomatic individuals those at risk of developing MS, potentially including genetic investigation in first-degree relatives of MS patients[13].

Table 1. Clinically useful and validated CSF biomarker in MS

	Status	Function	Evidence
IgG OCB	Clinically useful	Diagnostic	Nearly 86% specificity and more than 95% sensitivity for the diagnosis of MS[19]. Implemented in 2017 McDonald criteria as indicator of DIT[20]
		Prognostic for conversion	Associated with higher risk of conversion in MS when detected in CIS[28,29] and RIS[30-32]
IgG index	Clinically useful	Diagnostic	Positive values found in 70-80% of MS patients[18]. Useful as a complementary tool, without replacing CSF IgG OCB[41]
		Disease-activity	Associated with MRI activity[45]
		Prognostic for conversion	Associated with higher risk of conversion in MS when detected in CIS[43]
		Prognostic for progression	Associated with disability progression[44]
KFLC	Validated	Diagnostic	Useful for the diagnosis of MS[49,51,53,54,58]. Increased levels detected in MS patients with no IgG OCB[50,55,62]
		Prognostic for conversion	Associated with higher risk of conversion in MS when detected in CIS[43,60]
		Prognostic for progression	Associated with disability progression[60,64-66]
IgM OCB	Validated	Disease-activity	Associated with aggressive disease course[248,250]
		Prognostic for conversion	Lipid-specific IgM OCB are associated with higher risk of conversion in CIS patients[252,253]
		Prognostic for progression	Associated with disability progression and conversion to SPMS[247,248,256]
		Treatment-response	Lipid-specific IgM OCB predict a decreased response to IFN-β[256]
N-CAM	Validated	Diagnostic	Lower levels detected in MS patients and in PPMS compared with RRMS ones. Considered as an indicator of poor remyelination and repair[180,181]
		Disease-activity	Increased levels detected after relapses, especially under steroid treatment, and related to clinical remission[183]
CHI3L1	Validated	Diagnostic	Increased levels in MS and NMO patients[185,188,189]
		Prognostic for conversion	Associated with higher risk of conversion to MS in CIS patients[190,192]
		Disease-activity	Increased levels associated with higher clinical and MRI disease-activity[190,193]
		Treatment-response	Increased levels in non-responder patients under IFN-β treatment compared with responders[193]
NFs	Validated	Prognostic for conversion	In RIS increased CSF NF-L are an independent risk factor for the conversion into CIS and MS, with greater values related to shorter times of conversion[32]. Associated with higher risk of conversion to MS in CIS patients[224,234]
		Disease-activity	Double NF-L levels in relapsing patients compared with remitting ones[228]. CSF NF-L levels correlate with NEDA-3, MRI activity and brain atrophy[11]. Serum NF-L in early phases contributed to predict the lesion load and brain volume loss over a period of 10 years[238]
		Prognostic for progression	High NF-L concentrations associated with progression in both clinically stable patients and relapsing ones[226,227]. In CIS patients with optic neuritis, CSF NF-L predicted long-term cognitive and physical disability over a follow-up period ranging between 9-19 years[235]. Higher NF-H levels in SPMS patients[224,225]
		Treatment-response	NF-L concentrations decreased after 12-24 months of immunosuppressive therapy in active progressive MS patients[239], after switching from first-line therapies to fingolimod[240] and after 12 months of NTZ[241,242]
MBP	Validated	Disease-activity	Higher values detected in active RRMS compared with stable patients and progressive MS. Increased levels in MS are temporally related to relapses and detectable up to 5-6 weeks after, with greater values in polysymptomatic and severe exacerbations[158,159,166-168]. Reduced levels after steroid treatment[168,169]
GFAP	Validated	Prognostic for progression	Elevated levels in MS compared with controls[265-267], with higher values in patients with EDSS greater than 6.5[266]. Associated with greater EDSS score, longer disease duration and progressive course[268]. Increased levels of GFAP in MS predictive for the disability achieved 8-10 years later[267]
		Disease-activity	Associated with MRI parameters as infratentorial chronic lesion load and the intensity of Gd+ in both CIS and RRMS patients[269]
MMP-9	Validated	Disease-activity	Elevated values during clinical relapses, related to a greater number of MRI Gd+ lesions[144]. Higher values in MS compared with controls and in RRMS compared with PPMS[148]
		Treatment-response	Decreased levels after treatment with IFN-β[152-154] and NTZ[155]
CXCL13	Validated	Diagnostic	Higher levels in MS patients compared with controls, though low specificity[126-128]
		Prognostic for conversion	Associated with higher risk of conversion to MS in CIS patients[130]
		Disease-activity	Associated with clinical and radiological activity[126,127]. Decreased levels after steroid treatment[127]
		Treatment-response	Decreased levels after treatment with NTZ[127,132], RTX[129,131]

OPN	Validated	Diagnostic	Significantly greater levels in MS patients compared with controls[102,107,108,110]
		Disease-activity	In RRMS patients, higher levels detected in active disease compared with stable disease and during relapses compared with remission phases[100-103]
NO metabolites	Validated	Disease-activity	Increased levels in body fluids of MS patients, particularly RRMS compare with SPMS. Higher values detected during relapses[78,90]
MRZ reaction	Validated	Diagnostic	A humoral response against at least 2 of 3 viruses is detected in 78% of patients with MS with high specificity[73]
		Prognostic for conversion	Associated with higher risk of conversion in MS when detected in CIS[69,70]

MS: multiple sclerosis; CIS: clinically isolated syndrome; RIS: radiologically isolated syndrome; MRI: magnetic resonance imaging; OCB: oligoclonal bands; RRMS: relapsing-remitting multiple sclerosis; SPMS: secondary progressive multiple sclerosis; NMO: neuromyelitis optica; NEDA: no evidence of disease-activity; N-CAM: neuronal cell adhesion molecule; CHI3L1: chitinase-3-like-1; MBP: myelin basic protein; GFAP: glial fibrillary acidic protein; Gd+: gadolinium-enhancing; MMP-9: matrix metalloproteinase-9; CXCL13: C-X-C motif ligand 13; NFs: neurofilaments; NF-L: light chains of neurofilaments; NF-H: heavy chains of neurofilaments; OPN: osteopontin; NO: nitric oxid; MRZ: measles-rubella-varicella; NTZ: natalizumab; RTX: rituximab

Diagnostic biomarkers should be able to confirm the diagnosis of MS, improving diagnostic accuracy when applied together with clinical and MRI criteria. Thus, they can allow clinicians to exclude other possible differential diagnoses, including different autoimmune disorders and other neurological diseases. They should also ideally detect patients with clinically isolated syndrome (CIS) and radiologically isolated syndrome (RIS) and distinguish between different subtypes of the disease[5,13].

Monitoring biomarkers play a relevant role in MS, allowing neurologists to serially assess the status of the disease[13]. Particularly, disease activity biomarkers may crucially affect therapeutic decisions by detecting high disease activity and rapid disability worsening in early phases of MS[9,14]. Correlating with clinical and radiological activity, they may aid in identifying aggressive forms of MS and also provide an indirect assessment of low therapeutic response in patients under DMDs[1].

