The role of blood and CSF biomarkers in the evaluation of new treatments against multiple sclerosis

Jan Lycke[1], MD, PhD, and Henrik Zetterberg[2, 3, 4, 5], MD, PhD

[1]Department of Clinical Neuroscience, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

[2] Department of Psychiatry and Neurochemistry; Institute of Neuroscience and Physiology at Sahlgrenska Academy, University of Gothenburg

[3] Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden

[4] Department of Molecular Neuroscience, UCL Institute of Neurology, Queen Square, London, UK, [5] UK Dementia Research Institute, London, UK

Corresponding Authors:

Jan Lycke

Email: jan.lycke@neuro.gu.se

Address: Gröna stråket 11, 3 tr, Sahlgrenska University Hospital, 415 45 Gothenburg, Sweden

Telephone number: +46 733 109149

Henrik Zetterberg

Email: henrik.zetterberg@gu.se

Address: Clinical Neurochemistry Laboratory, House V, Sahlgrenska University Hospital, 431 80 Mölndal, Sweden

Telephone number: +46 31 3430025

Abstract

Introduction: Multiple sclerosis (MS) is an immune-mediated chronic neurodegenerative disease of the central nervous system (CNS). Therapeutic interventions with immunomodulatory agents reduce disease activity and disability development, which are monitored clinically and by magnetic resonance imaging (MRI). However, these measures largely lack information on the impact from these therapies on inflammation, demyelination and axonal injury, the essential pathophysiological features of MS. Several biomarkers for inflammation and neurodegeneration have been detected in cerebrospinal fluid (CSF). In MS, some of these biomarkers seem to reflect disease activity, disability progression, and therapeutic response.

Areas covered: In this review, we describe the most promising CSF biomarkers of inflammation and degeneration for monitoring therapeutic interventions in MS. We also describe the evolution of highly sensitive immunoassays that enable determination of neuron-specific biomarkers in blood.

Expert commentary: Together with clinical and MRI measures, CSF biomarkers may improve the assessment of therapeutic efficacy and make personalized treatment possible. One disadvantage has been the need of repetitive lumbar punctures to obtain CSF. However, the technical development of highly sensitive immunoassays allows determination of extremely low quantities of neuron-specific proteins in blood. This will potentially open a new era for monitoring disease activity and treatment response in MS.

Keywords: Biomarkers, blood, cerebrospinal fluid, multiple sclerosis, neurofilament light, plasma, serum, Single molecule array, treatment

1. Introduction

Multiple sclerosis (MS) is an inflammatory and degenerative disease of the central nervous system (CNS). There is accumulating evidence that the inflammatory activity is responsible for excessive nervous tissue degeneration and disability development, especially during the early phases of MS (1, 2). The rate of inflammation and disease activity are reflected in relapses and lesion formation on magnetic resonance imaging (MRI) and subsequently in residual disability and atrophy development. Experience from two decades of therapeutic interventions in relapsing-remitting (RR) MS shows that immunomodulatory and immunosuppressive drugs reduce disease activity. Additionally, there seems to be an effect on the rate of degeneration (3). Thus, disease-modifying therapies (DMT) reduce CNS inflammation and change the clinical course and prognosis of MS.

To monitor disease activity, disability development, degeneration and therapeutic response, the development of biomarkers that reflect different parts of the pathophysiology of MS is essential. Currently, patient assessments for treatment decisions are based essentially on clinical and MRI measures. However, they largely lack information about the inflammatory cascade, its damaging effect of the CNS and repairing processes that may occur in response to the MS disease process. Moreover, MS is considered heterogeneous regarding clinical course and clinical manifestations, pathological mechanisms, and treatment responses. Although there are clinical and MRI measures and also cerebrospinal fluid (CSF) biomarkers that to some extent can predict the course and severity of MS, their estimated risk is low and their influence on the individual level is difficult to discern (4). The therapeutic options constantly increase, and the variety of their mechanism of action and efficacy has reached an impressive range. There is an unmet need to develop biomarkers that have the potential to reflect several of these aspects. In this review, we present data on biomarkers obtained from CSF that may add important knowledge on the pathogenesis of MS and the effect of treatment intervention. We also show that the development of extremely sensitive immunoassays has made it possible to determine some of these biomarkers in blood. This may open a new era for monitoring treatment efficacy in MS.

1.1 Overview of multiple sclerosis

MS is a common disabling neurological disease in young adults, affecting women more than twice as often as men; it is most prevalent in North America (140 cases per 100,000) and

Europe (108 cases per 100,000); the prevalence is lowest in sub-Saharan Africa (2.1 cases per 100,000) and East Asia (2.2 cases per 100,000) (5). There are no symptoms, clinical findings or diagnostic tests that are specific for MS. Instead, the diagnosis is determined essentially by a combination of clinical characteristics and MRI findings to establish dissemination of demyelinating lesions of the CNS (6). There is accumulating evidence that the cause of MS is multifactorial and depending on the interplay between risk genes, environmental and life-style risk factors. Although MS is considered an immune-mediated disease of the CNS, there is degeneration of both white and grey matter already at the clinical onset of MS, and during later stages of MS, atrophy of the brain and spinal cord often becomes extensive. The pathology of MS is characterized by blood-brain barrier impairment, multiple focal inflammatory infiltrates, demyelination, axonal loss, and astrogliosis. The course of the disease is highly variable and the revised categories or phenotypes have included the presence of inflammatory activity as an important feature (7). In 85% of the patients, MS starts with a transient episode of neurological symptoms, lasting for days to months, followed by remission, a period of clinical stability (8, 9). This onset is usually followed by recurrent relapses, on average 0.5-1 per year. After 15-20 years, the course of relapses and remissions (RRMS) converts into a secondary progressive course (SPMS) characterized by slowly increasing disability, with or without superimposed relapses. In 15% of MS patients, the course is primary progressive (PPMS), *i.e.*, progressive from onset, and essentially without any relapses.

1.2 Clinical and MRI monitoring of disease course

In clinical practice and in randomised controlled trials (RCT) the most common clinical measures are annual relapse rate (ARR) and confirmed disability progression (CDP), *i.e.*, a significant increase of the Expanded Disability Status Scale (EDSS) (10) that is sustained after 3 or 6 months of follow-up (11, 12). However, in recent RCTs, the ARR is relatively low, making it insensitive as a marker of disease activity. Moreover, the sensitivity and specificity of ARR and CDP are highly dependent on the frequency of visits, the experience of the EDSS rater, and the EDSS inter- and intra-rater variability (13). Even after 3 and 6 months of follow-up, EDSS may change and a follow-up period of 12 or 24 months may be necessary to achieve stability in the progression assessment (13, 14). Thus, established outcomes as ARR and CDP have shortcomings even when used under optimal conditions, which must be taken into consideration in the assessment of patients in clinical practice. In RCT and in the

neurology outpatient clinic, common MS symptoms, *e.g.*, fatigue, cognitive dysfunction and psychiatric symptoms, are often not apparent and therefore neglected or not taken into account (15, 16). Thus, the basis for evaluating the clinical course and disability development is sometimes inexact.

MRI has become the basis in MS diagnostics (6) and there are several guidelines of the use of MRI in monitoring MS (17-20). In contrast to relapse rate, serial MRI of the brain is sensitive for detecting disease activity. Over the same observational time, the number of new lesions on MRI is 4-12 times higher compared to relapse rate. MRI of the spinal cord, on the other hand, has several weaknesses for detecting new disease activity, in particular concerning gadolinium-enhancing lesions (21, 22). The reasons for not including serial MRI of the spinal cord for monitoring disease activity in MS include difficulties in MRI acquisition and MRI assessment and the relatively low frequency of new asymptomatic lesions of the spinal cord (23). MRI of the brain every 6-12 month usually includes gadolinium contrast-enhancing T1-weighted sequences to detect acute lesions (indicating blood-brain barrier disruption), active (new or enlarging) T2-weighted lesions and T1 hypo-intense lesions ("black holes") as a marker for degeneration if sustained for at least 6 months (24, 25). Although lesion load and brain atrophy development have been included in several RCT as measures of disease severity and neurodegeneration (26), they have not yet become useful measures of disease severity and degeneration in clinical practice.

