

# Biochemical and Immunological Markers of Multiple Sclerosis

[Multipl Sklerozun Biyokimyasal ve İmmunolojik Belirteçleri]

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## ABSTRACT

Multiple sclerosis is the most common neurological disease in young adults characterized by recurrent relapses and/or progression within the central nervous system. It is a complex disease in which several pathophysiological mechanisms such as axonal/neuronal damage, demyelination, inflammation, gliosis, remyelination and repair, oxidative stress and excitotoxicity, alteration of the immune system and disruption of blood-brain barrier are involved. Biological markers; reflecting the immunopathological process, indicating responses to therapeutic interventions and optimizing therapy; are needed for the development of process-specific therapies and the prevention of disability.

**Key Words:** Multiple sclerosis, biological marker, inflammation, demyelination, remyelination and repair, axonal damage, oxidative stress, immune system

## ÖZET

Multipl Skleroz Merkezi Sinir Sistemi'nde tekrarlayan relapslar ve/veya progresyon ile karakterize, genç erişkinlerde görülen nörolojik hastalıktır. Aksonal/nöronal hasar, demiyelinizasyon, inflamasyon, gliozis, remiyelinizasyon ve tamir, oksidatif stres, immün sistemde değişiklik ve kan beyin bariyerinin bozulması gibi çeşitli patofizyolojik mekanizmaların rol oynadığı kompleks bir hastalıktır. İmmunopatolojik süreci yansıtan, terapötik değişimlere yanıtı gösteren ve tedaviyi optimize eden biyolojik belirteçler, spesifik terapilerin gelişmesi ve kalıcı sekellerin önlenmesi için gereklidirler.

**Anahtar Kelimeler:** Multipl sklerozis, biyolojik marker, inflamasyon, demiyelinizasyon, remiyelinizasyon ve tamir, akson hasarı, oksidatif stres, immün sistem

## INTRODUCTION

Multiple sclerosis (MS) is the most important human inflammatory demyelinating disease characterized by recurrent neurological relapses and/or progression that occur from multifocal white matter and cortical lesions within the central nervous system (CNS) [1]. The prevalence of MS is 1 per 1000 people and the ratio of female to male patients is 1.5 to 1. MS has heterogeneous clinical presentations and courses, ranging from benign to classical relapsing remitting (RR; prevalence 45 %), primary progressive (PP; prevalence 20 %) and secondary progressive (SP; prevalence 45 %) or rare fulminant disease courses. Furthermore, the pathophysiological processes such as inflammation, demyelination, axonal damage, glial scarring and repair mechanisms of MS are not uniformly represented among patient groups, but can be selectively predominant in individual patients, thus contribute to the heterogeneity in phenotypic expression of the disease, its prognosis and response to disease modifying therapies. Moreover, a broad spectrum of genes are reported to be involved in MS susceptibility and disease progression, as well as in protective mechanisms [2]. Because of this heterogeneity of MS, a subtyping of patients by genetical, clinical, neuro-radiological, and neuroimmunological parameters will be necessary in future. Therefore the importance of identifying biological markers for MS has evolved over the past years [3].

In this review we will give an overview on the current status and potential applicability of biological markers for the diagnosis, classification, disease activity and prediction of clinical courses in MS.

The development of process-specific therapies will be impossible without the use of biological markers that can objectively reflect the targeted immunopathological process, select patients in which the pathogenic process predominates, indicate responses to therapeutic interventions, aid during the more rapid screening of therapeutic agents in the early phase of their development and provide a simple and less expensive monitoring tool in clinical trials and finally routine patient management [4].