The definition of prognostic biomarkers as a separate class is slightly more controversial, since a prognostic impact is recognized in other categories of biomarkers[1]. Indeed, those markers able to predict either the risk of relapses or progression or both would belong to this group[13]. However, this term is usually attributed to those molecules reflecting axonal damage, astrocyte activation and remyelination, prevailing in progressive phases of disease[4]. They would also be important in identifying transitional progressive forms of MS, since reliable indicators are not available[9]. Alongside these, many studies have considered those molecules that are predictive of conversion to clinically definite MS when detected in patients with CIS as being "prognostic". To distinguish these "prognostic for conversion" biomarkers from the aforementioned "prognostic for progression" ones, their role in this review will be discussed as belonging to the diagnostic category for conceptual similarity.

Finally, the monitoring of treatment response in terms of both efficacy and safety may be very important in personalizing therapies and planning switches whenever appropriate[5] and may be of benefit in the use of pharmacodynamic/response and safety biomarkers[13].

However, boundaries are blurred and some markers may exhibit more than one function. Moreover, to be validated, exploratory molecules have to be reproducible among independent studies, and only easily detectable and cost-effective ones truly impacting the diagnostic therapeutic processes would be used in clinical practice[5].

For both anatomic and physiological reasons, cerebrospinal fluid (CSF) represents the main source of potential biomarkers for MS among body fluids[4]. Indeed, its composition may reflect the impairment of brain metabolism, the breakdown of the blood-brain barrier (BBB) and many ongoing processes occurring in the central nervous system (CNS) with a consequent production of catabolites[15]. However, requiring less invasiveness and due to blood continuity with CSF, serum samples are being increasingly used and explored as a source of biomarkers[1].

Considering the extensive number of molecules that are currently under investigation and detected in body fluids, this review will focus on CSF biomarkers that are currently used in clinical practice, those that have not been clinically implemented although validated and those requiring further validation after proving to be useful in small exploratory single-site studies. Since some molecules may potentially fall in more than one category according to a functional classification, they will be classified according to their main recognized role.

DIAGNOSTIC BIOMARKERS

IgG oligoclonal bands

CSF IgG oligoclonal bands (IgG OCB) are detected in almost 90% of patients with MS and in nearly 70% of patients with CIS[16]. It does not seem that OCB-negative MS shows different characteristics, even though a different immunogenetic phenotype of HLA-DRB1 has been identified in some studies[17]. Among several techniques, isoelectric focusing followed by immunofixation in parallel CSF and serum samples is mainly used for their detection due to a high sensitivity[18,19]. Of all possible patterns, type 2 is detected when at least two bands of IgG are present in CSF but not in serum, which is suggestive of intrathecal IgG synthesis and thus of an inflammatory disease of the CNS[18].

As a qualitative assessment, CSF IgG OCB detection is actually considered a more reliable test than any quantitative assessments of intrathecal synthesis[20]. With nearly 86% specificity and more than 95% sensitivity, CSF IgG OCB do not represent a pathognomonic finding of MS, but they may strongly support the diagnosis of MS when other causes of CNS inflammation have been ruled out[19].

In 2017, the latest revised McDonald criteria gave great significance to CSF OCB as a substitute for dissemination in time[20], increasing sensitivity in the diagnosis of relapsing-remitting MS (RRMS) in patients with a first clinical event[21]. CSF IgG OCB already had a role as a diagnostic biomarker before, first in Poser's criteria (1983) to define "laboratory supported" definite and probable MS diagnosis[22]. Years later, both the McDonald *et al.*[23] and Polman *et al.*[24] criteria considered the presence of CSF IgG OCB as sufficient to provide the evidence of dissemination in space (DIS), together with the detection of at least two MRI lesions consistent with MS. Although not being included in 2010 revised criteria[25], CSF analysis with IgG OCB detection still represents a common step of the diagnostic program, particularly because it may allow us to exclude other diagnoses adding a different type of information compared with MRI, which may be unable to allow the distinction between MS and mimics at early stages[26]. Moreover, it has been argued that the presence of CSF OCB may increase the specificity of those criteria when considered together with DIS[27].

Concerning the diagnosis of primary progressive MS (PPMS), the presence of CSF OCB is one of the required criteria[20] and its role has been confirmed over time in the consecutive revisions after Poser's criteria[23-25]. In addition to a diagnostic role, CSF IgG OCB also has a prognostic role for conversion to MS, since their identification in patients with CIS increases the risk to convert into clinically definite MS with a negative predictive value of 88%[28]. In a prospective study conducted by Tintoré and coworkers in 572 patients with CIS, the detection of CSF IgG OCB almost doubled the risk of a second relapse, regardless of baseline MRI, without affecting disability outcomes during a follow-up of 50 months[29].

Despite recognizing the presence of CSF IgG OCB as one of the factors predicting an increased risk to develop MS in patients with RIS[30], specific criteria have not been established in the 2017 latest revision[20]. Results from a recent study showed that the presence of CSF OCB in children with RIS increased the risk to develop pediatric MS and improved specificity of MRI criteria in these patients[31]. In another study conducted in 75 patients with RIS, CSF OCB also proved to be an independent risk factor for the conversion to CIS and MS and were associated with shorter times of conversion[32].

It has been suggested that the presence of CSF OCB, indicative of intrathecal synthesis, may both directly and indirectly perpetuate the inflammatory damage through the chronic stimulation of microglia via immunoglobulin and immunocomplexes. Occurring even once acute perivascular inflammation has stopped, such an activation facilitates further and mutual activation of both microglia and astrocytes. As a result, antibody-mediated inflammation promotes a microenvironment of chronic inflammatory damage and neurodegeneration[33]. From this perspective, the administration of a drug able to affect local humoral production would be further useful. However, among the currently available drugs, only natalizumab (NTZ) and cladribine proved to affect intrathecal Ig synthesis, ultimately leading to CSF OCB disappearance in some cases[34-37]. Nevertheless, the presence of CSF OCB does not seem to be associated with an aggressive disease course or a faster disease progression in MS[17,38].

IgG OCB represent a validated and clinically implemented biomarker for both the diagnosis of MS and the detection of CIS converters. Its validity relies on numerous confirmatory studies conducted in more than 200 patients, thus providing a strong level of evidence[3].

IgG index

The ratio between IgG quotient and albumin quotient, known as the Link Index[39], is largely used to assess a quantitative evaluation of intrathecal synthesis, enough to be considered an alternative to the detection of CSF OCB in previous MS diagnostic criteria[22-24]. However, the latest revision of McDonald criteria clearly states that the identification of CSF IgG OCB is superior to any quantitative assessments, whose results have to be cautiously considered when isolated or conflicting with the aforementioned tool[20]. In addition, it has been clearly defined in two different consensus statements that IgG index and other quantitative assessments are just complementary tests, less sensitive than qualitative detection of CSF OCB[18,19]. A value greater than 0.70 is universally considered suggestive of pathological intrathecal synthesis for IgG index, with abnormal values detected respectively in 70%-80% of patients with clinically definite MS[18]. With a cut-off value of 0.7, a positive predictive value by 60% for the diagnosis of demyelinating CNS disease has been found[40]. Considering that a correlation exists between IgG index and positive predictive value for MS, increasing IgG index values correlate with a greater probability of MS diagnosis[40]. Nonetheless, abnormal values are rarely detected in MS patients with no CSF OCB[41].