1.3 Clinical and MRI monitoring of response to treatment

In phase III RCT of RRMS, the most used primary outcome has been annual relapse rate. Secondary outcomes often include confirmed disability development (EDSS) and MRI measures of disease activity (T1 gadolinium enhanced lesions and new or enlarging T2 lesions). In some trials, T1 hypo-intense lesions and measures of brain atrophy have been included as secondary or tertiary outcomes of degeneration. Over recent years, a composite measure has emerged, designated "No Evidence of Disease Activity" (NEDA). It includes relapse rate, disability development, and the appearance of T1 Gd contrast-enhancing lesions or new or enlarging T2 lesions on MRI (27, 28). It has been incorporated as an important outcome in clinical trials and has been suggested as a new treating target in clinical practice (29). In fact, the concept of not accepting any significant disease activity during treatment has received increasing acceptance. Signs of new disease activity in the

clinical assessment and on MRI have become a reason to switch to more effective treatments. However, it has become evident that only a minority of patients still fulfil NEDA after long-term of follow-up and hence the ability of NEDA to predict outcome has been questioned (30, 31). Although there have been attempts to include brain atrophy development in NEDA, other important disabilities, *e.g.*, cognitive dysfunction, may still evolve in patients with NEDA (32).

1.4 Unmet need in the clinical and MRI assessment of disease course and therapeutic efficacy

Most patients with active MS are monitored repeatedly with clinical examinations and MRI to detect disease activity, progression of disability, or adverse events from on-going DMT. However, standard 1.5 or 3T MRI almost exclusively detects only focal white matter lesions, while grey matter (GM) pathology and diffuse pathology of the normal appearing white matter (NAWM) usually remain undetected (33, 34). Asymptomatic lesions of the spinal cord may also escape detection, since serial MRI monitoring usually only involves scanning of the brain. This may be one reason for why relapses are not always associated with new lesions on brain MRI. Other explanations may be the small size of the symptomatic lesion, its location, or the high number of lesions, sometimes confluent, that make it impossible to distinguish a new lesion among already present ones. There is also the problem to discern if a new clinical event is a relapse, a pseudo-relapse or a recurrent symptom from a *locus minoris*. Moreover, development of some symptoms or disabilities, e.g. MS fatigue or cognitive dysfunction, may not be recognized at a standard clinical visit (16). Interestingly, GM lesions are associated with cognitive dysfunction (35) and correlate with the diffuse pathology found in NAWM of MS patients, which in turn seem to be involved in the progression of MS (36). Thus, there are several reasons to improve the assessment of MS patients to increase the possibilities to identify new disease activity and/or signs of neurodegeneration.

Another disadvantage of the clinical and MRI assessment of MS patients is their low ability to determine the nature of the pathological process that is involved in relapse, disability or formation of new lesions. Briefly, relapses and the appearance of new lesions on MRI are considered exacerbations of inflammatory activity and demyelination, contrast enhancement of T1 lesions is due to disruption of the BBB and a sign of ongoing immune

attack of the CNS, and sustained deterioration of disability and atrophy development are considered secondary to neurodegeneration.

The approved DMTs against MS seem to work by modulating the immune system, thereby influencing the inflammatory activity within the CNS. Some of these drugs have experimentally also shown neuroprotective or regenerative effects (37). There is accumulating evidence that degeneration, at least in early phases of relapsing MS, is essentially secondary to inflammation (1, 2). However, other processes of degeneration probably gain in importance during the course of MS and in particular in progressive MS (1, 2). It is also possible that degeneration also occurs in parallel with inflammatory-driven neuronal injury in relapsing MS. Although the target of the autoimmune attack in MS may be oligodendrocytes and myelin, the neuroaxonal damage and loss are probably the culprit of the CNS atrophy development and the accumulation of disability and impairment in MS over time (26, 38). There is also accumulating evidence that therapeutic intervention reduces the rate of atrophy (3). Thus, by use of biochemical biomarkers of inflammation and degeneration it may be possible to explore the kind of intervention that is associated with a specific treatment and the effect on degenerative processes.

2.1 Overview of biochemical biomarkers in multiple sclerosis

Biomarkers are characterized as indicators that can be objectively measured and used for evaluation of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention (39). In MS, the outcome from neurological examinations, tests of impairment and disability, and MRI measures constitutes the basis for the assessment of patients and the response to therapeutic interventions. Biochemical biomarkers in MS can be categorised into diagnostic, predictive, disease activity, and treatment response biomarkers (40). Although, they have been extensively explored, there is currently no fluid biomarkers that are validated to be used for evaluating disease activity, progression of disability or therapeutic response (40).

In MS, CSF has been the body fluid of particular interest due to its proximity to the CNS. Blood-based biomarkers have essentially been used only for differential diagnostic tests and for safety concerns associated with DMT treatment. With the discovery of an increasing number of biomarkers that may be relevant in MS, guidelines for obtaining and storing CSF

(41) and for selecting and defining control groups (42) have been established. The examined CSF biomarkers in MS research may roughly be divided into inflammatory, including cytokines and chemokines, and degenerative, reflecting different pathological processes, *e.g.*, axonal degeneration, astrogliosis and oxidative stress. Since the currently approved DMTs for treatment of MS are all immunomodulatory or immunosuppressive, the inhibiting effect on inflammatory mediators, such as cytokines and chemokines, is expected. However, much more important is to establish if also damage and degeneration of the CNS are reduced during intervention with DMT. Demyelination, oligodendrocyte loss, neuroaxonal damage, glial damage, and astrogliosis all belong to the pathological processes that are active in MS. In the following section, we review CSF biomarkers that reflect either inflammatory disease activity (relapse rate, MRI lesion formation) or disability development (including brain atrophy development) and have shown altered concentrations following DMT treatment in MS.

2.2 Inflammatory biomarkers in cerebrospinal fluid

There is a plethora of inflammatory biomarkers in CSF that have been explored in MS and some of them are associated with disease activity (43). Altered levels of inflammatory and regulatory cytokines, chemokines, immunoglobulins, and lymphocyte surface markers indicate that the immune attack of the CNS in MS involves a cascade of events that act in parallel. Here, we review the inflammatory biomarkers in CSF that have been examined in relation to disease activity and therapeutic response.

2..2.1 C-X-C motif chemokine 13

C-X-C motif chemokine 13 (CXCL13) is the ligand of the B-cell receptor CXCR5 and is the most potent B-cell chemoattractant (44). Elevated concentrations of CXCL13 were found in patients with MS compared to controls and they correlated with relapse rate (45), disability score (EDSS), and formation of new lesions on MRI (46). CSF CXCL13 concentration was also higher in patients with IgG oligoclonal bands (OCB) compared to OCB-negative patients and may have a predictive role since higher CXCL13 levels were found in CIS patients converting to MS compared to non-converters (46). In 25 CIS and RRMS patients, methylprednisolone treatment (n=12) significantly decreased CSF levels of CXCL13 compared with placebo (n=13). Further, 12 months of natalizumab treatment of 20 RRMS patients reduced CXCL13

to undetectable levels in 18 of them (45). In a phase 2 open label trial with natalizumab in 24 PMS, 17 completed the trial. They demonstrated reduced mean CXCL13 levels after 60 weeks of treatment (47). Similar results had previously been reported in 22 PMS patients after 2 years of mitoxantrone treatment (48). We recently showed that treatment-naïve RRMS patients (n=7) and RRMS patients on interferon beta (n=33) had significantly higher CXCL13 levels in CSF than patients treated with natalizumab (n=19) (49) and that 4-12 months of fingolimod treatment of 43 RRMS patients reduced CXCL13 levels in CSF to similar levels of that of healthy controls (50). Although DMTs seem to reduce CSF CXCL13 concentrations irrespective of MS phenotype, the disadvantage with CXCL13 is that the concentration is frequently below the lower limit of quantification of the CXCL13 assay (50).

2.2.2 Osteopontin

Osteopontin (OPN) is a pro-inflammatory cytokine involved in pleiotrophic physiological and pathological processes including inflammation and immunity. The protein is expressed in most tissues and body fluids. Elevated levels of OPN in CSF were initially confined to active RRMS (51, 52), but elevated CSF levels have also been reported in PPMS and were associated with subsequent disability (53). Higher OPN levels were found during relapse but rescue treatment with methylprednisolone did not influence the concentration (53). Further, 60 weeks of monthly methylprednisolone therapy of 30 PMS patients did not influence the CSF OPN level either, although clinical and MRI measures improved (54). In contrast, 60 weeks of natalizumab treatment of 17 PMS patients reduced OPN significantly (47). Thus, there are contradictory results and the value of determining OPN in CSF is still uncertain.

