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Body fluid biomarkers in multiple sclerosis: how far we have come and how they could affect the clinic now and in the future

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Abstract

Multiple Sclerosis (MS) is an autoimmune inflammatory disease of the central nervous system (CNS) which affects over 2.5 million people worldwide. Although MS has been extensively studied, many challenges still remain in regards to treatment, diagnosis, and prognosis. Typically, prognosis and individual responses to treatment are evaluated by clinical tests such the expanded disability status scale (EDSS), magnetic resonance imaging (MRI), and presence of oligoclonal bands (OCB) in the cerebrospinal fluid (CSF). However, none of these measures correlate strongly with treatment efficacy or disease progression across heterogeneous patient populations and subtypes of MS. Numerous studies over the past decades have attempted to identify sensitive and specific biomarkers for diagnosis, prognosis, and treatment efficacy of MS. The objective of this article is to review and discuss the current literature on body fluid biomarkers in MS, including research on potential biomarker candidates in the areas of microRNA, messenger RNA, lipids, and proteins.

Keywords

Biomarker; Multiple sclerosis; experimental autoimmune encephalomyelitis; autoimmunity; prognosis; therapy

Introduction

Multiple Sclerosis (MS) is an autoimmune inflammatory disease of the central nervous system (CNS) which affects over 2.5 million people worldwide. The disease is more prevalent in women, with first clinical symptoms typically occurring in early adulthood. MS is characterized by inflammation of the CNS leading to demyelination, axonal damage and lesion (scar) formation. As a result, these changes lead to neurological impairment and a variety of clinical symptoms [1-3].

MS may encompass more than one single disease entity because it presents with a wide range of clinical and pathological features and its etiology is unknown [4]. MS is classified

into the four following subtypes: (1) primary progressive MS (PPMS) exhibits gradual, continuously increasing symptoms with minor fluctuations; (2) relapsing-remitting MS (RRMS) is characterized by episodes of acute symptoms of diverse neurological dysfunction followed by periodic remissions and a variable degree of recovery; (3) secondary-progressive MS (SPMS) initially presents as RRMS but progresses to a steady worsening of clinical symptoms with or without clinical attacks during the progressive phase; (4) progressive-relapsing MS (PRMS) is characterized by distinct relapsing-remitting periods with progressive chronic worsening between periods. Approximately 85% of MS patients are initially diagnosed with the relapsing-remitting form of the disease, and approximately half of these individuals will progress to SPMS [5]. The different types of MS create major challenges for biomarker research. For instance, different pathophysiological mechanisms which are involved in the different subtypes may result in differential modulation of a biological marker. Thus, using a biomarker for one disease type may not be useful for a different type.

Although MS has been extensively studied over the years, many clinical challenges still remain in regards to treatment, diagnosis, and prognosis. Currently, there is no cure for MS and available treatments help to improve patients disease symptoms [6]. Additionally, there are no precise biochemical or cytological diagnostic tools for MS and diagnosis is achieved by a wide variety of tests to eliminate other neurological disorders and confirm MS [5]. Typically, prognosis and individual responses to treatment are evaluated by clinical measures of disease progression such as the expanded disability status scale (EDSS) [7,8], presence of oligoclonal bands (OCB) in the cerebrospinal fluid (CSF) [9], and establishing brain volume and number of lesions by MRI [10-13]. However, none of these measures correlate strongly with treatment efficacy or disease progression across heterogeneous patient populations and subtypes of MS.

Prior to definite diagnosis of MS, patients often present with a first episode of neurological symptoms, termed clinically isolated syndrome (CIS). When CIS is accompanied by CNS lesions, as demonstrated by magnetic resonance imaging (MRI), the patient is considered to be at high risk of developing MS [14-16]. A pre-defined number of MRI lesions in pre-defined anatomical locations qualify patients as having MS at the time of the initial clinical episode (attack) [17]. In the remaining patients, an additional attack with evidence of at least two brain lesions in separate areas of the CNS will convert CIS to a diagnosis of clinically definite MS (CDMS). Approximately 60 - 80% of CIS patients are at high risk of developing MS. Importantly, clinical studies have suggested that early treatment can delay, or even prevent, conversion of CIS to CDMS. Importantly, there are no current means to accurately predict a response to, or an optimal dose for, a particular drug treatment, including: natalizumab, beta-interferons (IFN- β) or glatiramer acetate (GA) [14-16]. Furthermore, no clinical test is available to measure the efficacy of currently available drugs on the progression of MS [18]. Thus, diagnostic tests that can identify nominally healthy individuals with an increased risk for developing MS, or predictive tests that can identify patients with an increased risk for reoccurring clinical attacks, as well as responses to treatment, are urgently needed [19].