Nephelometry is the most used technique to measure albumin in CSF and serum as well, to provide a quotient that is a reliable measure of blood-CSF barrier function, especially when age-related[18,19]. This is crucial, since the increased concentration of a substance in CSF can be the result of either intrathecal synthesis or increased permeability of the blood-CSF barrier. Moreover, interindividual variability in serum IgG concentrations is similarly reduced by using a CSF/serum IgG quotient[18].

Applying different mathematical models, several indices have been derived[42], including Tourtellotte's, Reiber's, Link's and intrathecal IgG fraction. Actually, although the IgG index is the most commonly used quantitative measure of intrathecal synthesis in clinical practice, other indices using hyperbolic mathematical functions, such as Reiber's index, are considered more accurate, resulting in few false positives[18,19]. Senel and coworkers found 43% sensitivity and 64% specificity for IgG index regarding conversion of CIS to clinically definite MS, with a positive predictive value of 53% and a negative one of 54%[43].

As a prognostic biomarker, a very high IgG index has been related to a major disability progression with greater values in secondary progressive MS (SPMS) compared with PPMS and RRMS patients in a study conducted by Izquierdo and coworkers[44].

Finally, a recent retrospective study involving 149 patients with CIS and MS investigated a possible

association between CSF parameters and MRI activity. IgG index was highly correlated with the detection of new cerebral lesions on MRI scan and proved to be an independent predictor of future MRI activity[45].

Currently, IgG index is clinically implemented as additional evidence of CNS local humoral response, with the advantage of being based on easily achievable information from a simple CSF analysis[46]. Thus, it is useful in supporting the diagnosis of MS and could represent a complementary screening test in patients suspected of MS, without replacing the diagnostic value of CSF OCB[41].

Kappa free light chains and kappa index

CSF kappa free light chains (KFLC) result from intrathecal humoral activity of plasma cells. Being normal constituents of human Ig structure together with lambda light chains, they tend to accumulate together with Ig in inflammatory disease of CNS[47] and can be detected by ELISA, Western blotting[48] or nephelometry[49-51]. Several studies reported an increased concentration of free light chains in CSF of patients with MS[49,51]. As for IgG index, the use of a ratio between KFLC CSF/serum quotient and albumin quotient has been considered by the majority as the best method to represent intrathecal FLC synthesis[47,51], with some exceptions[49,52]. Conversely, lambda FLC Index did not prove to have comparable values of sensitivity and specificity, and it is currently not considered a potential diagnostic biomarker for MS[49,53].

KFLC index has been explored as a diagnostic biomarker, despite the lack of an unequivocal cut-off value currently causes some difficulties in comparing results from several studies [Table 2]. As an indicator of intrathecal synthesis, KFLC index correlates well with IgG index[54], using a cut-off value of 5, although showing greater sensitivity (more than 96% *vs*. nearly 50%) for CSF IgG OCB identification and MS diagnosis and according to higher negative predictive values, with comparable specificity.

Although different thresholds have been used in several studies, ranging from 4.25[55] to 12.3[53], KFLC index extensively proved to have a higher sensitivity and a lower specificity with a similar diagnostic accuracy compared with IgG OCB in discriminating MS and controls[53,55-59]. In a recent study by Gaetani and coworkers, KFLC index distinguished precisely as did IgG OCB between MS and non-inflammatory diseases using a cut-off value of 7.83[56]. It has been suggested that a higher cut-off value (10.6) could be useful to differentiate MS from other inflammatory diseases by increasing specificity and to predict conversion in CIS with greater accuracy as compared with OCB[56]. Similarly, high levels of CSF KFLC have also been demonstrated in CIS patients, showing a correlation with the risk of conversion to clinically definite MS within 2 years[43,60]. Moreover, unlike KFLC index threshold, a cut-off value for intrathecal KFLC synthesis has proved to be more reproducible[58,61,62].

Noteworthy, KFLC index proved to be increased in MS patients with no evidence of IgG OCB, amounting to almost 5% of cases[50,55,62], showing a greater sensitivity but a less specificity by using a threshold of 5.9. In a recent study by Ferraro and coworkers, a KFLC index ≥ 5.8 was detected in 25% of OCB-negative MS patients and in 98% of OCB-positive ones[63].

It has been hypothesized that KFLC index may replace IgG index as a first-line test, but some disagreement remains about the need to determine both KFLC index and IgG OCB in patients with suspected MS[63] or to use them sequentially[56]. Probably, the higher sensitivity of KFLC index compared with IgG OCB would allow clinicians to screen patients in a shorter time, with lower costs and the advantage of a quantitative assessment[49,58], restricting the use of IgG OCB to patients with positive KFLC index. Such a diagnostic route would allow clinicians to reduce false positive results when faced with an inflammatory disease of the CNS. Showing a comparable or higher specificity[50,54], IgG index could still have a role as a screening test complementary to KFLC index for the detection of intrathecal Ig synthesis. However, KFLC index currently shows an intermediate level of evidence as a diagnostic biomarker, requiring other confirmatory studies in larger cohorts[3].

Table 2. Different cut-off values for kappa index and characteristics of study cohorts

	Study cohort (number of analyzed paired serum and CSF samples)	True positives	True negatives	Cut-off	Sensitivity	Specificity	McDonald's diagnostic criteria
Crespi *et al.*[54]	385	MS (127)	Other neurological diseases: IND (117) NIND (141)	≥ 5	96	78	2017
Gaetani *et al.*[56]	170	RIS, CIS, MS (64)	Other neurological diseases (106): IND (24) NIND (82)	≥ 7.83	89	81	2010
Gurtner *et al.*[57]	320	RIS, CIS, MS (67)	Other neurological diseases (258): autoimmune (53), NIND (50), IND (38), degenerative (28), peripheral neuropathy (24), infection (13), cancer (11), neuromyelitis optica (10), others (31)	≥ 10.5	87	76	2010
Leurs *et al.*[59]	745 (from 18 centers)	CIS, MS (526)	Controls (219): IND (67) NIND (76) Symptomatic controls (49) Healthy controls (27)	≥ 6.6	88 93 (MS and controls)	83 83 (MS and controls)	2010 (84%) 2005 (16%)
Pieri *et al.*[53]	176	MS (71)	Other neurological diseases: IND (33) NIND (72)	≥ 12.3	93	100	2010
Presslauer *et al.*[58]	438 (from 4 centers)	CIS/MS (70)	Other neurological diseases (368), including meningitis/ encephalitis (41) Guillain-Barré (15) Neuroborreliosis (15) CIDP (7)	≥ 5.9	96	86	2010
Puthenparampil *et al.*[55]	137	MS (70)	Healthy controls (symtpomatic despite no neurological and systemic disorders) (37)	≥ 4.25	94	100	2017

MS: multiple sclerosis; CIS: clinically isolated syndrome; RIS: radiologically isolated syndrome; IND: inflammatory neurological diseases; NIND: non-inflammatory neurological diseases; CIDP: chronic inflammatory demyelinating polyneuropathy

It has also been pointed out that higher values of KFLC index are associated with greater disability[60,64-66], even though previous authors did not go in the same direction but hypothesizing a prognostic role for this marker[61,67].