2.2.3 Fetuin A protein

In the search for biomarkers in CSF from MS patients, a proteomic analysis found increased levels of fetuin A protein that appeared to correlate with disease activity (55). This association to disease activity (relapse, increase of EDSS, or new T2 or contrast-enhancing lesions on MRI) was confirmed in another study that included 100 MS patients (56). In patients treated with natalizumab for 6 and 12 months, CSF fetuin A protein levels decreased and the reduction was more pronounced in treatment responders (56). Similar effects on fetuin A protein were not seen in blood. Fetuin A protein is almost exclusively

expressed in the liver of normal adults but was found in demyelinated lesions in brains from MS patients (56) and has probably immune regulatory functions.

2.3.1 Degenerative biomarkers in cerebrospinal fluid

The most studied degenerative biomarkers in MS are neurofilaments (NFs) and glial fibrillary acidic protein (GFAP). They are essentially CNS-specific and they represent two major pathological processes in MS: NF is elevated in CSF due to axonal damage and increased GFAP represents astrocyte activation and astrogliosis. There are several other degenerative biomarkers that have been explored in MS but the results have been contradictory or the evidence has not convincingly supported an association between therapeutic intervention and change of their levels in CSF.

2.3.2 Neurofilament proteins

NFs are the major structural components of the axonal and dendritic cytoskeleton and are composed of four subunits, the triplet of neurofilaments known as light (NFL), medium (NFM), and heavy (NFH), alpha-internexin in the central or peripherin in the peripheral nervous system (57). NFL is the most abundant (ratio: 4:2:1, NFL:NFM:NFH) and soluble subunit. It constitutes the backbone of the NF core, to which NFM and NFH copolymerize (58). NFH is the most phosphorylated protein of the brain, involved in the regulation of cell homeostasis and axonal transport (59). Except for traumatic brain injury (60), the clinical relevance of NFM in neurological diseases has not yet been explored. In contrast, the role of NFH and in particular NFL as biomarkers in various neurological diseases, including MS, has become more and more established over recent years. NFs are released into the extracellular fluid after axonal injury (61), and their levels in CSF are thought to reflect the amount of the axonal injury. In MS, NFL has been suggested a biomarker of disease activity and NFH of progression (62, 63), however there is considerable overlap between data, making such categorization questionable. When comparing NFL and NFH, the former seems more reliable for discriminating patients with MS or CIS from controls (64) and superior for monitoring treatment efficacy in RRMS (64). It has been claimed that NFL, due to being less phosphorylated, is susceptible for proteases in CSF and therefore not stable at room temperature (65). However, investigation of pre-analytical stress conditions of CSF samples have shown that delay of sample processing at room temperature for 24 h, up to four freeze-thaw cycles, and blood contamination of 0.5% had only marginal effects on the

stability of NFL and NFH (66). In fact, NFL seems to be stable for up to 8 days at room temperature (64).

2.3.3 Neurofilament light protein

The concentration of CSF NFL seems to be increased in all phenotypes of MS (67, 68). However, during relapse CSF NFL concentration is 3- to 10-fold (68-70) higher than in remission. After relapse, CSF NFL concentration peaks after approximately 2 weeks and thereafter returns to low levels after 2-3 months (67, 69). NFL also increases in conjunction with contrast-enhancing lesions on MRI (67, 69, 71, 72) and when new T2 lesions appear on sequential MRI examination (49, 62). There is a strong correlation between NFL levels and both relapse rate and lesion formation (new T2 lesions on MRI) (49, 62). Higher NFL levels are associated with increased CSF cell count, oligoclonal bands, and conversion from CIS to MS (62, 68). No or only weak association has been found between progression or disability and NFL (62, 67, 73). Thus, NFL in CSF seems to be a reliable marker of disease activity in MS, indicating that axonal injury is a major feature in active relapsing MS. In fact, the first paper on increased NFL levels in CSF from RRMS patients demonstrated high levels of NFL in CSF during relapse (69) and this observation coincided with the often cited paper by Trapp et al. that reported a high rate of transected axons in active lesions of MS patients, determined by staining of nonphosphorylated neurofilament in ovoid swellings of axons (34). There may, however, be additional mechanisms than axonal injury, *e.g.*, increased neuronal secretion, contributing to the NFL increase in active MS. Anyhow, the clinical relevance of determing NFL in MS has been explored and there is evidence that increased CSF NFL concentration may have both diagnostic (62, 74-76) and predictive value (76, 77).

2.3.4 Neurofilament light protein as biomarker for therapeutic response

Accumulating data show reduction in NFL levels during treatment with DMT in RRMS (49, 50, 78, 79) but also in PMS (71). In an early study on this topic, natalizumab treatment during 6 to 12 months reduced mean NFL level three-fold from 1300 (SD, 2200) ng/l to 400 (SD, 270) ng/l. ($p<0.001$) (72). Most patients had switched to natalizumab from first line DMD (interferon beta or glatiramer acetate, n=73) because of the appearance of new disease activity. In fact, natalizumab treatment reduced NFL levels to similar values obtained in CSF of healthy controls. In line with previous pivotal and observational studies on natalizumab treatment in RRMS (80-83), natalizumab-treated patients showed reduced

relapse rate, disability and inflammatory CSF markers (inflammatory cell counts, IgG indices). The anti-inflammatory effect of natalizumab treatment on cytokine and chemokine levels in the CSF compartment had been reported previously (45, 84, 85) but the normalization of the NFL level indicated for the first time that natalizumab treatment may influence axonal damage. Reduction of NFL was also noted in RRMS patients in remission indicating that axonal damage is prevalent in clinically stable patients. Interestingly, 9 of the natalizumab-treated patients had SPMS and they also showed significantly reduction of NFL. However, post-treatment NFL levels of this sub-group were still significantly higher compared to healthy controls. Similar results have been reported in RRMS patients treated with fingolimod (78, 79), patients (80% RRMS) treated with rituximab (86), and also in PMS treated with mitoxantrone or rituximab (71). Patients with breakthrough disease on first-line therapies showed reduced NFL levels after switching to fingolimod, *i.e.*, a second line DMT, and this reduction correlated with reduction in relapse rate and MRI measures (79). Included patients tended to be in an active phase of the disease, suggesting that the decrease in NFL value could partly be explained by regression to the mean. However, a similar reduction of NFL levels was also shown in CSF obtained from patients participating in a randomised controlled trial (87), comparing fingolimod treatment with placebo in RRMS. In a sub-group of this study-population, CSF was obtained prior to and 12 months after treatment initiation. At baseline, CSF NFL concentrations did not differ between placebo, fingolimod 0.5 mg and fingolimod 1.25mg treated patients, but 12 months of fingolimod therapy significantly reduced NFL levels compared with those in the placebo group (78). A S similar influence on NFL values was seen in patients, mostly of RRMS course with breakthrough disease on interferon beta or glatiramer acetate, who had rituximab as an add-on treatment (86), and in PMS treated with mitoxantrone or rituximab (71). Most of the included PMS patients showed clinical activity or evidence of activity on MRI prior to treatment. Interestingly, significantly lower NFL levels were found before starting mitoxantrone or rituximab treatment in PMS patients previously treated with interferon beta compared to treatment naïve patients, indicating an effect on axonal degeneration also with first line DMT during progressive MS.

2.3.5 Neurofilament heavy protein

In contrast to NFL, NFH seems not to reflect disease activity in RRMS, but several studies demonstrate a relationship between CSF NFH levels and disability (62, 88, 89). Despite this

association, a clear difference between different MS phentypes has not been seen. There are only two studies that include NFH in CSF as a marker of DMT intervention (86, 90). However, the reduction of CSF NFH levels was not statistically significant and less pronounced than for NFL in both the natalizumab-treated patients (90) and in the study in which rituximab was tested as an add-on therapy to interferon beta or glatiramer acetate (86). Thus, NFH seems to be a better marker to reflect axonal degeneration during progression (63).