On the basis of analysis of the published studies examining pathophysiological mechanisms in multiple sclerosis [5-10] all biomarkers proposed so far can be classified into one of these seven categories:

1. Biomarkers reflecting alteration of the immune system
2. Biomarkers of axonal/neuronal damage
3. Biomarkers of blood-brain barrier (BBB) disruption
4. Biomarkers of demyelination
5. Biomarkers of oxidative stress and excitotoxicity
6. Biomarkers of gliosis
7. Biomarkers of remyelination and repair

## I. Biomarkers Reflecting Alteration of the Immune System

### *I.1. Cytokines and their receptors*

MS is considered a T cell-mediated autoimmune disease in which Th1 cytokines play a crucial role. Patients with active MS are reported to have high circulating and cerebrospinal fluid (CSF) levels of tumor necrosis factor (TNF)- $\alpha$  compared with the stable phase of MS. Blood and CSF levels of interleukin (IL)-1 $\beta$ , interferon (IFN)- $\gamma$ , IL-2, IL-6 and IL-12 (p40) are shown to be increased in MS with respect to healthy controls. However, Th2 cytokines (IL-10, TGF- $\beta$ , IL-4) are downregulated during active phase whereas upregulated during disease remission. IL-12 (p70)/IL-23 can differentiate between RR and SP-MS stages [4].

### *I.2. Chemokines and their receptors*

CCR5, CXCR3, CXCL10, CCR2/CCL2 may aid in studying disease heterogeneity and proof-of-principle therapy trials. CCR5 is suggested as a candidate biomarker of Th1 T cells and CXCR3/CXCL10 as markers of activated T cells [4].

### *I.3. Complement-related biomarkers*

C3, C4, activated neo-C9, regulators of complement activation (CD35, CD59) are needed to assess disease heterogeneity and develop novel therapies. Activated neo-C9 reflects the formation of membrane-attack complex (MAC) that contributes to demyelination [4].

### *I.4. Adhesion molecules*

Adhesion molecule-mediated leukocyte migration into the CNS is considered to be a critical step in the pathogenesis of MS. E-selectin, L-selectin, ICAM-1, VCAM-1, CD31, surface expression of LFA-1 and VLA-4 are the most important adhesion molecules [4]. Increased serum levels of PECAM-1, VCAM-1, ICAM-1 and E-selectin are reported in MS patients [11].

### *I.5. Biomarkers reflective of antigen-processing and presentation*

CD40/CD40L, CD80, CD86, heat shock proteins (hsp) represent a very important but little explored category; CD40/CD40L is suggested as a candidate biomarker that can differentiate between RR and SP-MS stages. Dysregulation in the hsp system is the most prominent and consistent finding of gene expression studies in MS [4].

### *I.6. Cell-Cycle and apoptosis-related biomarkers*

Fas (CD95) and Fas-L, FLIP, Bcl-2 and TRAIL reflect both a defect in the regulation of immune cells and proapoptotic properties of CNS components. FLIP is an antiapoptotic protein overexpressed in MS, TRAIL reflects clinical response to IFN- $\beta$  therapy in MS [4].

### *I.7. Antibodies*

Autoantibodies reflect the presence, nature, and intensity of a certain autoimmune response [12]. However,

antibodies may have different biological functions in MS ranging from a primary involvement at different stages of the immunopathogenic cascade such as demyelination, facilitation of repair mechanisms and balancing of the natural autoimmunity system. Since the finding of elevated immunoglobulins (Ig), mainly IgG1 and IgG3 isotypes in the cerebrospinal fluid (CSF) of more than 90 % of MS patients [13], detection of oligoclonal Ig is an important diagnostic marker in MS [14–16]; although the antigen-specificities of CSF oligoclonal Ig bands still remain to be defined in MS. Most antibodies detected in MS are also found in other neurological and systemic conditions as well as to a lower extent in healthy controls. Antibodies play an important role in the immunopathogenesis in a major subset of MS patients which may benefit from B cell directed therapies such as plasmapheresis [17]. Despite the fact that antibodies lack diagnostic specificity in MS yet, antibodies may serve as biological markers for either prognostic purposes, monitoring disease progression or distinction of immunopathogenetic subtypes of MS [18].

