Biomarkers in Multiple Sclerosis

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ABSTRACT. Multiple sclerosis (MS) is considered a complex genetic disease, and a large number of quantitative trait loci contribute to MS risk. Disease expression is further influenced by environmental risk factors such as EBV infection, lack of vitamin D, smoking and others. The complex etiology translates into autoimmune inflammation, demyelination, glial proliferation, and axonal/neuronal damage, which are found to different extents in the individual patient. At the clinical level MS also shows considerable heterogeneity with respect to course, signs and symptoms, and response to treatment. In order to gain a better understanding of the underlying pathogenetic processes we need biomarkers that are associated with immune alterations, damage of CNS cells and tissue or other important disease mechanisms and eventually reflect clinically relevant changes during the disease. In this chapter we describe the current status of biomarker research in MS and discuss the usefulness of individual markers with respect to clinical course or response to treatment.

Keywords: treatment response, prognosis, inflammation, neurodegeneration.

RESUMEN. La esclerosis múltiple (EM) se considera una enfermedad genética compleja, y un número grande de loci cuantitativos de riesgo contribuyen al riesgo. La expresión de la enfermedad está, además, influenciada por factores de riesgo ambientales tales como la infección por VEB, la falta de vitamina D, el tabaco y otros. Esta etiología compleja se traduce en una inflamación autoinmune, desmielinización, proliferación glial y daño neuronal/axonal, que se encuentra en diferentes grados en los pacientes individuales. En el plano clínico, la EM también muestra una gran heterogeneidad con respecto al curso, los síntomas y signos, y la respuesta al tratamiento. Con el fin de tener una mejor comprensión de los procesos patogénicos subyacentes, necesitamos biomarcadores que se asocien con las alteraciones inmunes, el daño a las células y tejidos del SNC y otros importantes mecanismos de la enfermedad y que eventualmente reflejen cambios clínicamente relevantes durante la enfermedad. En este artículo describimos el estado actual de la investigación de los biomarcadores en la EM y discutimos la utilidad de los marcadores individuales con respecto al curso clínico o respuesta al tratamiento.

Palabras clave: respuesta terapéutica, pronóstico, inflamación, neurodegeneración.

What is a biomarker?

A Biomarker is a feature that can be measured in an objective way and can be evaluated as an indicator of biological processes. Biomarkers can be classified in two different types:

Type 0 biomarkers are markers of the natural history of a disease, i.e. of pathogenic processes without therapeutic intervention, and correlate longitudinally with clinical parameters.

Type 1 biomarkers are indicators of pharmacological responses and are in accordance with the mechanism of action of the therapy. In clinical trials, the outcome designed to be evaluated are clinically meaningful measures called clinical endpoints. When a type 1 biomarker is able to provide information about the clinical efficacy of a therapy in a significantly shorter time than a clinical endpoint, it can substitute the clinical endpoint and become a surrogate endpoint.

Why biomarkers in Multiple Sclerosis?

Multiple Sclerosis is a very complex disease, in which different pathological processes including inflammation, demyelination, neuro-axonal loss, gliosis and repair mechanisms are involved. These processes are not uniformly represented across patient populations resulting in a variable clinical presentation. MS patients show substantial heterogeneity in the clinical course, manifestations of the disease, prognosis and response to therapies. It has become clear during the recent past years, that an early diagnosis and appropriately timely therapeutic intervention are critical for managing this disease. However, the abovementioned heterogeneity together with the lack of a “diagnostic” laboratory test complicates clinical decisions as well as estimation of prognosis.

Most of the clinical endpoints used in MS, such as exacerbation rate or accumulation of irreversible disability, are standardized, meaningful in real life, and reasonably easy to test. However the main problem is that these endpoints are not too useful in early diagnosis, early prognosis or in monitoring early response to therapies. In this context, the development of biomarkers that are able to provide information about prognosis or efficacy of a therapy in a significantly shorter time and relevant to the different pathological processes playing a role in MS, would hold great potential for:
1. - MS diagnosis and identification of disease stages and subcategories.
2. - Prediction of onset and disease course.
3. - Treatment selection and improved prognosis of treatment success.
4. - Evaluation of new therapies.