2.3.6 Glial fibrillary acidic protein

Glial fibrillary acidic protein (GFAP) is a structural intermediate filament of the cytoskeleton of astrocytes. It is generally considered a highly CNS-specific protein though studies have indicated the presence of it in extracerebral tissues (91). GFAP was originally isolated and characterized in MS-lesions with severe astrogliosis (92). In MS, no relation to relapse rate has been found but augmented concentrations of GFAP correlated with increased disability in RRMS (93) and the highest association is found in SPMS (67). CSF GFAP concentration is age-dependent. The association of CSF GFAP levels with disability was further investigated in MS patients and healthy controls in a long-term follow-up study of 9 years (73). Again, the highest GFAP levels were found in patients with SPMS. No effect on CSF GFAP levels were found in RRMS patients after 6 to 12 months of natalizumab treatment (72). Unchanged GFAP levels were also found in progressive MS after 12 to 24 months of mitoxantrone (n=30) or rituximab (n=5) treatment (71).

2.3.7 Chitinase 3-like 1 protein

Chitinase 3-like 1 protein (CHI3L1), also designated YKL-40, is up-regulated in inflamed tissues and altered expression has been detected in several CNS disorders, including MS. The level of CHI3L1 increases with age. In the CNS, CHI3L1 is mostly related to astrocytes and to some extent also activated macrophages and microglia (94). The function of CHI3L1 is unknown but it might be involved in regulation of neurotrophic factors and thereby tissue regeneration during inflammation. The transcription of CHI3L1 is associated with reactive gliosis and more pronounced in conditions of neuroinflammation than in degenerativ diseases (94). Elevated CSF CHI3L1 levels were found in CIS patients and in patients with optic neuritis (ON) who converted to MS compared to non-conveters (75, 95). Increased CHI3L1 levels were found in both RRMS (50, 94, 96, 97) and SPMS (97, 98) and have been

associated with relapses and number of contrast-enhancing lesions on MRI (50, 97, 98), but also with disability progression (75, 76). Treatment of MS patients (n=19; 17 RRMS, 2 SPMS) with natalizumab during approximately one year significantly reduced CSF CHI3L1 levels (97, 99). Similar reductions were found in RRMS patients switching from first line DMT to fingolimod (n=26). After 4-12 months of fingolimod treatment, CHI3L1 levels decreased but did not reach those of healthy controls (79). Daclizumab given intravenously or subcutaneously to 40 RRMS patients, participating in two open-label studies, significantly reduced CSF CHI3L1 levels at long-term follow-up (100). Interestingly, mitoxantrone treatment of MS patients (n=22; 3 RRMS, 19 SPMS), reduced CHI3L1 conscentrations in CSF (97). Thus, CHI3L1 is not confined to a specific phenotype of MS, but is rather associated with disease activity and progression of disability in a manner that may be modified by DMTs.

3.1 Moving from CSF to blood

In MS and other CNS disorders, pathological processes are more likely to be reflected in CSF than in blood due to its proximity to the affected tissue. However, the need for lumbar puncture constitutes a major barrier for more wide-spread use, especially when repeated lumbar punctures are needed. This is often the case in MS, when inflammatory disease activity, progression of degeneration, and DMT efficacy are monitored. Thus, moving from CSF- to blood-based biomarkers would be a major step for monitoring disease activity in MS. However, a general challenge with blood-based biomarker analyses when examining CNS diseases is that the biomarker concentration may reflect release from peripheral tissues. In MS, it has proven very hard to detect an inflammatory profile in the blood that reflects the inflammatory process in the brain. More sensitive assays are unlikely to solve this problem. Moreover, all DMTs modulate or suppress the immune system and their mechanisms of action differ between them. Hence, blood-based inflammatory biomarkers may reflect the therapeutic intervention of the immune system but not necessary the effect of DMT on the pathological processes in the brain. However, CNS-enriched proteins that are not expressed in peripheral tissues, or are expressed at much lower levels than in the brain, may be released into the bloodstream so that they can be measured at very low concentrations, provided the assay is sensitive enough. This development of highly sensitive immunoassays has just started and amongst the data on serum or plasma derived biomarkers, NFL is recognised as the most promising biomarker that could potentially be included as an

outcome marker in clinical trials and even as a tool for evaluation of MS patients in the clinic.

3.2 The evolvement of immunoassays to facilitate blood analyses

Most biomarker assays of relevance to MS research are immunochemical, *i.e.*, utilise antibodies to quantify a substance in a sample. The most common assay format is the sandwich enzyme-linked immunosorbent assay (ELISA) in which the target analyte is captured between two antibodies in a complex and one of the antibodies carries a signal generator, *i.e.*, an enzyme that converts a substrate into a detectable form (coloured, fluorescent or luminescent), which, in combination with a calibrator curve (derived from artificial samples with known analyte concentrations), allows for quantification of the analyte of interest. ELISA is a theme with many variations, such as the choice of signal generator where the enzyme can be replaced by, *e.g.*, a fluorophore or a DNA-based detection system. For a regular ELISA, it is rare to reach an analytical sensitivity below 10-50 pg/mL. In CSF, this may be enough, but to measure CNS-derived proteins in the blood, the low concentrations of them demand much higher sensitivity for detection. However, new ultrasensitive technologies have largely solved this problem. Most of these technologies rely on antibody-based detection of the target molecule, but in Single molecule array (Simoa), the detection reaction is compartmentalized into a small volume (50 femtolitres), so that the reporter molecule accumulates at a very high concentration (101), in Single molecule counting (SMC), the labelled detection antibodies, specifically captured by the target molecule/capture antibody complex, are released and counted one by one in a small detection cell, which allows for a single molecule read-out (102), and in proximity extension assay (PEA), partly overlapping complementary DNA strands are attached to the different antibodies allowing the strands to form a polymerase chain reaction-amplifiable template if immobilised close to each other on the same molecule (103). These variations in signal generation/detection may result in assays that can be 100- to a 1000-fold as sensitive as the corresponding regular ELISA using the same antibody pair.

3.3 An ultrasensitive assay for NFL

Increased levels of blood derived NFL were originally detected in MS with an electrochemiluminescence-based method for NFL (104). However, the analytical sensitivity

of that method limited the quantitation to samples with moderately to severely increased concentrations of blood NFL and could not quantify normal levels or detect mild increases (105). We recently developed an immunoassay for NFL based on Simoa technology that allows quantification down to subfemtomolar concentrations (below 1 pg/mL) of the analyte (106) and is 25-fold as sensitive as the previous electrochemiluminescence-based method for NFL (104). Serum NFL concentrations measured using Simoa correlate closely with CSF concentrations and are increased in HIV-associated dementia, progressive supranuclear palsy and frontotemporal dementia (106, 107). In fact, the Simoa method for NFL in blood samples (serum or plasma; either sample type works just as well), has markedly improved analytical sensitivity as compared to standard ELISA or electrochemiluminescence immunoassays, allowing the accurate measurement of NFL in blood down to concentrations occurring in healthy persons (108).

3.4 Serum or plasma NFL for monitoring disease activity and treatment response in MS

In MS populations, there is high correlation between blood and CSF NFL concentrations (104, 109-112). By use of the electrochemiluminescence immunoassays or the Simoa method for blood NFL, increased levels correlate with relapses, EDSS assessments, cerebral MRI measures such as number of cerebral or spinal lesions as well as cerebral lesion volumes , and the occurrence of contrast enhancing lesions (104, 109, 111-113).

DMT of MS has previously been shown to reduce levels of NFL in CSF (71, 72, 78). Recently, we investigated the effect on plasma NFL in 243 patients following 12 and 24 months of fingolimod treatment. They had previously been treated with first generation of DMT (interferon beta, galiramer acetate) and switched to fingolimod mostly due to lack of effect. After 12 months of fingolimod treatment, the mean reduction of plasma NFL was 34% and it remained stable at this level also after 24 months of treatment (111). In another study, similar reductions of serum NFL were reported in patients treated with interferon beta/glatirameracetate (n=39), fingolimod (n=136), natalizumab (n=21), and rituximab (n=16) (109). We showed in a large set up of paired serum and CSF samples obtained from RRMS (n=204) and PMS (n=82) from real life MS cohorts that the NFL levels were altered similarily in serum and CSF following DMT intervention. Thus, the DMT efficacy was reflected by NFL concentrations, and serum NFL was as reliable as CSF NFL to detect the change. While patients treated with first generation DMT reduced their serum NFL levels

after switching to more efficacious treatment, patients had stable serum NFL levels if they remained on a DMT of similar efficacy. Moreover, in RRMS patients who were treated with effective DMT, we confirmed our previous finding in CSF, that the NFL concentration in serum was not different from that in healthy controls (50, 72). These results support the value of blood-based NFL as a sensitive and clinically meaningful blood biomarker to monitor disease activity and the effects of therapies in MS.