As MS lacks definite biomarkers, numerous studies over the past decades have attempted to identify sensitive and specific biomarkers for this disease. By definition, a biomarker is an (laboratory) indicator that reflects a normal biological process, activity of a disease, or pharmacological response to therapeutic intervention [20,21]. The present review focuses on biomarkers in body fluids including microRNA, messenger RNA, lipids, and proteins and discusses their potential impact on MS diagnostics and treatment. Biomarkers using imaging and electrophysiology methodologies have been comprehensively reviewed elsewhere [22].

Part I: MS history, current biomarkers and challenges

History of MS and its first biomarkers

MS was first termed and described in detail by the French neurologist Jean-Martin Charcot, who followed patients with varying neurological symptoms, such as muscle spasms and walking difficulties. Autopsies revealed “multiple plaques” or scars along the nerves, which correlated to the clinical features of the disease. In 1868, he named the condition *la sclérose en plaques*, i.e. multiple sclerosis [23]. However, these type of lesions along the spinal cord in conjunction with brain atrophy had been described earlier, in 1838, by the Scottish pathologist Sir Robert Carswell [24,25]. The discovery of the myelin sheath by Rudolf-Carl Virchow in 1854, the discovery of Ranvier nodes, Schwann cells and the characterization of the nerve-fiber by Louis-Antoine Ranvier, as well as the discovery of oligodendrocytes in the late 1920's, shed light on the pathophysiology of MS and provided an explanation for the wide range of clinical symptoms [26,27].

Another major step in the understanding of MS was achieved in the early 1930's by Thomas Rivers and colleagues [28-30]. Their experiments showed that healthy monkeys injected with sterile brain extract developed CNS disease with immune cell infiltration and demyelinating lesions. Their experiments explained why Pasteur's vaccinations using emulsified extracts of brain tissue from rabies virus infected rabbits sometimes resulted in CNS inflammation and demyelinating disease akin to MS [31]. Additionally, these experiments established what is now known as the most studied model for MS and autoimmunity, termed experimental autoimmune encephalomyelitis (EAE). The EAE model took another step forward in 1947 when Elvin Kabat and Isabel Morgan demonstrated in parallel that a few injections of CNS tissue extract, emulsified in Jules Freunds' adjuvant, rapidly and reproducibly produced EAE disease, thereby providing the foundation for today's studies based on this disease model [32,33]. Since then, the number of publications using the EAE model has grown exponentially and has led to the discovery of many mechanisms contributing to the pathogenicity of inflammatory and demyelinating CNS diseases [34,35].

In addition to the development of the EAE model, a major contribution by Kabat and colleagues was the discovery that the composition of IgG in the CSF of MS patients differed from that of serum IgG [36]. Prior to this, scientists attempted to use colloidal gold activity to analyze CSF proteins, a test that was used to diagnose neurosyphilis. Although some MS patients displayed abnormal protein patterns using this test, it had poor sensitivity and specificity and results varied largely among laboratories [37-39]. Thus, the diagnosis of MS depended for many decades predominantly on clinical appearance and postmortem

autopsies. The work by Kabat and colleagues set the groundwork for the discovery of OCB in the 1960's by Lowenthal and colleagues [40]. Subsequently, OCB became an important supporting laboratory test for the diagnosis of MS due to its high sensitivity (~80%) [41]. However, Lowenthal also demonstrated that OCB could be detected in patients with other neurological conditions and that they were not specific for MS [40]. Eventually, the most important advance for the diagnosis of MS to date was provided in 1961 by Young and colleagues who compared the three imaging techniques X-ray, computed tomography (CT), and nuclear magnetic resonance (NMR) as potential tools to confirm the disease. They showed that NMR was the most sensitive technique to determine abnormalities (i.e. lesions) in the CNS of MS patients [42]. NMR and, later, magnetic resonance imaging (MRI, a more advanced and sensitive variation of NMR), together with OCB, became the gold standard for diagnosing and prognosing MS [43].