**Biomarkers in Multiple Sclerosis. Imaging methods**

**MRI measures**

Biomarkers based on Magnetic Resonance Imaging (MRI) findings of the CNS have been extensively used in the diagnostic of MS patients, in prognosis and also to monitor disease activity in clinical trials. Among the main advantages of using MRI as a monitoring tool are its non-invasiveness, sensitivity to detect disease activity and ability to produce outcomes that are both objective and quantitative. In recent years tremendous progress has been made in the field of MS imaging with the incorporation of newer MRI techniques, such as magnetization transfer (MT) imaging, MR spectroscopy (MRS) and diffusion weighted imaging (DWI). Nevertheless, MR imaging still has many limitations, mainly a lack in understanding, which imaging measures reflect what type of pathogenic processes.

**Optical coherence tomography (OCT)**

Optical coherence tomography (OCT) is another non-invasive imaging tool that measures the thickness of the retinal nerve fiber layer. Thinning of the retinal nerve fiber layer seems to be associated with brain atrophy and in consequence this un-expensive, non-invasive and practical imaging technique could become a useful biomarker for neuro-axonal damage.

**Biological markers**

**Specimens**

Biomarkers in MS have been identified in different body fluids such as urine, blood, CSF and tears. Each type of specimen has advantages and disadvantages.

- **CSF** - Since MS lesions are rarely biopsied, the CSF analysis remains the closest to the pathology and may better reflect the relevant inflammatory processes. Different types of measurements can be done in CSF including both soluble markers as well as cell populations. The main disadvantage is that CSF collection is an invasive procedure that is therefore can only be sampled for a limited number of time points.

- **Blood** - The main advantage of blood is that it is relatively simple to collect. However, the diurnal variation of many soluble markers represents an important disadvantage. Furthermore, levels of measured biomarkers can be affected by systemic infections, by degradation in the liver or by excretion in the kidney.

Urine - The main advantage is also the non-invasive collection and the main disadvantage is that chronic urinary tract infections and bacterial colonization of the bladder that are common in more disabled MS patients can affect the results. Furthermore, patients with bladder problems may restrict their fluid intake with resulting artificial hypohydration.

**Biomarkers reflecting alteration of the immune system**

**Cytokines and their receptors**

Cytokines are among the most intensively studied biomarkers in MS. As results of inflammation in MS lesions various cytokines are elevated in MS patients. However, such alterations are not specific for MS patients and can be also detected in other inflammatory CNS diseases. Th1 pro-inflammatory cytokines such as IFN-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-12 have been shown to be elevated in MS relapses, whereas anti-inflammatory cytokines such as the Th2 cytokine IL-4 and IL-10 and transforming growth factor (TGF)-β, have been associated with clinical remissions and a stable course of relapsing-remitting MS (RR-MS). IL-12 has been considered as biomarker suitable to distinguish progressive -from relapsing-remitting disease course and as biomarker of disease activity in progressive MS^1-5^.

Recently, a new Th lineage relevant for MS, the Th17 lineage, has been discovered^6^. The production of IL-17, the signature cytokine of Th17 cells, and IL-10 and transforming growth factor (TGF)-β, have been associated with clinical remissions and a stable course of relapsing-remitting MS (RR-MS)^7^.

**Chemokines and their receptors**

Chemokines are chemotactic cytokines involved in the recruitment of immune cells into lymphoid organs and to sites of inflammation. The chemokine receptor CCR5 has been described to correlate with symptomatic relapses^8-10^.

In addition, CXCR3
expression in circulating T cells from MS patients was increased. Immunohistochemical analyses of autopsy brain sections containing active MS lesions have described CXCR3 expression on virtually all tissue-infiltrating T cells. These CXCR3 positive perivascular cell infiltrates are uncommon in control brain specimens. It was suggested that the retention of CXCR3+ T cells in patients with MS is due to the presence of its ligand (IP-10) and CXCR3 cells, in the absence of ligand, recirculate. In active inflammatory MS lesions, CCR1+/CCR5+ expressing hematogenous monocytes have been abundantly found in perivascular cell cuffs and at the demyelinating edges of evolving lesions together with CCR+ microglia, but not in non-inflamed brain sections. Patients with MS show an enrichment of CCR7+ memory T cells co-expressing CCR5, a candidate biomarker for Th1 cells, and CXCR3 in their CSF. Chemo- kines and their receptors may become important in studying disease heterogeneity and thus, need to be validated in larger cohorts, but they are unlikely candidates for surrogate endpoints in MS.