Measles-rubella-varicella-zoster reaction

In the 1994 consensus report about CSF analysis in the diagnosis of MS, the detection of intrathecal Ig synthesis against neurotrophic viruses, such as measles, rubella and varicella-zoster, was considered a complementary diagnostic test for MS[18]. Such kind of local humoral response, called measles-rubella-varicella-zoster (MRZ) reaction (MRZR), has been reported in up to 94% of patients with MS if at least one intrathecal virus-specific response is detected[68], with anti-measles response as the most frequent one[69-71]. However, MRZR is usually considered positive if a humoral response against at least 2 of 3 viruses is reported, with a commonly used cut-off value of 1.5 for antibody index[72,73]. The reason for this local humoral response, which occurs without active replication of the virus[74], has not been entirely clarified[75]. An involvement of T lymphocytes promoting the differentiation of memory B cells into antibody secreting ones has been suggested[70].

High specificity of up to 97% for MRZR was also reported by Jarius and coworkers, who found a positive reaction in 78% of patients with MS compared to 3% of controls. Moreover, MRZR has proved to be able to

distinguish between MS and other diseases, such as neuromyelitis optica (NMO)[73], anti-MOG associated encephalomyelitis[73], and primary CNS lymphoma[72].

In a prospective 2-year study involving 89 patients with CIS, MRZ reaction was associated with a greater risk to convert to clinically definite MS, showing a greater positive predictive value (70%) than OCB (64%) and MRI (64%)[70]. In patients with acute optic neuritis with positive MRZR and MRI, conversion to clinically definite MS occurred in 86% of them after 4 years, with a prevalence of 73% for MRZR in those who converted[69]. Thus, MRZR can further support the diagnosis at onset and assist in discrimination between MS and other clinically similar inflammatory diseases, representing a complementary diagnostic biomarker with an intermediate level of evidence[3,74]. Nevertheless, further studies in additional cohorts are required[3].

DISEASE ACTIVITY BIOMARKERS

Nitric oxide metabolites

Due to the role of oxidative stress in MS pathogenesis, nitrate and nitrite have been investigated as disease activity biomarkers[76]. Indeed inflammatory processes produce, as a result of the activation of immune cells, reactive oxygen species, including nitrogen-based oxidants[76]. Moreover, Nitric oxide (NO) seems to have much more roles than being a blood flow controller and a synaptic transmitter, regulating the permeability of the BBB, exerting immunomodulatory properties and mediating axonal damage and demyelination[77].

Increased levels of nitrate and nitrite have been identified in body fluids of MS patients in several studies. Particularly, many studies have reported greater concentrations of these molecules in CSF[78-80], serum[81,82] and urine[83] of MS patients compared with controls. Accordingly, the inducible form of nitric oxide synthase has been detected in CSF of MS patients, while not in healthy controls[84], and its mRNA has been found in cerebral tissue of MS patients[77]. Interestingly, interferon-beta (IFN-β) has proved to exert a remarkable inhibition of inducible NO synthase expression in astrocytes[85].

Meanwhile, it is still controversial whether the concentration of NO metabolites is significantly different in RRMS compared with PMS. Indeed, some studies found higher CSF and serum levels of NO metabolites in RRMS compared with SPMS[86,87], while others did not detect any differences[80,88].

Speculating a role as a disease activity biomarker, the association between NO metabolites and the occurrence of relapses in RRMS patients has been explored, and several studies have confirmed this hypothesis[78,89-91], but longitudinal and multicenter studies are needed.

In a study by Yamashita *et al.*[78], significantly higher nitrite and nitrate levels were detected among patients in relapse compared with those in remission and patients treated with steroid in the previous 1-2 months. Acar *et al.*[90] found higher nitrate and nitrite concentrations in relapsing patients than in remitting ones, with the latter ones still showing greater values than controls. Accordingly, NO metabolites predicted disease activity with 71% specificity and 66% sensitivity. In contrast, few studies reported evidence of an association between NO metabolites and MRI findings[90,92], as well as between the development of disability and EDSS progression[92].

Osteopontin

Osteopontin (OPN) is a sialoprotein, whose role in bone remodeling has long been known[93]. Beyond this, it is closely linked to the immune system, since it mediates chemotaxis, cell adhesion and signaling, and it also promotes cytokine and interleukin (IL) function, inducing IL-12 and inhibiting IL-10 among others. In its soluble form, indeed, it is secreted by and also interacts with macrophages and activated leukocytes, reduces the inducible form of NO synthase, promoting inflammation. In its intracellular

form, it is expressed by dendritic cells and promotes Th17 and Treg differentiation[94]. Moreover, it is thought to mediate the upregulation of Th1 and Th17 cytokines, mainly IFN-γ and IL-17[95], and the inhibition of pro-apoptotic proteins, favoring T cell survival[96]. It has been suggested that a specific subset of Th1 cells, particularly arising in CSF during relapses, produces OPN, high levels of IFN-γ and matrix metalloproteinase-9 (MMP-9) after polyclonal stimulation, playing a pathogenetic role[97].

In experimental models of relapsing-remitting experimental autoimmune encephalomyelitis (EAE), OPN expression was constantly evidenced in microglia next to periventricular lesions and in neurons limited to the relapse phase, which increased in mice with greater disease severity[98]. Moreover, when recombinant OPN was given to mice, severe relapses occurred after 1-3 days. Conversely, knockout mice for OPN seemed to be protected from the development of severe EAE[96].

Accordingly, immunohistochemistry analysis of MS brain lesions in humans identified marked OPN expression immediately near the lesions, in vascular endothelial cells, microglia and astrocytes, which was greater in more active lesions[98,99].

High levels of OPN have been found in plasma of RRMS patients, with greater concentrations in patients with active disease compared with those without exacerbations[100-103] and during relapses compared with remissions[102,104,105]. Similar results were found in other studies[96,101,106], with significantly higher CSF and serum OPN levels in MS patients compared with controls[102,107-110]. A positive correlation between IL-17 and both OPN and IL-23 concentrations has also been found[106]. Moreover, CSF concentrations of OPN in MS patients, re-evaluated 5 years after sampling, proved to be not only elevated but also related to the occurence of relapses and to clinical severity[111]. It has been supposed that the increase in OPN during relapses has an inverse correlation with the concentration of serum extracellular proteasome, with marked effects on chemotaxis[112]. However, other studies did not find a clear association between OPN levels and disease activity[107,113].

According to some studies, SPMS patients exhibited elevated OPN values as well compared with controls[102,107], while a significant difference was not reported by other studies[104]. In a recent meta-analysis by Agah and coworkers, all MS patient subtypes showed higher OPN levels compared with controls, except for CIS[101]. However, greater concentrations were found in RRMS patients compared with all other groups and in those with exacerbations compared with patients with stable disease.