History of the current diagnostic criteria for MS

As outlined earlier, MS is a heterogeneous disease and no singular feature or test can differentiate it from other neurologic diseases with similar symptoms [44]. Historically, the earliest set of diagnostic criteria designed to clinically differentiate MS from other diseases prior to the discovery of OCB and MRI were the Schumacher criteria established in 1965 [45]. Thus, clinical information alone had to suffice for diagnosis [45]. The Schumacher criteria have been revised over the years in an attempt to provide earlier diagnosis with greater sensitivity and specificity [46]. The Schumacher criteria were superseded by the Poser criteria that include the appearance of CSF OCB [19]. The current criteria, commonly called the McDonald criteria [47], were established in 2001 and revised in 2005 and 2010 by Polman and colleagues [17,48]. The basic criteria state that a positive diagnosis of MS, as opposed to CIS, must have two or more lesions in separate locations within the CNS (DIS – dissemination in space) and two or more demyelinating events that occurred at separate times (DIT – dissemination in time). DIS criteria state that lesions must appear within at least two of the following CNS locations: juxtacortical, periventricular, infratentorial and spinal cord. DIT criteria can be met by two or more clinical attacks, MRI evidence of both gadolinium-enhanced lesions and non-enhancing lesions (indicating two separate events) or a combination of attacks and lesions indicating two separate events. Evidence of CSF IgG OCB is no longer required for diagnosis, but OCB can still be a useful tool to support diagnosis and to possibly indicate the PPMS subtype. The new criteria still need to be evaluated in coordination with other clinical symptomatic evaluations to provide the highest specificity [17,46,49]. Although CSF analysis is no longer mandatory for diagnosis of RRMS, it may still be an important tool for diagnosis of patients with negative MRI finding [50,51]. MS subtypes were revised in 2013 by Lublin and colleagues to include descriptors of disease activity and progression based on clinical relapse rate and imaging findings [52]. In this revision, the subtypes of MS are further divided into active vs. non-active and progressive vs. non-progressive subtypes [52,53].

Part II: Discovery of novel (non-imaging) biomarkers

MicroRNA (miRNA)

miRNAs are small non-coding RNAs that regulate gene expression [54]. Additionally, miRNAs have been found to regulate key processes in immune cells [55], T cell activation [56], as well as cellular processes in the CNS in health and disease [57]. Importantly, miRNAs can be secreted by cells for paracrine signaling and were detected in many different biological fluids, including CSF, serum, urine and saliva [58,59]. Growing evidence shows that miRNA dysregulation may contribute to human autoimmune pathology. As discussed below and shown in Table 1, a number of miRNA expression profiling data sets have been generated to better understand disease mechanisms and evaluate this group of molecules as potential prognostic and diagnostic biomarkers.

In one of the early reports, Otaegui *et al.* identified miRNAs differentially expressed in peripheral blood mononuclear cells (PBMC) of RRMS patient. They identified differences in miRNA expression profiles between patients in remission, relapsing, and in healthy individuals. Specifically, miR-18b and miR-599 were shown to be increased during relapses as compared with both healthy individuals and patients in remission. Additionally, miR-193a was shown to increase in patients in remission [60]. A recent report showed that miR-193a is increased in CD4+ T lymphocytes of RRMS patients upon stimulation [61]. Keller *et al.* compared the miRNA expression profiles in whole blood samples of patients with RRMS to healthy individuals [62]. They identified a miRNA expression profile that can discriminate between RRMS patients and healthy individuals. This includes an increased expression of miR-422a and miR-223. Additionally, miR-422a was reported to be decreased in plasma of MS patients, and miR-223 was reported to decrease in the serum of RRMS and PPMS patients [63,64]. Fenoglio *et al.* showed a decrease in miR-15b and miR-23a in serum of RRMS and PPMS patients [64]. These two miRNAs were reported by Ridolfi *et al.* to be decreased in serum from RRMS patients in remission and in PPMS [65]. Importantly, the levels of the miRNA expression correlated with EDSS disability scores, particularly in PPMS. Thus miR-15b and miR-23a may be useful as potential biomarkers to indicate progression.