4. Conclusions

MS is a heterogeneous immune-mediated disease with considerable elements of degeneration in its pathogenesis (1, 2). The growing possibility to treat MS has raised demands for accessible, accurate and validated biomarkers for prediction of disease severity and for monitoring disease activity, disability progression, and the effect of therapeutic intervention. The goal is to offer patients and clinicians tools to enable personalized treatment to prevent further injury to the CNS and improve function. Up to now, the DMTs are essentially immunomodulatory or immunosuppressive and with few exceptions approved for relapsing MS. Current biomarkers for monitoring therapeutic response rely on clinical and MRI measures. However, in conventional MS care they may not capture subclinical processes, more subtle dysfunctions, *e.g.*, cognitive dysfunction, and low-grade neurodegeneration. Biochemical biomarkers in CSF and blood may add important information on both inflammation and neurodegeneration in MS and may improve our knowledge about MS pathogenesis. Cytokines, chemokines and other inflammatory biomarkers in CSF may reflect the diversity of the involvement of the immune system in MS and its activity. However, these biomarkers are not CNS-specific and other diseases, *e.g.*, infections and treatments that affect the immune system, may influence biomarker concentrations. Moreover, DMTs have different mechanisms of action, influencing the inflammatory biomarkers in the CSF differently from what might happen in blood. It has also become evident that inflammatory biomarkers are often sensitive to degradation by proteases and therefore demand adequate sample handling and storage. The inflammatory biomarkers CXCL13, osteopontin, and feutin A protein were selected in this review since they all have shown association to disease activity and were influenced by DMT intervention. However, the evidence is mostly based on studies of limited size, the results are sometimes contradictory and the sensitivity of current assays may not allow detection of the biomarker in patients with low to moderate disease activity. In contrast, CNS-derived

proteins are less influenced by pathological processes in other tissues or directly by immunomodulatory or immunosuppressive therapies. Although, they may not be the target of the immune attack in MS, they are released extracellularly in response to inflammation-mediated damage to the CNS. NFL and NFH reflect the rate of axonal damage and GFAP and CHI3L1 represent astrogliosis and astrocyte or microglia involvement. While NFs and CHI3L1 correlate mostly with disease activity, GFAP seems to be a marker of disease progression in MS. The latter marker appears to be less responsive to DMTs or may possibly require considerably longer follow-up than two years (71, 72). It is obvious from current data on CSF biomarkers that NFL is the most promising biomarker in MS and that it is already useful for evaluation of disease intensity at the clinical onset and for monitoring therapeutic response. The development of highly sensitive immunoassays has enabled determination of extremely low concentrations of NFL in blood. The accumulating data on blood-based NFL in MS show that it may be possible to switch from CSF to blood tests for this marker. This will become an important contribution for monitoring therapies in RCT and, when the Simoa method and other methods with similar analytical sensitivity have become more available, also in conventional MS care.

5. Expert commentary

Monitoring of therapeutic response in MS would improve if current clinical and MRI measures were expanded with determination of biochemical biomarkers in CSF and blood. This would improve the guidance for therapeutic decisions. By combining inflammatory and degenerative biomarkers, the possibility increases to better define the effect of DMT intervention in MS. Several studies that have had this approach show that altered CSF levels of inflammatory biomarkers (*e.g.*, CXCL13) correlate with those determined in CSF for CHI3L1 and NFL (50, 79), indicating that the reduction of inflammation is accompanied by decreased glial activity and axonal degeneration. In fact, these studies strongly support that DMT treatment also have profound effects on the progressive changes in MS.

The most promising biochemical biomarker in MS is NFL, reflecting the rate of axonal damage. Axonal loss is the main source of brain atrophy and disability development in MS and increased NFL levels are found in MS irrespective of phenotype, is associated with relapse and formation of lesions on MRI, but less to disability progression. The response to DMT is reflected in CIS, RRMS and inflammatory active PMS. However, the invasiveness and

discomfort of lumbar puncture, especially if repeated for monitoring therapeutic response, has limited the usefulness of NFL and other biochemical biomarkers. The development of an ultrasensitive immunoassay for NFL has made NFL a more accessible biomarker. The high correlation between NFL in CSF and serum/plasma (111, 112) and their similar outcome following DMT treatment suggest that blood-based NFL can replace CSF (112). NFL in plasma/serum is robust and the concentration is only slightly reduced when samples were transported by post during several days (111). Thus, NFL seems to become the first blood-based test that can be used on a large scale for monitoring treatment efficacy in MS.

6. Five year commentary

The Simoa technique and other platforms with similar analytical sensitivity are rapidly disseminated over the world, making it possible to offer blood-based NFL for monitoring therapeutic response in MS patients. Other CNS-enriched proteins may follow, and thereby extend the characterisation of pathological processes in MS and the impact from DMT on them. Over recent years the therapeutic alternatives have increased. Today, almost all MS patients with activity are treated with DMT and early and effective intervention should be mandatory. NFL should become an accessible blood test for monitoring DMTs in conventional care and should be included together with clinical and MRI measures as a biomarker for disease activity. In treated MS patients who reach traditionally defined NEDA, NFL may even replace other measures of disease activity. In the futrue, a blood test every third month for monitoring the NFL level may become the only surveillance for disease activity needed in such patients.

7. Key issues

- Cerebrospinal fluid biomarkers may detect disease activity in multiple sclerosis, improve the assessment of therapeutic efficacy and make personalized treatment possible.
- Highly sensitive immunoassays allow for the determination of extremely low concentrations of neuronal proteins in blood; the most promising blood-based biomarker for disease activity in multiple sclerosis is neurofilament light.
- Blood-based tests of disease activity will potentially open a new era for monitoring disease activity and treatment response in multiple sclerosis.

8. Review criteria box

We searched PubMed for English language articles on multiple sclerosis and biomarkers using the keyword "multiple sclerosis" together with other keywords, including: "biomarker", "diagnosis", "prognosis", "imaging", "MRI", "treatment", and several other keywords relevant to every section (*e.g.*, biomarker names). We largely selected publications in the past 5 years, but did not exclude important older publications. Selection criteria also included a judgment on the novelty of studies and their relevance for the well-informed neurologist/MS researcher.

Conflict of interests/disclosures

JL has received travel support and/or lecture honoraria from Biogen, Novartis, Teva, and Genzyme/Sanofi Aventis; has served on scientific advisory boards for Almirall, Teva, Biogen, Novartis, and Genzyme/Sanofi Aventis; serves on the editorial board of the Acta Neurologica Scandinavica. HZ is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg, and has served at advisory boards of Eli Lilly, Roche Diagnostics, Lundbeck, and Pharmasum Therapeutics. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Funding

No specific funding was used to prepare this manuscript.

References

1. Friese MA, Schattling B, Fugger L. Mechanisms of neurodegeneration and axonal dysfunction in multiple sclerosis. Nat Rev Neurol. 2014;10(4):225-38.
2. Lassmann H. Pathology and disease mechanisms in different stages of multiple sclerosis. J Neurol Sci. 2013;333(1-2):1-4.
3. Sormani MP, Arnold DL, De Stefano N. Treatment effect on brain atrophy correlates with treatment effect on disability in multiple sclerosis. Annals of neurology. 2014;75(1):43-9.
4. Gajofatto A, Calabrese M, Benedetti MD, Monaco S. Clinical, MRI, and CSF markers of disability progression in multiple sclerosis. Dis Markers. 2013;35(6):687-99.
5. Doshi A, Chataway J. Multiple sclerosis, a treatable disease. Clin Med (Lond). 2016 Dec;16(Suppl 6):s53-s59.

6. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann Neurol. 2011;69(2):292-302.
7. Lublin FD, Reingold SC, Cohen JA, Cutter GR, Sorensen PS, Thompson AJ, et al. Defining the clinical course of multiple sclerosis: the 2013 revisions. Neurology. 2014;83(3):278-86.
8. Confavreux C, Vukusic S. Natural history of multiple sclerosis: a unifying concept. Brain. 2006;129(Pt 3):606-16.
9. Scalfari A, Neuhaus A, Degenhardt A, Rice GP, Muraro PA, Daumer M, et al. The natural history of multiple sclerosis: a geographically based study 10: relapses and long-term disability. Brain. 2010;133(Pt 7):1914-29.
10. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). Neurology. 1983;33:1444-52.
11. Filippini G, Del Giovane C, Vacchi L, D'Amico R, Di Pietrantonj C, Beecher D, et al. Immunomodulators and immunosuppressants for multiple sclerosis: a network meta-analysis. Cochrane Database Syst Rev. 2013(6):CD008933.
12. Pozzilli C, Prosperini L. Clinical markers of therapeutic response to disease modifying drugs. Neurol Sci. 2008;29 Suppl 2:S211-3.
13. Noseworthy JH, Vandervoort MK, Wong CJ, Ebers GC. Interrater variability with the Expanded Disability Status Scale (EDSS) and Functional Systems (FS) in a multiple sclerosis clinical trial. The Canadian Cooperation MS Study Group. Neurology. 1990;40(6):971-5.
14. Kalincik T, Cutter G, Spelman T, Jokubaitis V, Havrdova E, Horakova D, et al. Defining reliable disability outcomes in multiple sclerosis. Brain. 2015;138(Pt 11):3287-98.
15. Amato MP, Ponziani G, Siracusa G, Sorbi S. Cognitive dysfunction in early-onset multiple sclerosis: a reappraisal after 10 years. Arch Neurol. 2001;58(10):1602-6.
16. Penner IK. Evaluation of cognition and fatigue in multiple sclerosis: daily practice and future directions. Acta Neurol Scand. 2016;134 Suppl 200:19-23.
17. Filippi M, Rocca MA, Ciccarelli O, De Stefano N, Evangelou N, Kappos L, et al. MRI criteria for the diagnosis of multiple sclerosis: MAGNIMS consensus guidelines. Lancet Neurol. 2016;15(3):292-303.
18. Traboulsee A, Simon JH, Stone L, Fisher E, Jones DE, Malhotra A, et al. Revised Recommendations of the Consortium of MS Centers Task Force for a Standardized MRI Protocol and Clinical Guidelines for the Diagnosis and Follow-Up of Multiple Sclerosis. AJNR Am J Neuroradiol. 2016;37(3):394-401.
19. Vagberg M, Axelsson M, Birgander R, Burman J, Cananau C, Forslin Y, et al. Guidelines for the use of magnetic resonance imaging in diagnosing and monitoring the treatment of multiple sclerosis: recommendations of the Swedish Multiple Sclerosis Association and the Swedish Neuroradiological Society. Acta Neurol Scand. 2017;135(1):17-24.
20. Wattjes MP, Rovira A, Miller D, Yousry TA, Sormani MP, de Stefano MP, et al. Evidence-based guidelines: MAGNIMS consensus guidelines on the use of MRI in multiple sclerosis--establishing disease prognosis and monitoring patients. Nat Rev Neurol. 2015;11(10):597-606.
21. Silver NC, Good CD, Sormani MP, MacManus DG, Thompson AJ, Filippi M, et al. A modified protocol to improve the detection of enhancing brain and spinal cord lesions in multiple sclerosis. J Neurol. 2001;248(3):215-24.
22. Thorpe JW, Kidd D, Moseley IF, Kenndall BE, Thompson AJ, MacManus DG, et al. Serial gadolinium-enhanced MRI of the brain and spinal cord in early relapsing-remitting multiple sclerosis. Neurology. 1996;46(2):373-8.

23. Lycklama G, Thompson A, Filippi M, Miller D, Polman C, Fazekas F, et al. Spinal-cord MRI in multiple sclerosis. Lancet Neurol. 2003;2(9):555-62.
24. Giorgio A, Stromillo ML, Bartolozzi ML, Rossi F, Battaglini M, De Leucio A, et al. Relevance of hypointense brain MRI lesions for long-term worsening of clinical disability in relapsing multiple sclerosis. Mult Scler. 2014;20(2):214-9.
25. Mitjana R, Tintore M, Rocca MA, Auger C, Barkhof F, Filippi M, et al. Diagnostic value of brain chronic black holes on T1-weighted MR images in clinically isolated syndromes. Mult Scler. 2014;20(11):1471-7.
26. De Stefano N, Airas L, Grigoriadis N, Mattle HP, O'Riordan J, Oreja-Guevara C, et al. Clinical relevance of brain volume measures in multiple sclerosis. CNS Drugs. 2014;28(2):147-56.
27. Havrdova E, Galetta S, Hutchinson M, Stefoski D, Bates D, Polman CH, et al. Effect of natalizumab on clinical and radiological disease activity in multiple sclerosis: a retrospective analysis of the Natalizumab Safety and Efficacy in Relapsing-Remitting Multiple Sclerosis (AFFIRM) study. Lancet Neurol. 2009;8(3):254-60.
28. Lublin FD. Disease activity free status in MS. Mult Scler Relat Disord. 2012;1(1):6-7.
29. Giovannoni G, Turner B, Gnanapavan S, Offiah C, Schmierer K, Marta M. Is it time to target no evident disease activity (NEDA) in multiple sclerosis? Mult Scler Relat Disord. 2015;4(4):329-33.
30. Bevan CJ, Cree BA. Disease activity free status: a new end point for a new era in multiple sclerosis clinical research? JAMA Neurol. 2014;71(3):269-70.
31. Rotstein DL, Healy BC, Malik MT, Chitnis T, Weiner HL. Evaluation of no evidence of disease activity in a 7-year longitudinal multiple sclerosis cohort. JAMA Neurol. 2015;72(2):152-8.
32. Damasceno A, Damasceno BP, Cendes F. No evidence of disease activity in multiple sclerosis: Implications on cognition and brain atrophy. Mult Scler. 2016;22(1):64-72.
33. Calabrese M, Magliozzi R, Ciccarelli O, Geurts JJ, Reynolds R, Martin R. Exploring the origins of grey matter damage in multiple sclerosis. Nat Rev Neurosci. 2015;16(3):147-58.
34. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. Axonal transection in the lesions of multiple sclerosis. N Engl J Med. 1998;338(5):278-85.
35. Geurts JJ, Calabrese M, Fisher E, Rudick RA. Measurement and clinical effect of grey matter pathology in multiple sclerosis. Lancet Neurol. 2012;11(12):1082-92.
36. Mistry N, Abdel-Fahim R, Mougin O, Tench C, Gowland P, Evangelou N. Cortical lesion load correlates with diffuse injury of multiple sclerosis normal appearing white matter. Mult Scler. 2014;20(2):227-33.
37. Anastasiadou S, Knoll B. The multiple sclerosis drug fingolimod (FTY720) stimulates neuronal gene expression, axonal growth and regeneration. Exp Neurol. 2016;279:243-60.
38. Filippi M, Rocca MA, Barkhof F, Bruck W, Chen JT, Comi G, et al. Association between pathological and MRI findings in multiple sclerosis. Lancet Neurol. 2012;11(4):349-60.
39. Biomarkers Definitions Working G. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther. 2001;69(3):89-95.
40. Comabella M, Montalban X. Body fluid biomarkers in multiple sclerosis. Lancet Neurol. 2014;13(1):113-26.