Oligoclonal bands and antibodies

Currently, the only biomarker apart from MRI that is accepted in the diagnosis of MS is the detection of CSF oligoclonal bands (OCB), immunoglobulins separated by isoelectric focusing, which are found in approximately 90-95% of MS patients. Six decades after their discovery, the precise origin and meaning of OCB is not yet understood, but their presence is strongly associated with MS. OCB are also found in infectious and inflammatory neurological diseases such as Lyme disease, syphilis, subacute sclerosing panencephalitis and fungal meningoencephalitis, and in these conditions, most if not all immunoglobulin bands are directed against the respective pathogen. Once present, OCB persist in the CSF of MS patients, pointing to a stable B cell-mediated intrathecal immune response in MS. The sensitivity of OCB detection in IEF is higher than 85% with a specificity of 92% and a positive and negative predictive value of over 86.5% and 90%, respectively. A recent study combining IEF of oligoclonal immunoglobulin G (IgG) with alkaline phosphatase immunodetection showed a higher sensitivity and specificity than MRI criteria. Using this approach, it has been possible to reliably predict a second attack in patients with a clinically isolated demyelinating syndrome and thus, OCB is a promising surrogate endpoint for MS.

<table>
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<tr>
<th>Biomarker</th>
<th>Sample</th>
<th>Method</th>
<th>Diagnosis</th>
<th>Prognosis</th>
<th>Disease activity</th>
<th>Treatment response / effect</th>
<th>Validation</th>
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<tr>
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<td>Immunofluorescence, FACS, RBA</td>
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<td>-</td>
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<td>+</td>
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<td>RT-PCR</td>
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<td>-</td>
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+: the biomarker has been analyzed for respective outcome; -: the biomarker has been analyzed for respective outcome; b.a.: marker for bioavailability of a therapeutic; t.r.: marker of treatment response; p.m.: predictive marker of treatment response;*: the respective outcome has been re-analyzed in at least one independent population; ~: the biomarker has been analyzed in at least one independent population with conflicting results; n: the biomarker has not been validated in an independent population.
the conversion into clinically definite multiple sclerosis (CDMS). Not only oligoclonal IgG, but also oligoclonal IgM may be detected by IEF (Table 1)2. The potential pathophysiological importance of IgM is highlighted by the fact that IgM is the most potent activator of complement, which co-localizes with demyelinated areas in MS and NMO33, 34. Early reports about oligoclonal IgM as a strong predictor of an earlier conversion to CDMS and a more aggressive disease course have so far not been replicated in other studies25, 30.

The pathologic role of autoantibodies in autoimmune disease is widely accepted. Among the potential autoantigens in MS, myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), are of particular interest due to their location in compact myelin (MBP) and the outer surface of the myelin sheath (MOG), and to their pathogenicity in the experimental autoimmune encephalomyelitis (EAE) model. In a recent report, the presence of serum IgM auto-antibodies specific for the extracellular domain of MOG and antibodies specific for MBP in CIS patients was highly predictive for the conversion into CDMS31. The subsequent analysis of the prognostic value of anti-MOG and anti-MBP in eight different CIS cohorts has shown controversial results with correlations ranging from highly significant32-33, significant in a sub analysis34-36, to not significant at all37-38. Since the same type of method, i.e. immunoblotting to recombinant MOG and human myelin-derived MBP, was used in all studies, the controversial results may primarily reflect differences in study populations (e.g. different genetic backgrounds) rather than methodological problems (Table I).

It is a matter of ongoing debate, whether pathogenic autoantibodies are reliably detected by binding to conformational or linear epitopes or glycosylated proteins39. Recently, high-sensitivity bioassays for detection of autoantibodies that bind to the extracellular part of native MOG have been reported40-41. To obtain MOG in its native form with all posttranslational modifications, the full-length human MOG cDNA was expressed in a human glioblastoma cell line and serum antibodies directed to the conformational epitopes of the extracellular domain of native MOG were detected by flow cytometry42. Using this approach, anti-MOG antibodies have been found significantly elevated in the sera of MS patients compared to controls. These autoantibodies exerted cytotoxicity in vitro and enhanced demyelination as well as axonal damage after injection in susceptible rats43. It is not yet known whether the development of autoantibodies in MS patients reflects a response to myelin injury or is the cause for the insult. Further studies are necessary to elucidate whether these autoantibodies correlate with pathological and clinical parameters.