A study by Du *et al.* reported a critical role for miR-326 in the regulation of Th17 cells [66]. Interestingly, miR-326 was up-regulated in peripheral blood leukocytes from RRMS patients and its expression correlated to disease severity both in MS and EAE, suggesting it as a potential biomarker for disease severity. An additional report showed an increase of miR-326 in active lesions but not in inactive lesions, further supporting the role of miR-326 as a biomarker for disease activity [67]. A report by Cox *et al.* identified miR-17 and miR-20a to be significantly decreased in peripheral blood cells from RRMS, SPMS and PPMS subtypes and that these miRNAs modulate T cell activation genes [68]. Furthermore, a report by the Sievers *et al.* showed that miR-17 and miR-20a (miR-17-92 cluster) expression was decreased in peripheral blood B lymphocytes following natalizumab treatment in RRMS patients [69]. Haghikia *et al.* identified a miRNA profile in the CSF which was differentially regulated in patients with MS as compared with other neurological diseases (OND) [70]. Importantly, the report showed that miR-181c and miR-633 in CSF

could differentiate relapsing-remitting from secondary progressive MS courses with 82% specificity and 69% sensitivity.

Lovett-Racke and colleagues reported that miR-128 and miR-27b were increased in naïve CD4⁺ T cells and miR-340 was increased in memory CD4⁺ T cells from patients with MS [71]. This was done in an attempt to establish factors driving the susceptibility to and development of MS. The miRNAs reported were shown to inhibit Th2 cell development and favor pro-inflammatory Th1 responses by targeting downstream genes such as IL-4. Furthermore, targeting these miRNAs led to the restoration of Th2 responses, which illustrate a therapeutic potential of these miRNAs for MS. In addition to MS studies, several reports studying EAE further implicated a possible role for some miRNAs as biomarkers [72].

In summary, the literature reviewed here shows that changes in miRNA expression may be associated with MS and could potentially be used as biomarkers for this disease. Notably, many of the miRNA reported by these studies have been shown to be differentially regulated in other tissues outside the CNS during inflammation. Thus, the specificity of such biomarkers requires further validation [73-75]. Furthermore, few of the reports showed overlap in the altered miRNAs and results were not reproducible between studies. However, several miRNAs still stand out, including miR-193a, miR-223 and miR-326. Additionally, evidence that miRNAs can be measured in many body fluids suggests clinical feasibility of these molecules as potential biomarkers for MS.

Messenger RNA (mRNA)

Early epidemiology studies focused on understanding the genetics of MS. The strongest genetic link for MS susceptibility was established with the genes of the human leukocyte antigen (HLA)-DR15 haplotype [76]. Advances in genome studies such as the human genome sequence project, identification of disease-related SNPs, and whole genome admixture studies and next-generation sequencing methodologies have provided new molecular targets for mechanistic investigations and as potential biomarkers [77]. More recently, transcriptome studies have investigated gene transcription (mRNA expression) in MS using cDNA microarrays to interrogate the broad expression of mRNAs in particular cell populations. It has been suggested that this approach could identify new potential biomarkers in MS [78].

Table 1 summarizes recent MS transcriptomics studies. The majority of these reports have noted complex changes in gene expression profiles in MS patients [78]. These expression profiles include genes that encode proteins involved in apoptosis, cell cycle, inflammation, cell adhesion, heat shock/oxidative stress and matrix metalloproteinase pathways [78,79]. Bompreszi *et al.* identified changes in gene expression profiles in PBMC that distinguished between MS patients and healthy controls [80]. Importantly, they observed a significant increase in the IL-7 receptor transcript in MS, a gene that is linked to increased MS susceptibility [81]. Subsequent studies showed expression changes between RRMS patients in remission versus active relapse [82,83], between SPMS and PPMS patients versus healthy controls [84], and between RRMS patients with varied disease outcomes ranging from good to poor [85]. Other studies have begun to reveal specific mechanisms involved in

progression of MS using transcriptomics to identify specific cell cellular processes influencing the transcription profile. For instance, Satoh *et al.* identified aberrant expression of apoptosis and DNA damage-regulating genes in MS PBMC, whereas Romme-Christianson and colleagues identified cytokine expression profiles in CSF and whole blood (WB) in RRMS patients [86,87]. Hecker *et al.* identified two gene transcripts that had potential as individual biomarkers: GPR3 and IL17RC [88]. Both were increased in WB of patients with good outcomes and decreased differentially in those with poor and very poor outcomes [89]. Other studies have used transcriptomics to determine changes in gene expression in relation to some of the currently used therapies for MS, including IFN-beta (β), glatiramer acetate (GA) and natalizumab [90-97]. Most of these studies were focused on determining the efficacy of the drug treatment and/or the metabolic pathways by which the drugs operate (i.e., mechanisms of action). Myxovirus resistance protein 1 (Mx1, also known as MxA) and 2'-5'-oligoadenylate synthetase (OAS) have been identified as potential biomarkers for IFN- β treatment response [90,91,94]. Levels of Mx1 mRNA in PBMC are indicative of biological activity of IFN- β treatment since they measure a downstream product (Mx1) of the IFN- β receptor signaling pathway [98,99]. It was shown that Mx1 mRNA levels are negatively correlated with MRI activity and neutralizing antibodies of injectable IFN- β [100-102]. Thus, measurement of Mx1 mRNA following IFN- β treatment might be a potential biomarker to measure the biological activity of this drug in patients [99]. mRNA expression changes in CSF for IFN- γ and IL-23 were shown to correlate to GA treatment [97]. Additional studies identified expression fingerprints that determined drug responses for individual patients. For instance, Sturzebecher *et al.* reported a change in the expression of 125 genes in patients that showed good IFN- β 1 responses [93].