### ***1.7.1. Anti-myelin oligodendrocyte glycoprotein (mog) antibodies***

Several CNS antigens including myelin basic protein (MBP), proteolipid-protein (PLP), myelin-associated-glycoprotein (MAG) and 2',3'-cyclic-nucleotide 3'-phosphodiesterase have been described as targets for auto-antibodies in MS, but their role in disease pathogenesis is obscure. Myelin oligodendrocyte glycoprotein (MOG) is a quantitatively minor type I transmembran protein, which is exclusively expressed in the CNS on the outermost surface of the myelin sheath and oligodendrocyte plasmamembrane. MOG is initially identified as a dominant target antigen for demyelinating antibodies in experimental autoimmune encephalomyelitis (EAE) induced by CNS tissue homogenates [19-20]. Findings of the studies related to anti-MOG antibodies in CSF and serum of MS patients are very controversial, with frequencies ranging from 0–80 % in MS patients and 0–60 % in healthy controls. A recent study indicating that IgG antibodies directed against native membrane-bound, glycosylated MOG are serologic markers for early inflammation in MS provides evidence for a possible prognostic role of anti-MOG antibodies [21]. Furthermore, the presence of MOG-specific auto-antibodies associated or not with anti-MBP auto-antibodies in patients with a first demyelinating event is reported to be highly predictive of definite MS [22].

### ***1.7.2. Antibodies against aquaporin-4***

The NMO antibody (NMO-Ig) is directed at the aquaporin-4 water-channel, a component of the dystroglycan-protein complex, located in astrocyte foot processes at the blood–brain-barrier [23,24]. If the sensitivity and specificity of NMO-IgG antibodies are confirmed, it will be possible to classify a subgroup of patients within the heterogenous disease complex of MS, and to stratify

NMO patients for specific treatments, such as plasmapheresis [25] or rituximab, a selective anti-CD20 monoclonal antibody [26].

### ***1.7.3. Antibodies to myelin lipids and glycopeptides***

An important myelin lipid antigen that may serve as a target for an autoantibody response is galactocerebroside (GalC), which accounts for 32 % of the CNS myelin lipid content. Anti-galactocerebroside (a-GalC) antibodies have been suggested to be MS-specific and are not found in healthy subjects. Anti-GalC antibodies identify mostly RR MS patients and, thus, have been proposed as an indicator of ongoing disease activity. Recently it is demonstrated that CSF114(Glc), a structure-based designed glycopeptide, is recognized by specific IgM serum antibodies in MS patients but not in blood donors and other autoimmune conditions [27]. Serum IgG and IgM anti-CSF114(Glc) antibodies are found to be correlated with clinical activity and cerebral MRI lesions. These findings are consistent with other recent studies demonstrating an enhanced antibody response in MS patients to other glycosylated antigens such as anti-Glc (alpha1,4)Glc(alpha) [28].

### ***1.7.4. Antibodies to axonal antigens***

A promising marker for monitoring axonal damage and thus for the conversion to chronic progressive MS are the cytoskeletal neurofilament proteins. Although most investigations concentrated on neurofilament proteins released into the CSF and/or serum upon axonal damage, increased levels of antibodies to the neurofilaments light subunit have also been found in primary or secondary chronic progressive MS [29,30] and show good correlations with clinical disability and brain atrophy. Moreover, antibodies to various gangliosides [31] and other neuronal antigens [32] have been described to be associated with disease progression in MS patients.

### ***1.7.5. Antibodies as biological markers for remyelination and repair***

Nogo-A, a protein associated with CNS myelin, impairs regenerative responses and suppresses sprouting and plastic changes of synaptic terminals [33]. The growth inhibitory effect of Nogo-A can be blocked by anti-Nogo-A antibodies that emerge as a challenging repair concept [34]. The protective and regenerative role of anti-Nogo-A antibodies has been shown in EAE [35]. Significantly elevated serum anti-Nogo-A IgM antibody levels are reported in MS [36].