In another study, using a novel tetramer radioimmunoassay more sensitive for MOG autoantibody detection than other methodologies, it was shown that autoantibodies from patients suffering from acute disseminated encephalomyelitis (ADEM) selectively bound conformational MOG tetramers, whereas antibodies derived from adult-onset MS cases bound only rarely44. Aberrant N-glycosylation has been recently shown to be a fundamental determinant of autoantibody recognition in MS patients as compared to controls45. A synthetic glycosylated peptide antigen called CSF114(Glc) was designed on the structural basis of myelin epitopes and proved to bind by ELISA specific IgM autoantibodies in the sera of MS patients, but not in healthy donors and other autoimmune conditions. The induction of anti-CSF114(Glc) antibodies correlated with clinical activity and brain lesions on MRI. So far, this novel immunoassay seems to represent a promising diagnostic and prognostic marker in a subgroup of MS patients. Other promising candidate autoantibodies have been described such as those against neurofascin46, which appear to be involved in axonal injury, however validation in independent studies is required.

Among the potential environmental triggers in MS, recent data indicate that Epstein-Barr virus (EBV) may play an important role47. Despite the fact that the specificity spectrum of OCB is as yet poorly understood and a clear association of OCB with the pathogenesis of MS lacking, EBV appears to be one of the targets of OCB. An attempt to assess the specificity of OCB by screening protein expression arrays containing 37,000 tagged proteins lead to the identification of two high-affinity epitopes derived from Epstein-Barr virus (EBV) proteins48. The frequency of the immunoreactivities towards these EBV proteins, BRRF2 and EBNA1, was significantly higher in the serum and CSF of MS patients than in those of control donors. These findings point to a perturbed EBV-specific immune response in MS patients and corroborate the long time discussed association of EBV infection with the development of MS.

Further evidence for the importance of EBV stems from epidemiological studies investigating EBNA1 specific antibodies. The analysis of blood samples collected before the onset of disease revealed that antibody titers to the EBNA complex and EBNA1 were elevated in individuals who later developed MS compared to those who remained healthy and that elevated titers persisted after MS onset49-50. The risk of MS increases thereby with higher antibody titers49. Teenagers and young adults experiencing infectious mononucleosis, an acute EBV-trig-
Aquaporin-4 is located in astrocytic foot processes at the blood-brain barrier and is the most abundant water channel in the brain exhibiting an important role in brain water homeostasis. Although abundant in optic nerve and spinal cord aquaporin-4 is found throughout the healthy brain and is completely lost in NMO lesions as opposed to MS lesions. Thus, NMO-IgG antibodies represent a novel disease-specific biomarker, the first discovered for any demyelinating disease affecting the human CNS, and help distinguish NMO patients from those with classical MS and other inflammatory demyelinating variants of MS. NMO-IgG antibodies enable clinicians to reliably identify a subgroup of patients within the heterogeneous disease complex of MS before fulfillment of all traditional clinical diagnostic criteria and to direct them to early and specific treatments, such as plasmapheresis or B cell depletion by rituximab, a selective anti-CD20 (B-cell) monoclonal antibody. However, it needs to be stressed that not all patients who fulfill clinical NMO criteria are NMO antibody positive.

Complement-related biomarkers

Several studies have established a role of complement in the pathology of MS and in consequence several complement proteins have been investigated as potential biomarkers of diseases activity although with conflicting results. Proteomic analysis has identified changes in levels of specific complement proteins (factor I, C3, clusterin) in CSF samples in patients with MS compared with healthy donors and recently the complement regulator factor H has been identified as a serum biomarker of disease activity. Serum factor H levels were significantly higher in progressive disease compared to controls and relapsing patients.