IFN-β proved to downregulate OPN and IL-17 in MS patients and to decrease the incidence of EAE and the amount of Th1 and Th17 cells in mice[114,115]. Indeed, RRMS patients treated with IFN-β showed OPN at similar levels compared to untreated patients in remission phase[96]. Glatiramer acetate and NTZ lead to the decrease of plasma OPN levels as well[107,116]. Several polymorphisms of the OPN gene have been investigated to find an association with disease course or activity[117-120]. A few have been correlated with the level of disability[118], with disease course and risk for conversion to SPMS[119,120], and with susceptibility to MS development and relapse rate[121].

Additional studies are needed to confirm the role of OPN as a useful disease activity biomarker.

C-X-C motif ligand 13

C-X-C motif ligand 13 (CXCL13), also known as B cell attracting chemokine (BCA-1), is a protein favoring the chemotaxis of mature B lymphocytes by interaction with its receptor CXCR5. This receptor is also expressed by CD4+ T follicular helper cells, CD4+ Th17 cells, activated Treg cells and a subgroup of CD8+ T cells[122].

Together with other lymphoid chemokines, it favors the organization of germinal centers in lymphoid follicles, including meningeal tertiary lymphoid organs in the CNS[122]. Indeed, CXCL13 has been found to be overexpressed in active MS lesions and in intrameningeal B-cell follicles of chronic white matter lesions, sustaining humoral autoimmunity and disease activity[122,123]. Not coincidentally, mice lacking CXCL13 develop milder forms of disease[124], and its expression correlates with intrathecal Ig syntesis[125].

In a recent meta-analysis conducted on 226 studies about the role of several cytokines in patients with MS, CSF CXCL13 levels proved to differentiate well between patients with MS and controls and to decrease after DMDs[126,127]. Accordingly, in a study by Khademi and coworkers, CSF CXCL13 was found to be significantly higher in infectious neurological diseases and MS. The latter group showed significantly higher values than CIS and other controls[128]. However, its lack of high specificity was confirmed by overexpression of CXCL13 in the CNS in other diseases, such as neuroborreliosis and primary CNS lymphoma[129]. Next to its diagnostic role, it also proved to be higher in CIS converting to clinically definite MS[130] and to correlate with both clinical and radiological disease activity[127,128]. Currently, its role as predictive for CIS conversion has an intermediate level of evidence, needing replication in additional cohorts[3].

Elevated levels of CSF CXCL13 also seem to decrease after B-cell depleting treatment such as rituximab[129,131], after methylprednisolone[127] and NTZ[127,132]. High CSF CXCL13 levels also correlated with low expression of immunoregulatory IL-10 and TGF-β1[127]. On the basis of this evidence, CSF CXCL13 has been mainly suggested as a disease activity and treatment response biomarker.

MMP-9

MMPs are zinc-endopeptidases, able to catalyze the cleavage of many substrates in several physiological and physiopathological processes. Indeed, MMPs play a role in tissue remodeling, angiogenesis and cell migration, but also in inflammation, wound healing and malignancies[133]. During inflammation, many molecules are able to activate MMPs, including reactive oxygen species and both TNF-α and IL-17 via NF-κB[134,135]. MMPs, in turn, are able to activate cytokines, adhesion molecules, receptors and microglia[136,137]. Moreover, MMPs may determine BBB dysfunction by proteolyzing capillary basement membrane and tight junction proteins between endothelial cells[133,138].

MMPs seem to be involved in several neurological diseases, such as MS, Alzheimer's disease, Parkinson's disease, cancer and cerebrovascular diseases[133]. In EAE, elevated levels of several MMPs have been found, considered responsible for major severity of the disease[133,139,140]. It has been supposed that MMPs may act in MS through the digestion of myelin basic protein (MBP) as well, besides favoring leukocyte leakage at post-capillary venules[138]. Among six subfamilies, gelatinases (MMP-2 and MMP-9) are constitutively expressed in brain and best explored in MS pathogenesis[133]. Particularly, there is slightly more evidence about MMP-9 as a disease activity biomarker in MS, while results on MMP-2 are more controversial[141,142].

Elevated levels of MMP-9 have been detected in serum and CSF of patients affected by MS and other neurological diseases compared with controls, showing an association with disease activity[143-149]. In a study by Lee and coworkers, higher values of MMP-9 were found during clinical relapses, also related to a greater number of MRI gadolinium-enhancing (Gd+) lesions[144]. Similarly, another study confirmed higher concentrations of CSF MMP-9 in MS patients compared with controls, more in RRMS compared with PPMS ones, but there was no unequivocal association with clinical disease activity[148].

Considering the role in MMP inhibition played by tissue inhibitors of MMPs (TIMPs), the ratio MMP-9/TIMPs has also been considered as an equally valid biomarker and has been found to be increased in the serum of MS patients compared with controls, accordingly to elevated MMP-9 levels[149].

An increased expression of MMP-9 in active MS lesions and in active borders of chronic lesions has been found in some studies employing brain tissue from MS patients[150,151], confirming previous results and corroborating MMP-9 as a potentially valid disease activity biomarker.

Some studies have explored the variations of MMPs levels in patients under DMDs. A significant decrease in serum MMP-9 mRNA in RRMS patients under IFN-β has been noted after a 12-month follow-up by Galboiz and coworkers[152] and confirmed by other studies[153]. Among these, changes in MMP-9 levels occurred under IFN-β-treatment in a study by Comabella and coworkers, with a trend of reduction during the first 3 months and then an increase until reaching baseline values. Worthy of note, a significant increase in TIMP-1 concentrations occurred in the responder group compared with non-responders[154]. A possible response to NTZ treatment has also been explored. Balasa and coworkers reported a significant decrease in serum MMP-9 after 8 months of treatment and a good correlation between the biomarker and disease activity[155], but this finding was not confirmed by other studies[156]. In NTZ-treated patients, decreased baseline levels of MMP-9 were found in patients who developed progressive multifocal leukoencephalopathy compared with those who did not[157].

However, additional studies are needed for its validation, providing evidence of its role as a potential disease activity biomarker for MS.

Myelin basic protein

It has long been known that MBP is a potential disease activity biomarker for MS[158], since it displays an acute damage to CNS myelin, despite not being specific for the disease[159]. MBP is a polypeptide that assures the preservation of myelin structure and membrane compaction[160]. Four human isoforms are known, one of them prevailing in adult CNS myelin as a polypeptide containing 170 amino acid residues[161]. MBP contains multiple epitopes, with the ones recognized by monoclonal and polyclonal antibodies mainly allocated in 80-100 residues[161,162]. MBP-specific effector T lymphocytes have proved to play an essential role in the pathogenesis of experimental EAE models[163], which is rather suppressed when T cells are inhibited by MBP-specific Tregs[164].