### ***1.7.6. Antibodies to viral antigens***

The involvement of microbial infections in the pathogenesis of MS has been recognized and substantiated by data from epidemiological and biological studies [37]. It has been proposed that infections contribute to the etiology of MS but there is no convincing evidence for a unique disease-specific microbe. Several viruses and bacteria

have been associated with MS. Of these, some herpes viruses such as Epstein-Barr virus (EBV) and human herpesvirus type 6 (HHV-6) have been repeatedly associated with MS [38]. An immune response to HHV-6 and EBV was clearly detected in the early phases of the disease [39]. Furthermore, testing for anti-HHV-6 IgM antibodies can possibly identify patients who will develop a second relapse. An increased immune response to EBV has been demonstrated in MS patients [40]. Specifically, it has been found that immunoreactivities to the EBV proteins, BRRF2 and EBNA-1, are significantly higher in the serum and CSF of MS patients than in those of control donors. Antibodies to EBV and HHV-6 may be considered as prime candidates for potential biomarkers in MS [41].

## **II. Biomarkers of Axonal/Neuronal Damage**

Axonal damage can be induced by T cells, especially those positive for CD8, [42] and by microglia and macrophages, [43] through defects in calcium homeostasis [44] or excitotoxic mechanisms [45]. Axonal damage may also be an indirect effect of demyelination over a long period and leads to transitions from RR to SP subtype [46].

### ***II.1. Axon cytoskeleton markers in serum and CSF***

#### ***II.1.1. Neurofilaments***

Neurofilaments, the major axonal cytoskeleton proteins, consist of three components that differ in molecular size: a light chain (N-L), an intermediate chain (N-M), and a heavy chain (N-H). Increased immunoreactivity of non-phosphorylated neurofilament has been observed, especially within active MS lesions [5]. Neurofilaments and their differential state of phosphorylation in body fluids are potential markers for neurodegeneration in MS. Increased concentrations of N-L in CSF have been reported in patients with RR and progressive MS compared with healthy people and patients with inflammatory and non-inflammatory neurological disorders [47-50]. During relapse, concentrations are reported to make a peak in the third week after onset of the previous relapse, suggesting a delayed relation with disease activity [47,48]. N-L concentrations are reported to be independent of age, sex, and disease duration [47,48,51].

#### ***II.1.2. Actin and tubulin***

The second major component of the axonal cytoskeleton is the microtubule, which is up to 100  $\mu\text{m}$  in length and consists of tubulin ( $\alpha$  and  $\beta$ ) subunits. Actin is the major component of the microfilaments. Increased actin and tubulin concentrations are found in CSF of 19 patients with progressive MS compared with 16 patients with RR MS [50]. No difference in concentrations of tubulin antibodies are reported between 67 MS patients and controls [30].

#### ***II.1.3. Tau proteins***

The microtubule-associated tau proteins (55–74 kDa) are involved in stabilisation and assembly of axonal microtubuli behaving as a railway sleeper in a railway track [52]. Elevated concentrations of tau protein have been found in the CSF of MS patients, with a bimodal distribution reflecting significantly higher values in RR MS compared to CP MS patients [53]. Correlation of CSF tau levels in RR MS patients with the IgG index suggests a strict connection of axonal damage with inflammatory processes. These results suggest that tau proteins merit further studies in serum as well as in CSF in the context of MS [54].

### ***II.2. Markers of membrane homeostasis***

#### ***II.2.1. 24S-Hydroxycholesterol***

Axons contain a large volume of membranes because of their elongated shape. Cholesterol is the main lipid in these membranes, and 24S-hydroxycholesterol is a cholesterol metabolite specific to the brain. Serum 24S-hydroxycholesterol is a likely marker for changes in brain cholesterol turnover caused by demyelination or neurodegeneration [55]. Decreased serum 24S-hydroxycholesterol concentrations are reported in MS patients aged 50–70 years, the reduction being most pronounced in the PP clinical subtype [56]. The decreased serum or plasma 24S hydroxycholesterol concentration may be a marker for axonal loss in MS. Thus, 24S hydroxycholesterol is the only marker related to neuronal damage that has shown promising results in peripheral blood [54].