Adhesion molecules as markers of blood brain barrier disruption

Several adhesion molecules are involved in the transendothelial migration of leukocytes into the CNS, a central process in MS pathogenesis. Cytokines, which are secreted within an inflammatory focus in MS, induce the up-regulation of adhesion molecules prior to blood-brain-barrier (BBB) disruption. Adhesion molecules may also be released in a soluble form from activated endothelial cells,
leukocytes and platelets into serum and CSF. Recently, plasma levels of soluble adhesion molecules (sPECAM-1, sP-Selectin and sE-Selectin) have been shown to be increased in RR-MS patients compared to chronic progressive MS patients. In addition, soluble levels of sPECAM-1, sP-Selectin and sE-Selectin were increased during relapse, suggesting that these molecules might be useful paraclinical markers of disease activity in MS with restriction to the clinical course of the disease (Table I). Furthermore, increased levels of soluble intercellular adhesion molecule-1 (sICAM-1) were found in the serum of MS patients during a clinical relapse or with active MRI scans, and an association between high intrathecal sICAM-1 levels and IgG indices in RR-MS patients has been reported. The increase of soluble vascular cell adhesion molecule-1 (sVCAM-1) has been reported to be associated with a decrease in MRI lesions in MS patients treated with IFN-β-1b. In SPMS early upregulation (1-6 months) of sVCAM-1 is associated with MRI activity in the 19-24 months treatment interval in the IFN-β1b treated group. Similar results have been reported in RR-MS patients treated with IFN-β1a. However, downregulation of very late antigen-4 (VLA-4) showed a higher sensitivity in predicting a favorable treatment response compared to VCAM-1 upregulation, and VLA-4 was significantly up-regulated during relapses (Table I).

Matrix metalloproteases (MMPs) comprise a family of endopeptidases that degrade extracellular proteins and are involved in the desintegration of the subendothelial basement membrane of the BBB and thus CNS lesion formation in MS. Several MMP family members (MMP-2, -3, -7, -9) have been detected in autopsied brains of MS patients, and MMP-9 expression has been reported to correlate directly with a resistance towards activation-induced cell death. Altered T and B cell apoptosis seems to be involved in MS pathogenesis. An increased gene expression of anti-apoptotic mediators has been demonstrated in a microarray analysis of blood cells from patients with RR-MS (Blevins et al., in preparation). The proapoptotic factor and bcl-2 family member Bcl-X(L) has been reported to be expressed at increased levels in peripheral blood cells of MS patients and to correlate directly with a resistance towards activation-induced T cell death. Consistent with these findings, the expression of bcl-2 family members in blood cells from MS patients correlated with clinical features of disease activity, such as the number of gadolinium-enhancing MRI lesions and clinical relapses. Apart from bcl-2-related proteins, the analysis of expression levels of the death inducing ligand CD95 and the TNF-related apoptosis inducing ligand (TRAIL) - either in their membrane-bound or soluble form - has given controversial results in different MS populations.

In a longitudinal gene and protein expression analysis of patients under IFN-β treatment, drug-responders have been distinguished from non-responders by early and sustained induction of TRAIL. Increased concentrations of soluble TRAIL in serum predicted treatment to IFN-β response even before treatment was started. Thus, TRAIL may be used as a prognostic marker of treatment response to IFN-β in MS (Table I). However, the reported findings still await confirmation in a larger cohort of MS patients, and the significance of TRAIL in the pathogenesis of MS needs to be further clarified.

Changes in cellular populations
Various changes related to T cells, B cells, NK cells or other immune cells can be analyzed today involving a variety of different parameters such as phenotype, antigen specificity, frequency, proliferation capacity, activation status, susceptibility to apoptosis, expression of specific surface molecules, cytokine production and many more. There are numerous interesting candidates among these parameters, but unfortunately, the assessment of most of them involves rather complex immunological assays, which are difficult to perform, expensive and often not standardized among different labs. In consequence, all these analyses may only be performed in laboratories with expertise in this field and they are not suited for the routine use in clinical laboratories. Thus, observations on biomarkers for cellular immune function not only have to be validated in independent experiments by other groups, but it is essential to define the respective methods precisely and to standardize them, i.e. develop standard operating procedures.
The putative role of CD4+ T cells in the pathogenesis of MS is supported by many findings, which are summarized in detail elsewhere. Since the above difficulties with respect to validation and standardization apply to the majority of them and due to the sheer amount of studies, we will not list them here.

Mature B cells and plasma blasts in the CSF have been reported to correlate with acute brain inflammation measured by MRI and with inflammatory CSF parameters in CIS and RRMS, but not in chronic progressive MS. Clonally expanded B cells, mainly of a memory phenotype, accumulate in chronic MS lesions and in the CSF of MS patients. A subset of B cells, short lived plasma blasts, have been reported to be more frequent in the CSF of MS patients compared to other inflammatory and non-inflammatory diseases, and the numbers of these plasma blasts strongly correlated with intrathecal IgG synthesis and inflammatory parenchymal disease activity as shown by MRI. About 45% of MS patients with fulminant attacks, that are unresponsive to corticosteroids, improve after therapeutic plasma exchange (TPE). TPE removes pathogenic humoral and plasma factors, suggesting B cell-mediated pathomechanisms in MS. Recently, lesion biopsies of 53% of a small cohort of RR-MS patients unresponsive to corticosteroids have been classified as histopathological pattern type II, according to the classification by Lucchinetti and colleagues. The same patients experienced significant clinical improvement after TPE, but none with pattern I or pattern III. Since pattern type II is characterized by antibody/complement-associated demyelination, these findings clearly link TPE responsiveness in corticosteroids non-responders to a MS-subtype, in which the pathophysiology is predominantly B cell driven. Patients with this subtype of disease could benefit from TPE or even Rituximab treatment prior to application of corticosteroids. Whether the subgroup of MS patients with histopathological pattern type II can be identified without brain biopsies, but by alternative biomarkers using imaging or biochemical/immunological constituents, remains to be shown.