41. Teunissen CE, Petzold A, Bennett JL, Berven FS, Brundin L, Comabella M, et al. A consensus protocol for the standardization of cerebrospinal fluid collection and biobanking. Neurology. 2009;73(22):1914-22.
42. Teunissen C, Menge T, Altintas A, Alvarez-Cermeno JC, Bertolotto A, Berven FS, et al. Consensus definitions and application guidelines for control groups in cerebrospinal fluid biomarker studies in multiple sclerosis. Mult Scler. 2013;19(13):1802-9.
43. Tomioka R, Matsui M. Biomarkers for multiple sclerosis. Intern Med. 2014;53(5):361-5.
44. Aloisi F, Columba-Cabezas S, Franciotta D, Rosicarelli B, Magliozzi R, Reynolds R, et al. Lymphoid chemokines in chronic neuroinflammation. J Neuroimmunol. 2008;198(1-2):106-12.
45. Sellebjerg F, Bornsen L, Khademi M, Krakauer M, Olsson T, Frederiksen JL, et al. Increased cerebrospinal fluid concentrations of the chemokine CXCL13 in active MS. Neurology. 2009;73(23):2003-10.
46. Khademi M, Kockum I, Andersson ML, Iacobaeus E, Brundin L, Sellebjerg F, et al. Cerebrospinal fluid CXCL13 in multiple sclerosis: a suggestive prognostic marker for the disease course. Mult Scler. 2011;17(3):335-43.
47. Romme Christensen J, Ratzer R, Bornsen L, Lyksborg M, Garde E, Dyrby TB, et al. Natalizumab in progressive MS: results of an open-label, phase 2A, proof-of-concept trial. Neurology. 2014;82(17):1499-507.
48. Axelsson M, Mattsson N, Malmestrom C, Zetterberg H, Lycke J. The influence of disease duration, clinical course, and immunosuppressive therapy on the synthesis of intrathecal oligoclonal IgG bands in multiple sclerosis. J Neuroimmunol. 2013;264(1-2):100-5.
49. Novakova L, Axelsson M, Khademi M, Zetterberg H, Blennow K, Malmestrom C, et al. Cerebrospinal fluid biomarkers as a measure of disease activity and treatment efficacy in relapsing-remitting multiple sclerosis. J Neurochem. 2017;141(2):296-304.
50. Novakova L, Axelsson M, Khademi M, Zetterberg H, Blennow K, Malmestrom C, et al. Cerebrospinal fluid biomarkers as a measure of disease activity and treatment efficacy in relapsing-remitting multiple sclerosis. J Neurochem. 2016.
51. Braitch M, Nunan R, Niepel G, Edwards LJ, Constantinescu CS. Increased osteopontin levels in the cerebrospinal fluid of patients with multiple sclerosis. Arch Neurol. 2008;65(5):633-5.
52. Chowdhury SA, Lin J, Sadiq SA. Specificity and correlation with disease activity of cerebrospinal fluid osteopontin levels in patients with multiple sclerosis. Arch Neurol. 2008;65(2):232-5.
53. Bornsen L, Khademi M, Olsson T, Sorensen PS, Sellebjerg F. Osteopontin concentrations are increased in cerebrospinal fluid during attacks of multiple sclerosis. Mult Scler. 2011;17(1):32-42.
54. Ratzer R, Iversen P, Bornsen L, Dyrby TB, Romme Christensen J, Ammitzboll C, et al. Monthly oral methylprednisolone pulse treatment in progressive multiple sclerosis. Mult Scler. 2016;22(7):926-34.
55. Harris VK, Diamanduros A, Good P, Zakin E, Chalivendra V, Sadiq SA. Bri2-23 is a potential cerebrospinal fluid biomarker in multiple sclerosis. Neurobiol Dis. 2010;40(1):331-9.
56. Harris VK, Donelan N, Yan QJ, Clark K, Touray A, Rammal M, et al. Cerebrospinal fluid fetuin-A is a biomarker of active multiple sclerosis. Mult Scler. 2013;19(11):1462-72.

57. Lee MK, Cleveland DW. Neuronal intermediate filaments. Annu Rev Neurosci. 1996;19:187-217.
58. Lee MK, Xu Z, Wong PC, Cleveland DW. Neurofilaments are obligate heteropolymers in vivo. J Cell Biol. 1993;122(6):1337-50.
59. Petzold A. Neurofilament phosphoforms: surrogate markers for axonal injury, degeneration and loss. J Neurol Sci. 2005;233(1-2):183-98.
60. Martinez-Morillo E, Childs C, Garcia BP, Alvarez Menendez FV, Romaschin AD, Cervellin G, et al. Neurofilament medium polypeptide (NFM) protein concentration is increased in CSF and serum samples from patients with brain injury. Clin Chem Lab Med. 2015;53(10):1575-84.
61. Norgren N, Rosengren L, Stigbrand T. Elevated neurofilament levels in neurological diseases. Brain Res. 2003;987(1):25-31.
62. Teunissen CE, Iacobaeus E, Khademi M, Brundin L, Norgren N, Koel-Simmelink MJ, et al. Combination of CSF N-acetylaspartate and neurofilaments in multiple sclerosis. Neurology. 2009;72(15):1322-9.
63. Teunissen CE, Khalil M. Neurofilaments as biomarkers in multiple sclerosis. Mult Scler. 2012;18(5):552-6.
64. Kuhle J, Plattner K, Bestwick JP, Lindberg RL, Ramagopalan SV, Norgren N, et al. A comparative study of CSF neurofilament light and heavy chain protein in MS. Mult Scler. 2013;19(12):1597-603.
65. Koel-Simmelink MJ, Teunissen CE, Behradkia P, Blankenstein MA, Petzold A. The neurofilament light chain is not stable in vitro. Annals of neurology. 2011;69(6):1065-6; author reply 6-7.
66. Koel-Simmelink MJ, Vennegoor A, Killestein J, Blankenstein MA, Norgren N, Korth C, et al. The impact of pre-analytical variables on the stability of neurofilament proteins in CSF, determined by a novel validated SinglePlex Luminex assay and ELISA. J Immunol Methods. 2014;402(1-2):43-9.
67. Malmestrom C, Haghighi S, Rosengren L, Andersen O, Lycke J. Neurofilament light protein and glial fibrillary acidic protein as biological markers in MS. Neurology. 2003;61(12):1720-5.
68. Norgren N, Sundstrom P, Svenningsson A, Rosengren L, Stigbrand T, Gunnarsson M. Neurofilament and glial fibrillary acidic protein in multiple sclerosis. Neurology. 2004;63(9):1586-90.
69. Lycke JN, Karlsson JE, Andersen O, Rosengren LE. Neurofilament protein in cerebrospinal fluid: a potential marker of activity in multiple sclerosis. J Neurol Neurosurg Psychiatry. 1998;64(3):402-4.
70. Lycke J, Malmestrom C, Stahle L. Acyclovir levels in serum and cerebrospinal fluid after oral administration of valacyclovir. Antimicrob Agents Chemother. 2003;47(8):2438-41.
71. Axelsson M, Malmestrom C, Gunnarsson M, Zetterberg H, Sundstrom P, Lycke J, et al. Immunosuppressive therapy reduces axonal damage in progressive multiple sclerosis. Mult Scler. 2014;20(1):43-50.
72. Gunnarsson M, Malmestrom C, Axelsson M, Sundstrom P, Dahle C, Vrethem M, et al. Axonal damage in relapsing multiple sclerosis is markedly reduced by natalizumab. Ann Neurol. 2011;69(1):83-9.
73. Axelsson M, Malmestrom C, Nilsson S, Haghighi S, Rosengren L, Lycke J. Glial fibrillary acidic protein: a potential biomarker for progression in multiple sclerosis. J Neurol. 2011;258(5):882-8.
74. Kuhle J, Disanto G, Dobson R, Adiutori R, Bianchi L, Topping J, et al. Conversion from clinically isolated syndrome to multiple sclerosis: A large multicentre study. Mult Scler. 2015;21(8):1013-24.