#### ***II.2.2. Apolipoprotein E***

Apolipoprotein E (APOE) is a polymorphic plasma protein involved in lipid transport between astrocytes and neurons and in the regeneration of axons and myelin after damage of the CNS. Although MS patients show a frequency of different alleles similar to that of the normal population, the  $\epsilon 4$  carriers have significantly more rapid clinical worsening and more severe lesion load on MRI [57,58], with an increasing axonal damage as measured by MRS [59]. Three studies have investigated the concentrations of apolipoprotein E in body fluids of patients with MS, [60-62] two of them reporting low CSF apolipoprotein E concentrations in MS, [60,62] one reporting the opposite. No correlation of serum / CSF apolipoprotein E concentrations with age, disease duration, or clinical decline is found [62].

### ***II.3. Other axonal markers associated with axonal damage***

#### ***II.3.1. Amyloid Precursor Protein (APP)***

APP accumulation in axonal ovoids is a sensitive marker for acute axonal injury in MS lesions [5,63,64]. In all types of MS lesions APP accumulation has been shown, though at a higher density in early active and late active lesions than in inactive and remyelinated lesions [43,63,65,66]. Because APP accumulation occurs at an

early stage during lesion formation, it might be useful to predict prognosis during early disease stages. No studies on APP or APP-derived soluble proteins in CSF or serum of patients with MS have been reported [54].

### **II.3.2. N-acetylaspartic acid**

N-acetylaspartic acid probably functions as a molecular water pump, in the osmoregulation of neurons. One molecule of N-acetylaspartic acid transports up to 32 molecules of water out of neurons against the water concentration gradient. N-acetylaspartic acid, as measured by MR spectroscopy, is used to estimate axonal reduction in MS. Decreases in N-acetylaspartic acid are reported early in lesion formation and in normal-appearing white matter and the concentrations correlate negatively with the expanded disability status scale (EDSS) scores [67], and axonal volume [46]. N-acetylaspartic acid can reflect both neuronal and oligodendrocyte damage, because of the recycling of this amino acid between neurons and oligodendrocytes. So far, no studies are known that measure N-acetylaspartic acid in serum or CSF of patients with MS.

### **II.3.3. 14–3–3 Protein**

The 14–3–3 proteins are expressed in all eukaryotic cells and are a group of multifunctional proteins that modulate the function of a wide array of other cellular proteins, including kinases, phosphatases and transmembrane receptors. Increased expression of 14-3-3 proteins in glial cells in MS lesions has been reported [68]. Thus, the detection of 14–3–3 protein in the CSF at the first neurological event suggestive of MS may be a useful predictor of short-term conversion to clinically definite MS and to MS with higher scores on EDSS [69]. These results still require validation by large studies, using sensitive methods.

### **II.3.4. Neuron-specific enolase**

Neuron-specific enolase (NSE) is a dimeric glycolytic enzyme proper of neuronal cells ( $\alpha\alpha$  dimer) and astrocytes ( $\alpha\alpha$  dimer). The Neuron-specific enolase- $\gamma$  isoform is specific for neurons and is present in cell bodies as well as in axons. Neuronal loss can be documented in several neurodegenerative diseases by increased CSF levels of NSE. No differences in mean Neuron-specific enolase concentrations in CSF are found between MS patients and healthy controls, or between patients with different clinical subtypes of MS [48,70-72].

## **III. Biomarkers of Blood-Brain Barrier (BBB) Disruption**

### **III.1. Matrix metalloproteinases**

Matrix metalloproteinases (MMPs) comprise a family of at least 23 zinc-containing endopeptidases that degrade extracellular proteins. Thus, they control cell migration across the blood-brain barrier (BBB) by disrupting the subendothelial basement membrane and eventually af-

fect tissue disruption in MS. Increased expression of various MMPs (MMP-2, -3, -7, -9) has been demonstrated in autopsied MS brains and MMP-9 has been detected in acute MS lesions. In MS, high MMP-9 activity has been shown in CSF in association with BBB breakdown. Similarly, elevated MMP-9 mRNA levels in peripheral blood mononuclear cells and raised MMP-9 levels in serum from MS patients have been detected and are associated with disease activity assessed clinically or by MRI. More recently, it has been proved that MMP activity (ratio between MMPs and tissue inhibitors of metalloproteinases levels) may mark different subtypes of the disease, increased MMP-2 levels in chronic progressive MS and increased MMP-9 in relapsing and active forms. Finally, following IFN- $\beta$  treatment, clinical improvement is paralleled by decreased serum levels of MMP-9 [73]. Although MMPs appear to be promising, it is unlikely that these markers will be more useful than MRI-based markers of BBB dysfunction.