A treatment study with Daclizumab, a monoclonal antibody directed against CD25, has shown a profound inhibition of brain inflammatory activity. The reduction of the inflammatory activity correlated highly with the absolute expansion of CD56bright NK cells (Table I), indicating an important immunoregulatory role for these cells in MS pathogenesis. The same population of NK cells turned out to be reduced in frequency in untreated RR-MS and CIS patients as compared to healthy donors. The same study revealed three distinct subpopulations of untreated RRMS patients by large-scale FACS based immunophenotyping. Furthermore, CD56dim NK cells have also been reported to increase in frequency during the last trimester of pregnancy, a time of reduced MS relapses, in women with MS. Similarily, an expansion of circulating CD56bright NK cells has been noted in subjects with RRMS following treatment initiation with IFN-β. The expansion of CXCR1+ NK cells, however, correlated positively with disease activity in RR-MS patients.

Changes in other immune system related soluble proteins

Within sites of inflammation, a broad panel of receptors involved in immune response and regulation are upregulated on the surface of various cell types. This process also involves exocytosis of inflammation-associated proteins, but also shedding of soluble forms of cell surface receptors. Besides their expression on the surface of most human cells, class I human leukocyte antigens (HLA class I) occur naturally also in the form of soluble HLA-A, B and C (sHLA class I) antigens in body fluids. Increased concentrations of sHLA class I have been observed in the CSF and serum of MS patients, and both sHLA class I and sHLA class II are influenced reciprocally by immunomodulatory treatment (Table I).

HLA-G is a nonclassic HLA class I molecule that differs from classical HLA class I by its limited polymorphism, the highly restricted tissue distribution and the pattern of alternative splicing. Expression of HLA-G can be induced in cancers, transplantation, inflammatory diseases and viral infections protecting target tissues from auto-aggressive inflammation and exerting immunotolerogenic function. Levels of soluble HLA-G (sHLA-G) are significantly increased in MS patients compared to other neurological diseases. Recently, it has been shown that a balance between intrathecal produced sHLA class I and sHLA-G, which are inversely related to MRI and clinical disease activity, may exist. It has been suggested that these molecules serve opposing roles in the balance between inflammation (sHLA class I) and immunomodulation (sHLA-G). Consequently, monitoring of sHLA molecules in sera and/or CSF of MS patients might be useful as biomarkers of disease activity.

CD14 is expressed on mononuclear cells and together with TLR4 represents a pattern recognition receptor of microbial products. Besides mediating inflammation in the context of the innate host defense CD14 has also been shown to be involved in the non-inflammatory phagocytosis of apoptotic cells by macrophages. A soluble form of CD14 (sCD14) can be detected in serum or plasma and elevated serum levels of sCD14 have been reported in MS pa-
tientes but also in other organ-specific autoimmune diseases
de MS patients. Corroborating this result, a decrease in sCD14 levels during relapse could be observed in longitudinally
analyzed patients. The role of sCD14 in autoimmune disease is unknown but the differences in sCD14 levels might reflect a differential activation and role of the innate immune system during inflammation in MS. Therapy with IFN-β induced higher sCD14 serum levels in RRMS patients. Further studies should validate these findings in different populations and correlate the biological data with MRI imaging of brain inflammation.

In a recent study using mass spectrometry-based proteomic approach (isobaric labeling) in CSF samples from patients with CIS, who subsequently converted to clinically definite MS and patients who remained as CIS, chitinase 3-like 1 has been identified as a new biomarker for conversion. Chitinase 3-1 like levels were increased in the CSF of patients who converted to clinically definite MS compared with patients who continued as CIS and controls. High CSF levels of chitinase 3-1 like significantly correlated with the number of gadolinium enhancing lesions and the number of T2 lesions observed in brain magnetic resonance imaging scans performed at baseline, and were associated with disability progression during follow-up and shorter time to clinically definite multiple sclerosis.