Several studies have found increased CSF levels of MBP in patients with MS, temporally related to relapses[158,159,165-167] and detectable up to 5-6 weeks later[168]. Accordingly, RRMS patients with disease activity show higher values than progressive MS and stable patients[165]. CSF MBP concentrations are also greater when polysymptomatic and severe relapses occur, correlating with EDSS score and MRI activity and decreasing after corticosteroid treatment[168,169]. Zhou *et al.*[170] explored the association between MBP gene variations and MS course in a 5-year prospective study involving 127 patients with CIS, identifying a risk genotype (CT+TT of rs12959006) for the risk of conversion to MS, disability progression and relapses. MBP-like material has been found in the urine of MS patients as well, although its concentration fluctuates and does not seem to be temporally related to acute myelin damage. Conversely, higher values have been found in SPMS patients and are supposed to be related to disease progression[161]. Considering the role of MBP in the pathogenesis of MS and its potential role as a therapeutic target, several clinical trials have been carried out or are currently ongoing to evaluate possible new drugs[171-174]. However, this biomarker has not been validated and the preliminary results need to be replicated in additional cohorts.

Neuronal cell adhesion molecule

Neuronal cell adhesion molecule (N-CAM) is considered a marker of repair and remyelination[175] and it is mainly expressed in the CNS, but its involvement in neoplastic diseases has also been documented[176]. During the development of the CNS, the polysialylated form of N-CAM is actively involved in myelination, axonal growth and neural cell migration[177]. It has been found to be expressed by neural precursors of oligodendrocytes, astrocytes and neurons, supporting the process of myelination in the olfactory bulb in mouse brain[177].

In animal models, increased N-CAM expression has been identified in astrocytes in acutely demyelinated areas[178] and, similarly, in areas damaged by kainic acid[179]. Soluble forms of N-CAM have also been found to be involved in peripheral nerve myelination and repair, with Schwann cells expressing specific receptors for the molecule[177]. Both soluble and membrane-bound forms of this molecule exist, with different and little known expression and specific functions, and N-CAM belongs to the immunoglobulin superfamily[180]. Normal CSF values of soluble N-CAM range between 460 and 1,060 ng/mL[177]. Among several neurological diseases, CSF levels were found to be reduced in MS patients, who showed a mean value of 250 ± 107 ng/mL, compared with healthy controls (mean value of 412 ± 109), with similar findings when comparing patients affected by Alzheimer's disease and meningitis with controls, regardless of age and gender[180]. Moreover, PPMS patients exhibited lower levels compared with RRMS ones[181]. These data confirmed the results of a previous study showing lower soluble N-CAM concentrations in non-acute phase MS patients compared with controls and acute-phase MS patients[182]. In the last group, indeed, increased CSF N-CAM levels were noted, gradually increasing in the first week after relapse and correlating well with the remission of symptoms[183]. Moreover, comparing acute-phase patients who underwent steroid treatment with those who did not, significantly greater values were recorded in the first group[183]. However, steroid treatment does not determine an increase in N-CAM levels in itself, and this finding was not reported in non-acute phase MS patients who were treated[183].

Among DMDs, NTZ and mitoxantrone proved to significantly increase N-CAM levels in MS patients, while fingolimod did not[181].

Considering the evidence of lower N-CAM levels in PPMS compared to RRMS[181], in RRMS compared to CIS, and in polyneuropathy compared to Guillain-Barré syndrome[180], this molecule is actually considered mainly as an indicator of scarce repair capability more than a marker of severe neuronal damage[180]. However, it is not currently used in clinical practice and needs further validation[1,184].

Chitinase-3-like-1

Chitinase-3-like-1 (CHI3L1) (or YKL-40) belongs to the family of chitinases, enzymes that catalyze the cleavage of chitin by hydrolysis. Its biological role in humans has not been definitely clarified, despite many proofs of its involvement in several processes exist, such as tissue remodeling, angiogenesis, tumorigenesis and inflammation[185]. Belonging to the same family, chitotriosidase is known to be associated with several diseases, including infectious and inflammatory ones[186].

Though it is not a specific marker for MS, CSF CHI3L1 levels have been found to be increased in RRMS and NMO patients compared with controls, including healthy people, patients suffering from other inflammatory diseases and SPMS patients[185,187,188]. Conversely, serum CHI3L1 levels were not significantly different between groups in the aforementioned studies[185,187]. Elevated levels of CHI3L1 were also detected in both PPMS and SPMS compared with healthy controls[189]. Patients who fulfilled diagnostic criteria for active progressive MS or showed elevated levels of MMP-9 and CXCL13 also had higher concentrations of CHI3L1[189]. However, as a diagnostic biomarker, CHI3L1 needs further replication in additional cohorts[3].

Particular attention has been given to the prognostic role of this molecule, whose CSF concentration has proved to be an independent predictor for the risk of conversion to clinically definite MS in CIS[187,190-192], but not in RIS[32]. In a study by Comabella and coworkers, CSF CHI3L1 levels additionally correlated with shorter latency time of conversion and with disability progression during follow-up and radiological disease activity[190].

In a large multicenter study involving 813 patients with CIS, the aforementioned results were confirmed. Not only CSF CHI3L1 concentration was associated with the risk of conversion to clinically definite MS,

but it also was correlated with shorter time to conversion and to disability worsening, for which it was an independent risk factor[192]. As a consequence, there is strong evidence of its role as a biomarker able to predict CIS conversion, and it should be assessed for clinical implementation[3].

As a treatment response biomarker, serum CHI3L1 levels were measured in 76 RRMS patients under IFN-β treatment and were found to be increased in the non-responder group compared with the responder one. As there was such a difference since baseline, it was suggested that non-responders had higher disease activity and accordingly greater CSF CHI3L1 levels[193].

Other biomarkers requiring further validation

Several T-cell cytokines have been explored as potential biomarkers for MS, but which are crucial in MS pathogenesis has not been entirely elucidated yet[194].

IL-12 and IL-23 respectively induce the differentiation of naive T cells in IFNγ-producing Th1 cells and IL-17-producing Th17 cells[195]. Both interleukins increase the encephalitogenic potential of T lymphocytes, but only IL-23 has been found to be a critical molecule in the development of EAE[196]. On the basis of results coming from EAE models, where animals improved after administration of neutralizing antibodies against the shared IL-12/IL-23 p40 subunit, a phase II double-blind placebo-controlled trial with the monoclonal antibody ustekinumab was conducted in 249 RRMS patients, although it did not show substantial efficacy[197].

Differently, IL-17 does not seem to be crucial to EAE development, though increasing its severity and atypical presentation, maybe through the recruiting of neutrophils and the effect of MMPs[194]. Nevertheless, increased IL-17 mRNA expression in mononuclear cells was found in MS lesions and in CSF and blood of MS patients[198,199], and Th17 cells were found to undergo a more marked increase in CSF during MS relapses than Th1 cells, which usually prevail in both blood and CSF[200]. A monoclonal antibody against IL-17A (secukinumab) has proved to reduce MRI activity in MS, but further studies are needed[201].

Tumor necrosis factor alpha (TNF-α) is a cytokine involved in the pathogenesis of several autoimmune diseases, including MS, wherein increased CSF and serum levels of this molecule have been detected[202-204]. Today, it is known that TNF-α may exert different biological effects, depending on the involved receptor, both stimulating inflammatory processes and apoptosis (via TNF receptor 1) or inducing a pro-survival pathway and reducing inflammation (via TNF receptor 2). This might explain the failure and the unexpected results of treatment approaches with unselective anti-TNF-α drugs in MS patients, which lead to an increase in disease activity in MS[205,206]. The modulation of TNF-α signaling has provided promising results in EAE, whose remission has been induced by selective inhibition of the soluble form of TNF-α, which mainly acts via TNF receptor 1[207].