## **IV. Biomarkers of Demyelination**

MBP and MBP-like material, proteolytic enzymes, endogenous pentapeptide QYNAD and gliotoxin may enhance the interpretation of MRI/pathological correlations and have a potential for partial surrogacy. QYNAD is an endogenous peptide in CSF with Na-channel blocking properties, originating from proteolytic cleavage during inflammatory process [4].

## **V. Biomarkers of Oxidative Stress and Excitotoxicity**

### **V.1. Nitric oxide derivatives**

Macrophages and activated microglia can release cytotoxic factors, including nitric oxide (NO), a non-specific mediator of inflammation involved in blood-brain barrier damage and demyelination. NO causes mitochondrial dysfunction and contributes to excitatory amino acid-induced neuronal injury (neuro-excitotoxicity), axonal loss and oligodendrocyte damage. The CSF levels of its oxidation products, nitrite and nitrates (NOx), are found to be 4-fold higher in patients with primary progressive MS and a correlation has been described between nitrite/nitrate ratio and relative brain atrophy [74], suggesting a relationship with neurodegenerative processes. However, other studies have reported the absence of correlation between NO metabolites and disease activity or elevated NO metabolites in control groups. Thus, the CSF levels of NO derivatives still require validation [75].

### **V.2. Isoprostanes**

Isoprostanes are emerging as a new class of biologically active products of AA metabolism with potential relevance to human neurodegenerative and demyelinating diseases. CSF 8-epi-PGF $2\beta$  levels in MS patients are found to be three times higher than in a benchmark group of subjects with other non-inflammatory neurological diseases, or in nonneurological patients under-

going subdural anesthesia [76]. Steroid-treated MS patients, who tend to have low EDSS scores [77], exhibit lower 8-epi-PGF2 $\alpha$  levels than those of untreated patients, and within the whole group of patients the levels of 8-epi-PGF2 $\alpha$  moderately correlate with the degree of disability. F2-isoprostanones may be preferable to neuroprostanes as markers of lipid peroxidation in demyelinating diseases [78].

## VI. Biomarkers of Gliosis

Glial fibrillary acid protein (GFAP), the constitutive protein of astrocyte intermediate filaments, is a classic marker of astrogliosis. Elevated CSF concentrations of GFAP have been found in MS patients, correlating with disability scales and the extent of neurologic deficits [79,80] and possibly suggesting irreversible tissue degeneration. In contrast, S-100 protein, a unique component of the nervous system, is considered a marker of astrocyte activation for the relapsing phase of the disease. It is found to be significantly lower in the CSF of patients with primary and secondary progressive MS with respect to RR MS patients [80].

## VII. Biomarkers of Remyelination and Repair

NCAM (neural cell adhesion molecule), CNTF (ciliary neurotrophic factor), MAP-2 + -13 (microtubule-associated protein-2 exon 13), CPK-BB (creatine phosphatase BB), PAM (peptidylglycine  $\alpha$ -amidating monooxygenase) are potential candidates, that are needed to guide development of repair-promoting strategies in MS and to help in disease heterogeneity studies [4].

## Conclusion

A perfect marker should be reliable, reproducible, non-invasive, sensitive and disease specific. Furthermore, it should optimize therapy and prevent disability. It is unlikely that a single biological marker can fulfill all the criteria of a surrogate endpoint in MS. Therefore it seems more conceivable to measure a panel of different markers in individual MS patients to reflect the various stages of inflammation, demyelination, axonal degeneration and remyelination.

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