**Biomarkers of demyelination**

Cholesterol is one of the main components of cell membranes, and 24S-hydroxysterol is a metabolite, which is exclusively synthesized in the brain and spinal cord and thus a marker for cell membrane homoeostasis within the CNS. Serum levels of 24S-hydroxycholesterol have been reported to be increased in the CSF of patients with RR-MS. The increase was most pronounced in patients with gadolinium-enhancing lesions compared to patients without active scans. In contrast, serum 24S-hydroxycholesterol levels have been found to be reduced in the primary progressive clinical subtype. Therefore, 24S-hydroxycholesterol may indicate the transition from a predominantly immune-mediated to a degenerative phase of the disease.

**Biomarkers of oxidative stress and excitotoxicity**

According to the histopathological classification by Lucchinetti and colleagues, the histopathologic pattern type III of demyelination is characterized by an oligodendrocyte dystrophy, principally affecting the most distant processes of these cells and leading to apoptosis of oligodendrocytes at later stages of lesion development. This pattern is associated with nuclear expression of hypoxia-inducible-factor-1α (HIF-1α) and mimics myelin destruction in acute white matter ischaemia. So far no MRI or clinical correlate to the histopathological classification of demyelination has been identified, but together with the latter HIF-1α clearly merits further exploration as a marker for pattern III MS.

Recently, a brain epitope (D-110), detectable by a monoclonal antibody against canine distemper virus and cross-reactive to an endogenous brain epitope, has been shown to be expressed at high levels in this specific subtype (type III) of actively demyelinating MS lesions but not or to a much lesser extent in other MS cases. The presence of D-110 was significantly associated with expression of HIF-1α within the lesions. In addition, D-110 is liberated into the CSF, where it can be detected by ELISA in about 17% of patients. This proportion is similar to the reported incidence of cases with type III lesions found in histopathological studies. Although the detection of elevated levels of D-110 is not specific for type III lesions, but also found in acute lesions of white matter ischemia, it may become a useful diagnostic tool to identify clinically a defined multiple sclerosis subtype (Table I). Further direct correlations between pathology and CSF values will be necessary to determine the reliability of this tool for diagnosis and subtyping of MS.

Since inflammation-induced hypoxia-like MS lesions occur in the absence of significant vascular pathology, these lesions have been suggested to result from a toxic hypoxia induced by mitochondrial damage. Among the various molecules, which are produced in effector cells in inflammatory lesions and mediate mitochondrial dysfunction, nitric oxide (NO) and reactive oxygen species are involved in axonal and myelin sheath vulnerability to hypoxia in MS pathogenesis. NO has also been linked to various beneficial and immunomodulatory effects in brain inflammation like inhibition of antigen presentation, T cell proliferation, induction of apoptosis in T cells and the downregulation of adhesion molecules. Elevated levels of the NO metabolites nitrite and nitrate have been found in CSF, serum and urine in MS patients (Table I). Intrathecal production of nitrite and nitrate was associated with clinical disease activity in the early phase of the disease as assessed by MRI parameters. Raised baseline levels of intrathecal NO metabolites were associated with clinical and MRI progression in MS patients over 3-year follow-up, suggesting that these could indicate a more aggressive disease course. In another study increased NO metabolites correlated with a presumed biomarker of axonal degeneration (neuro-
filaments) and clinical disability (EDSS), suggesting NO-mediated stress is related to the development of sustained disability in MS\(^{15}\). These results need to be replicated in larger cohorts with a high level of accuracy regarding the biochemical assessment of the metabolites, since it is strongly dependent on the sampling procedure of the probes. An alternative way, in which NO and other reactive oxygen species might affect the function of oligodendrocytes is by radical-mediated oxidation and breakdown of myelin components\(^{22}\). Cholesterol is the major component of the myelin sheath and its breakdown product 7-ketocholesterol can activate and attract microglial cells in brain tissue and thus lead to neuronal damage. Indeed, higher levels of 7-ketocholesterol can be detected in CSF of MS compared to controls with other non-inflammary neurological diseases\(^{12}\).