B cell-activating factor (BAFF), belonging to the TNF family, is a maturation and survival factor for B lymphocytes, whose serum levels have been found to be increased in several autoimmune diseases[208]. In MS, increased BAFF concentrations in CSF and in demyelinating lesions have been detected[209]. The association with disease activity has not been elucidated, since some controversial results have been reported[1,209]. Moreover, the clinical significance of increased BAFF levels under treatment with some DMDs is not clear[209].

PROGNOSTIC BIOMARKERS

Neurofilaments

Neurofilaments (NFs) are components of the neuronal cytoskeleton, responsible for the increase in nerve conduction velocity in myelinated fibers and for their structural support[210]. Consisting of heavy (NF-H),

medium (NF-M) and light (NF-L) chains[211], their detection in CSF and blood samples has been the subject of interest for years. Several studies investigated the increase in NFs in several neurological diseases[212], such as amyotrophic lateral sclerosis[213], Alzheimer's disease[214], frontotemporal dementia[215], stroke[216], MS[211], Huntington disease[217], atypical parkinsonian syndromes and neurocognitive impairment in HIV-positive individuals[218].

In most cases, NFs have been investigated as a potential prognostic and disease activity marker related to axonal damage, speculating a relation between the quantitative amount of CSF and serum NFs and the rate of neurodegeneration[218].

In MS, NFs have also been extensively examined as a diagnostic, disease activity and drug response biomarker. Moreover, serum NF-L levels, detected through a single molecule array (Simoa), appear strictly related to CSF levels[219-222], despite being approximately 42-fold lower[223]. Cut-off values have not been unequivocally established as for CSF ones. However, serum NF-L values between 16-20 pg/mL have been identified as a normal range among a heterogeneous group of healthy controls enrolled in various studies[211], without gender difference and with a trend to increase along with age-related physiological axonal damage[223].

Particularly, while the detection of higher CSF NF-H levels in SPMS patients suggests a major correlation with chronic axonal damage and is accordingly age-related[224,225], CSF NF-L seem to be better related to acute axonal damage due to inflammation. Indeed, increased levels of NF-L were found in CSF of MS patients compared with controls, with greater concentrations during exacerbations. Moreover, such high concentrations were associated with progression in both clinically stable patients and relapsing ones[226,227]. A recent meta-analysis confirmed these results, finding higher CSF NF-L levels in RRMS patients compared with PMS and double concentrations in relapsing patients compared with remitting ones[228].

In a longitudinal study involving 22 IFNβ-1a- and riluzole-treated patients and 20 IFNβ-1a- and placebo-treated ones with early MS, serum NF-L concentrations were assessed over a 24-month period, correlating well with EDSS changes, Gd+ lesions and the development of brain atrophy. Moreover, increased serum NF-L levels were associated with worse results in neuropsychological tests assessing visuospatial functioning, recall and both verbal and non-verbal episodic learning[229].

Similar results concerning the association between serum NF-L levels and cognitive impairment in early stages of MS[230] and between serum NF-L concentrations and EDSS changes[231] were confirmed by other studies, though not all agreed[232]. Despite correlating with EDSS in PMS patients, serum NF-L levels failed to correlate with EDSS progression in the previous year and during a median follow-up of 27 months. Particularly, serum NF-L increased in all PMS patients, including those who did not exhibit changes in EDSS or an increase in disability[232].

In patients with RIS, increased CSF NF-L levels were found to be an independent risk factor for the conversion to CIS and MS. Matute-Blanch and coworkers considered a cut-off value equal to 619 ng/L, since greater values were related to shorter times of conversion[32]. CSF NF-L concentrations have been found to be increased in patients with CIS as well[233], with greater ones in those who converted to clinically definite MS[224,234]. Despite these promising results, their role as prognostic biomarker for CIS conversion is still weak, and replication in larger cohorts is needed to confirm it[3].

In 86 CIS patients with optic neuritis as the first clinical event, CSF NF-L levels also predicted long-term cognitive and physical disability over a follow-up period ranging between 9 and 19 years[235].

As a disease activity and prognostic biomarker, the amount of CSF NF-L levels showed a significant association with NEDA-3 status, MRI activity and brain atrophy and significantly correlated with serum NF-L ones[11]. In several studies, serum NF-L also correlated with MRI activity, predicted the development of brain volume loss in a period of 2 years and decreased under DMDs[236,237]. A recent study obtained similar results, with serum NF-L detected in early phases contributing to the prediction of lesion load and brain volume loss over a period of 10 years[238].

CSF NF-L concentrations proved to decrease after 12-24 months of immunosuppressive therapy in active progressive MS patients[239] and after switching from first-line therapies to fingolimod in RRMS ones[240]. Moreover, compared with NF-H, CSF NF-L has been found to be superior as a therapeutic biomarker after 12 months of NTZ-treatment in RRMS patients[241,242]. Nevertheless, the potential role of CSF NF-L as a treatment response biomarker is severely limited by the invasiveness of performing serial lumbar punctures. Conversely, serial serum NF-L assessments would represent a more easily detectable marker and a reliable indicator of CSF NF-L levels[219,221]. Results from a recent study conducted on 15 MS patients treated with alemtuzumab and monitored with serial serum NF-L measurements were significant[243]. Indeed, serum NF-L levels correlated well with clinical and radiological activity at baseline and during follow-up, decreasing within 6 months from drug administration until reaching stable values under 8 pg/ml in those patients who achieved NEDA-3. Moreover, patients who showed clinical and radiological disease activity during follow-up also exhibited increased levels of serum NF-L up to 5 months before relapses.

So far, several studies have confirmed the reliability of NF-L as a disease activity and treatment response biomarker for MS, even though it does not represent a MS-specific biomarker. However, a precise cut-off is still missing, precluding the chance to stratify the risk of clinical and radiological disease activity according to NF-L levels. The opportunity to consider only intra-individual values is still debated, without focusing on their deviation from values reported in healthy people[237].

Further replication in larger, multicenter cohorts is needed. A randomized controlled trial, prospectively recruiting 900 patients from 45 sites in the USA, will provide further information about the potential role of serum NF-L as a prognostic and treatment response biomarker for MS[244].

IgM oligoclonal bands

Unlike small and monomeric IgG, IgM are large molecules consisting of pentameter units and ten antigen-binding sites and are strong activators of complement[245]. In a similar way to CSF IgG OCB, their identification is considered a sign of intrathecal synthesis, suggesting an inflammatory disease of the CNS[246]. However, CSF IgM oligoclonal bands (IgM OCB) are mainly considered a prognostic and disease activity biomarker than a diagnostic one, though not routinely used in clinical practice[1].

In a study involving 29 MS patients who were followed-up for 5 to 16 years, the presence of CSF IgM OCB was strongly associated with conversion to SPMS and the achievement of greater EDSS scores[247]. In a similar way, IgM OCB-positivity strongly predicted a severe disease course influencing the probability of developing greater disability in a cohort of 64 MS patients[248].