75. Modvig S, Degn M, Roed H, Sorensen TL, Larsson HB, Langkilde AR, et al. Cerebrospinal fluid levels of chitinase 3-like 1 and neurofilament light chain predict multiple sclerosis development and disability after optic neuritis. Mult Scler. 2015;21(14):1761-70.
76. Martinez MA, Olsson B, Bau L, Matas E, Cobo Calvo A, Andreasson U, et al. Glial and neuronal markers in cerebrospinal fluid predict progression in multiple sclerosis. Mult Scler. 2015;21(5):550-61.
77. Salzer J, Svenningsson A, Sundstrom P. Neurofilament light as a prognostic marker in multiple sclerosis. Mult Scler. 2010;16(3):287-92.
78. Kuhle J, Disanto G, Lorscheider J, Stites T, Chen Y, Dahlke F, et al. Fingolimod and CSF neurofilament light chain levels in relapsing-remitting multiple sclerosis. Neurology. 2015;84(16):1639-43.
79. Novakova L, Axelsson M, Khademi M, Zetterberg H, Blennow K, Malmestrom C, et al. Cerebrospinal fluid biomarkers of inflammation and degeneration as measures of fingolimod efficacy in multiple sclerosis. Mult Scler. 2017;23(1):62-71.
80. Polman CH, O'Connor PW, Havrdova E, Hutchinson M, Kappos L, Miller DH, et al. A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. N Engl J Med. 2006;354(9):899-910.
81. Putzki N, Kollia K, Woods S, Igwe E, Diener HC, Limmroth V. Natalizumab is effective as second line therapy in the treatment of relapsing remitting multiple sclerosis. Eur J Neurol. 2009;16(3):424-6.
82. Putzki N, Yaldizli O, Buhler R, Schwegler G, Curtius D, Tettenborn B. Natalizumab reduces clinical and MRI activity in multiple sclerosis patients with high disease activity: results from a multicenter study in Switzerland. Eur Neurol. 2010;63(2):101-6.
83. Rudick RA, Stuart WH, Calabresi PA, Confavreux C, Galetta SL, Radue EW, et al. Natalizumab plus interferon beta-1a for relapsing multiple sclerosis. N Engl J Med. 2006;354(9):911-23.
84. Khademi M, Bornsen L, Rafatnia F, Andersson M, Brundin L, Piehl F, et al. The effects of natalizumab on inflammatory mediators in multiple sclerosis: prospects for treatment-sensitive biomarkers. Eur J Neurol. 2009;16(4):528-36.
85. Mellergard J, Edstrom M, Vrethem M, Ernerudh J, Dahle C. Natalizumab treatment in multiple sclerosis: marked decline of chemokines and cytokines in cerebrospinal fluid. Mult Scler. 2010;16(2):208-17.
86. Alvarez E, Piccio L, Mikesell RJ, Trinkaus K, Parks BJ, Naismith RT, et al. Predicting optimal response to B-cell depletion with rituximab in multiple sclerosis using CXCL13 index, magnetic resonance imaging and clinical measures. Mult Scler J Exp Transl Clin. 2015;1:2055217315623800.
87. Kappos L, Radue EW, O'Connor P, Polman C, Hohlfeld R, Calabresi P, et al. A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. N Engl J Med. 2010;362(5):387-401.
88. Kuhle J, Leppert D, Petzold A, Regeniter A, Schindler C, Mehling M, et al. Neurofilament heavy chain in CSF correlates with relapses and disability in multiple sclerosis. Neurology. 2011;76(14):1206-13.
89. Petzold A, Eikelenboom MJ, Keir G, Grant D, Lazeron RH, Polman CH, et al. Axonal damage accumulates in the progressive phase of multiple sclerosis: three year follow up study. J Neurol Neurosurg Psychiatry. 2005;76(2):206-11.
90. Kuhle J, Malmestrom C, Axelsson M, Plattner K, Yaldizli O, Derfuss T, et al. Neurofilament light and heavy subunits compared as therapeutic biomarkers in multiple sclerosis. Acta Neurol Scand. 2013;128(6):e33-6.

91. Kepes JJ, Perentes E. Glial fibrillary acidic protein in chondrocytes of elastic cartilage in the human epiglottis: an immunohistochemical study with polyvalent and monoclonal antibodies. Anat Rec. 1988;220(3):296-9.
92. Eng LF, Ghirnikar RS, Lee YL. Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). Neurochem Res. 2000;25(9-10):1439-51.
93. Rosengren LE, Lycke J, Andersen O. Glial fibrillary acidic protein in CSF of multiple sclerosis patients: relation to neurological deficit. J Neurol Sci. 1995;133(1-2):61-5.
94. Bonneh-Barkay D, Wang G, Starkey A, Hamilton RL, Wiley CA. In vivo CHI3L1 (YKL-40) expression in astrocytes in acute and chronic neurological diseases. J Neuroinflammation. 2010;7:34.
95. Comabella M, Fernandez M, Martin R, Rivera-Vallve S, Borras E, Chiva C, et al. Cerebrospinal fluid chitinase 3-like 1 levels are associated with conversion to multiple sclerosis. Brain. 2010;133(Pt 4):1082-93.
96. Correale J, Fiol M. Chitinase effects on immune cell response in neuromyelitis optica and multiple sclerosis. Mult Scler. 2011;17(5):521-31.
97. Malmestrom C, Axelsson M, Lycke J, Zetterberg H, Blennow K, Olsson B. CSF levels of YKL-40 are increased in MS and replaces with immunosuppressive treatment. J Neuroimmunol. 2014;269(1-2):87-9.
98. Burman J, Raininko R, Blennow K, Zetterberg H, Axelsson M, Malmestrom C. YKL-40 is a CSF biomarker of intrathecal inflammation in secondary progressive multiple sclerosis. J Neuroimmunol. 2016;292:52-7.
99. Stoop MP, Singh V, Stingl C, Martin R, Khademi M, Olsson T, et al. Effects of natalizumab treatment on the cerebrospinal fluid proteome of multiple sclerosis patients. J Proteome Res. 2013;12(3):1101-7.
100. Komori M, Kosa P, Stein J, Zhao V, Blake A, Cherup J, et al. Pharmacodynamic effects of daclizumab in the intrathecal compartment. Ann Clin Transl Neurol. 2017;4(7):478-90.
101. Rissin DM, Kan CW, Campbell TG, Howes SC, Fournier DR, Song L, et al. Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. Nat Biotechnol. 2010;28(6):595-9.
102. Todd J, Freese B, Lu A, Held D, Morey J, Livingston R, et al. Ultrasensitive flow-based immunoassays using single-molecule counting. Clin Chem. 2007;53(11):1990-5.
103. Lundberg M, Eriksson A, Tran B, Assarsson E, Fredriksson S. Homogeneous antibody-based proximity extension assays provide sensitive and specific detection of low-abundant proteins in human blood. Nucleic Acids Res. 2011;39(15):e102.
104. Kuhle J, Barro C, Disanto G, Mathias A, Soneson C, Bonnier G, et al. Serum neurofilament light chain in early relapsing remitting MS is increased and correlates with CSF levels and with MRI measures of disease severity. Mult Scler. 2016;22(12):1550-9.
105. Gaiottino J, Norgren N, Dobson R, Topping J, Nissim A, Malaspina A, et al. Increased neurofilament light chain blood levels in neurodegenerative neurological diseases. PLoS ONE. 2013;8(9):e75091.
106. Gisslen M, Price RW, Andreasson U, Norgren N, Nilsson S, Hagberg L, et al. Plasma Concentration of the Neurofilament Light Protein (NFL) is a Biomarker of CNS Injury in HIV Infection: A Cross-Sectional Study. EBioMedicine. 2016;3:135-40.
107. Rojas JC, Karydas A, Bang J, Tsai RM, Blennow K, Liman V, et al. Plasma neurofilament light chain predicts progression in progressive supranuclear palsy. Ann Clin Transl Neurol. 2016;3(3):216-25.

108. Kuhle J, Barro C, Andreasson U, Derfuss T, Lindberg R, Sandelius A, et al. Comparison of three analytical platforms for quantification of the neurofilament light chain in blood samples: ELISA, electrochemiluminescence immunoassay and Simoa. Clin Chem Lab Med. 2016;54(10):1655-61.
109. Disanto G, Barro C, Benkert P, Naegelin Y, Schadelin S, Giardiello A, et al. Serum Neurofilament light: A biomarker of neuronal damage in multiple sclerosis. Annals of neurology. 2017;81(6):857-70.
110. Bergman J, Dring A, Zetterberg H, Blennow K, Norgren N, Gilthorpe J, et al. Neurofilament light in CSF and serum is a sensitive marker for axonal white matter injury in MS. Neurol Neuroimmunol Neuroinflamm. 2016;3(5):e271.
111. Piehl F, Kockum I, Khademi M, Blennow K, Lycke J, Zetterberg H, et al. Plasma neurofilament light chain levels in patients with MS switching from injectable therapies to fingolimod. Mult Scler. 2017:1352458517715132.
112. Novakova L, Zetterberg H, Sundstrom P, Axelsson M, Khademi M, Gunnarsson M, et al. Monitoring disease activity in multiple sclerosis using serum neurofilament light protein. Neurology. in press.
113. Kuhle J, Nourbakhsh B, Grant D, Morant S, Barro C, Yaldizli O, et al. Serum neurofilament is associated with progression of brain atrophy and disability in early MS. Neurology. 2017;88(9):826-31.