Oligodendrocytes may be also damaged by glutamate-mediated excitotoxicity mediated by the AMPA/kainate type of glutamate receptors as shown in the EAE model\(^{13}\). Glutamate is released in excessive amounts by activated leukocytes and microglia and leads to increased calcium fluxes and excitotoxic death of oligodendrocytes and neurons upon binding to AMPA/kainate receptors. Markers for glutamate production such as glutaminase are increased in macrophages and microglia in close proximity to dystrophic axons within active MS plaques\(^{133, 134}\). Treatment with Riluzole, a neuroprotective inhibitor of glutamate release from nerve terminals, reduced the rate of cervical cord atrophy and the development of T1 hypointense lesions\(^{135}\). However, due to the small sample size, lack of a placebo group, short follow-up and lack of statistical significance, these data have to be interpreted cautiously. How biomarkers for glutamate excitotoxicity should be defined and how they can be assessed in the clinical setting, remains to be determined.

**Biomarkers of axonal/neuronal damage, gliosis, remyelination and repair**

In recent years, there has been increasing interest in the identification of biomarkers of axonal damage in MS. While markers of inflammation often correlate poorly with disability, markers of axonal degeneration are expected to provide helpful information on future disability of MS patients.

Neurofilaments are the major axonal cytoskeleton proteins and consist of three components that differ in molecular size (a light chain Nf-L, an intermediate chain Nf-M, and a heavy chain Nf-H)\(^{136}\). Several studies have shown that the concentration of the Nf-L is increased in the CSF of MS patients compared with healthy people and patients with other inflammatory and non-inflammatory neurological disoders\(^{137}\). Increased CSF levels were most pronounced in RR-MS and peaked in the third week after relapse, which suggests a delayed relation with disease activity\(^{138, 139}\). However, the CSF concentrations of Nf-L correlate poorly with clinical disability measured by the EDSS. Apart from the protein itself, intrathecal anti-Nf-L autoantibodies have been shown to occur more frequently in PP-MS patients compared to RR-MS patients and healthy controls and patients with other neurological diseases (Table I)\(^{139-142}\). The positive correlation of anti-Nf-L autoantibodies with MRI measures of brain atrophy suggests a correlation with disease progression.

Glial fibrillary acidic protein (GFAP), a monomeric intermediate filament protein, is a structural component of the cytoskeleton highly expressed in astrocytes. GFAP is considered to be the morphological basis of astrogliosis, which is a prominent feature of MS\(^{143}\). Increased CSF levels of GFAP have been reported in MS patients, however results are inconsistent\(^{139, 143-146}\). Since it has also been found intrathecally in patients with other neurological diseases, GFAP represents a non-specific marker of CNS tissue injury, although CSF levels of GFAP correlated with disability in MS patients.

N-acetylaspartic acid (NAA) is an amino acid that is almost exclusively expressed in neurons. NAA is important in osmoregulation as it functions as a water pump by transporting water molecules out of neurons against the water concentration gradient. Due to its neuronal specificity NAA has been used as a marker of neuronal damage in pathology as well as magnetic resonance spectroscopy. Reduced neuronal levels of NAA, both in MS lesions and the normal appearing white matter, correlate inversely with the degree of disability\(^{137}\).

Nogo, a major component of CNS myelin, is a development-related molecule inhibiting axonal regeneration and sprouting of synaptic terminals. Three isoforms of Nogo exist (A, B, and C), generated by alternative splicing or differential promoter usage of a single gene. Intrathecal anti-Nogo-A autoantibodies are significantly more frequent in patients with RR-MS as compared to chronic progressive MS\(^{147}\). A study reporting an exclusive presence of soluble Nogo-A in the CSF of MS patients has been proven wrong subsequently\(^{148, 149}\).

Even though they require further validation, still, biomarker candidates for axonal damage are clearly promising to evolve into surrogate endpoints in MS.

**Future prospects**

After reading this review, the reader will note the discrepancy between the stressed urgency to deve-
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Balashov KE, Rottman JB, Weiner HL, Hancock WW. CCR5(+) and CXCR3(+) T cells are increased in...


90.- Huang WX, Huang MP, Gomes MA, Hillert J. Apoptosis mediators fasL and TRAIL are upregulated in peripheral blood mononuclear cells in MS. *Neurology* 2000; 55: 928-934.


129.- Yuceyar N, Taskiran D, Sagduyu A. Serum and cerebrospinal fluid nitrite and nitrate levels in relapsing-remitting and secondary progressive multiple sclerosis.