In patients with CIS, the identification of CSF lipid-specific IgM OCB was associated with greater MRI lesion load and brain atrophy at the first clinical event[249] and with an aggressive disease course[250]. Periventricular lesion load during the first years of disease proved to be related as well to the entity of IgM intrathecal synthesis in CIS patients, so that an active role of IgM in the development of demyelinating lesions has been supposed[251].

Moreover, both the risk of a second clinical event and its earliness were strongly increased when both CSF lipid-specific IgM OCB and IgG OCB were detected, as in 22% of 192 patients with CIS[252]. In another

study by Ferraro and coworkers, the identification of CSF IgM OCB in CIS patients was predictive of the occurrence of another relapse within a year[253]. Results from a blinded multicenter study involving 52 neurological patients and 13 centers confirmed the reproducibility of the test[254]. However, further confirmatory studies in additional cohorts are needed, and IgM OCB detection currently has an intermediate level of evidence as a predictive biomarker for CIS conversion[3].

The presence of CSF IgM OCB has been also associated with a severe disease course in RRMS patients, while it seems to be less frequent among PPMS compared with RRMS ones[255]. Strong evidence of its value as a prognostic biomarker for RRMS exists[3,249], so its potential clinical implementation has to be evaluated.

Finally, there is little evidence for the possible interactions between DMDs and CSF IgM OCB. The response to IFN-β treatment in RRMS seems to be reduced in patients exhibiting CSF lipid-specific IgM OCB, who showed a minor reduction in relapse rate and a higher probability of achieving greater EDSS values[256].

NTZ has proved to reduce serum IgM and IgG levels after 2 years of treatment in a time-dependent manner[257]. In a study by Villar and coworkers, NTZ determined a decrease in CSF IgM OCB in patients with no active disease, with complete disappearance in 70% of them, while no effects were reported in those with active disease[258].

Glial fibrillary acidic protein

Glial fibrillary acidic protein (GFAP) is highly expressed in the cytoskeleton of astrocytes, and it belongs to the family of intermediate filaments, which are highly cell type specific[259]. Since GFAP is upregulated in astroglial cell activation (astrogliosis), occurring in many inflammatory and non-inflammatory diseases[167,259-261], it has been explored as a biomarker for MS. Particularly, reactive astrocytes proved to be actively involved in neurodegenerative diseases, probably losing their protective role and developing neurotoxic functions[262-264]. The glial scar itself, which physically protects a damaged area, may also physically obstruct remyelination[264]. Finally, A1 astrocytes were found in MS lesions, as in EAE models where they were associated with neuronal and oligodendrocytic death[262]. On the basis of these remarks, an association between GFAP and disability in MS has been investigated. Elevated CSF GFAP levels were found in MS patients compared with controls[265-267], showing higher concentrations in patients with EDSS greater than 6.5 compared with those with minor disability[266]. In a study by Högel and coworkers, serum levels of GFAP proved to be associated with a greater EDSS score as well, but also with longer disease duration and progressive course[268]. On this issue, positive correlations have been found between CSF GFAP and disease duration, likewise between serum GFAP and disease severity, in a cohort of 93 PPMS patients[264]. In a study by Axelsson and coworkers, the increased levels of GFAP in MS patients were predictive for disability resulting 8-10 years later, confirming the association of this molecule with disability and progression in MS patients[267]. A similar result was obtained in a more recent longitudinal study involving 301 patients with CIS/MS with a mean follow-up time of 11 years, showing a correlation between GFAP levels and an early progression in the EDSS score[234]. However, further studies are needed to confirm its role as a prognostic biomarker for MS.

Evidence of an association between GFAP and high disease activity also exists, showing correlation with MRI parameters such as infratentorial chronic lesion load and the intensity of Gd+ in both CIS and RRMS patients[269]. Effectively, there is evidence that GFAP may increase in CSF and serum soon after (4-24 h) traumatic brain injuries, as a marker of acute lesion[261]. Due to its high cell type specificity and good correlation with neuronal degeneration, GFAP is currently considered a potential prognostic biomarker for progression[4].

CONCLUSION

Research on biological markers is very active and current. At present, there are few molecules available, considering the hundreds under investigation. But they are continuously increasing due to a greater knowledge of MS and its underlying physiopathology. For instance, there are no clinically useful disease activity biomarkers, despite the large number of exploratory molecules described for this functional group.

As for the group of diagnostic biomarkers, previously dominated by IgG OCB analysis, the possibility to rely on quantitative, less expensive and less time-consuming assessments as a first-line screening, is moving forward.

Though it is true that CSF is the most suitable means for getting information about CNS physiopathology[15], it is equally true that much of interest is moving towards serum biomarkers. For quite some time, their clinical use has been limited by both a greater variability and very low concentrations, a problem overcome by the introduction of increasingly sophisticated tools (e.g., the detection of serum NF-L levels through Simoa)[222]. Furthermore, treatment response biomarkers, such as anti-IFN-β and anti-NTZ antibodies, are mainly determined in serum and have not been included in this review, which is focused on CSF biomarkers. Despite requiring a more invasive approach, CSF still represents a unique source of data about the CNS, enough to have been defined as a "liquid biopsy" of CNS[4]. This is even more true since the histological analysis of brain tissue cannot be routinely performed and almost any study on new potential biomarkers has to start from CSF analysis. There is no doubt that we are now able to diagnose and treat patients in early phases and even wondering about treating asymptomatic patients with only radiological signs suggestive of the disease. Thinking of how MS diagnosis has been revolutionized by MRI in the last 20 years, it would not be impressive if new and promising biomarkers might lead to a new revolution in MS in the coming years.

HIGHLIGHTS

1. CSF is a unique source of potential biomarkers for MS, despite requiring a certain invasiveness for its collection.
2. Only CSF diagnostic biomarkers are currently used in clinical practice, though hundreds of molecules have been validated as disease activity and prognostic biomarkers.
3. IgG OCB maintain a prominent role as a validated diagnostic biomarker and are considered an alternative tool to MRI which can substitute for dissemination in time according to the 2017 revision of McDonald criteria. They also retain a prognostic role for conversion to MS when detected in patients with CIS.
4. NF-L has proved to be a useful biomarker as indicator of disease activity in MS. The possibility of measuring NF-L at different time points through serum detection makes it also suitable for the monitoring of treatment response.
5. KFLC index has proved to be a more sensitive but less specific diagnostic biomarker compared with IgG OCB, representing a potential first-line assessment in patients with suspected MS and reducing the request for IgG OCB analysis. It has a role as a prognostic for CIS conversion biomarker as well, but the lack of a universal cut-off value still represents a limit.
6. IgM OCB show good potential as a prognostic biomarker, since they are associated with an aggressive disease course, a higher risk of conversion to MS in CIS patients, disability progression and conversion to SPMS.
7. Several disease activity biomarkers seem promising, though requiring further validation. Increased levels of NO metabolites, OPN, MBP, MMP-9, N-CAM, CXCL13 and CHI3L1 have been detected in a close temporal correlation with relapses.

DECLARATIONS

Authors' contributions

The conception and design of the study, conducted the literature review, drafted the manuscript: Toscano S
The conception and design of the study, and provided critical revision and final approval of the article: Patti F

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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