Overall, the reports summarized here showed mixed results with very few overlapping gene transcripts between reports. In depth compilations and analyses are presented by Lindberg *et al.* [103], Comabella *et al.* [78] and Hecker *et al.* [85]. The differences in results may be attributable to a variety of factors: differences in MS patient subpopulations, differences in sample sources, differences in experimental design, and differences in microarray platforms [78]. Nonetheless, the transcriptomics studies performed to date have provided insights into which genes are being differentially expressed during various MS disease states and treatment courses. However, the rate of gene expression and transcription cannot necessarily be used to infer the resulting protein expression due to post-translational regulation, modification, and alternate mRNA splicing [104,105]. Along these lines, special microarrays have been used to detect differently spliced forms of mRNA. Interestingly, studies have demonstrated that genes without differential mRNA regulation can instead show differential splicing patterns between RRMS patients and healthy controls [106]. Advancements in next-generation RNA-sequencing might facilitate the search for mRNA biomarkers by aiding in the identification of differential mRNA splicing patterns and unknown gene products [107].

Lipids

Lipids serve various functions in tissues throughout in the body, for example as structural components (cell membranes, myelin), as energy storage, and for cellular signaling. Changes in concentrations of these molecules, their metabolites, or associated lipid species

could be the result of changes in the metabolic processes and/or rate of cell damage/death in MS patients [108-110]. Several types of lipids have been studied as a potential biomarker of MS, including sterols (i.e. cholesterol), oxysterols, and phospholipids. Lipidomics research using recent advancements in mass spectrometry techniques are being explored for the identification of potential lipid biomarkers [111].

I. Cholesterol—The brain contains a large amount of cholesterol (2-3% of the wet weight) [112], more than any other organ [113]. Importantly, the majority of cholesterol (as much as 70%) found in the CNS is contained within the myelin sheaths covering the neuronal axons [114]. Cholesterol can be detected in urine, blood, and CSF, making it a potential biomarker candidate for clinical application with minimal invasive techniques. Cholesterol found in the CNS is derived almost exclusively from *de novo* synthesis rather than from import across the blood-brain barrier (BBB) [113]. Using C¹⁴ labeling of CNS cholesterol during rat development, CNS-derived cholesterol metabolism products have been detected in urine [115], and changes in its levels were found in response to demyelination induced by chemical agents or during EAE [116]. Increased total cholesterol, high density lipoprotein (HDL) and low density lipoprotein (LDL) levels were found in the plasma during EAE [117]. A subsequent study in MS detected an association between progression of MS and increased levels in serum LDL, total cholesterol and triglycerides, whereas HDL levels were increased only in correlation with lower lesion volumes [118]. Giubilei *et al.* showed a positive correlation between plasma LDL levels and the number of active brain lesions of CIS patients [109]. Thus, cholesterol and LDL may be used as potential biomarkers to determine disease activity. However, changes in cholesterol index (cholesterol, HDL, LDL, triglycerides, etc.) can be the result of many normal cellular processes and biological variation [119]. Furthermore, It has been suggested that the widespread prescription of statins, used to control high cholesterol levels, make sensitive correlations of cholesterol index from blood and urine to MS disease onset or progression problematic [111]. Nevertheless, recent studies are investigating the possible use of statins as anti-inflammatory and immunomodulatory drugs in MS, thus cholesterol index is a potential predictive biomarker to measure the efficacy of statins in MS [120].

II. Oxysterols—To maintain cholesterol homeostasis, excess cholesterol must be removed from the CNS, enter the circulation, and be processed by the liver [114]. The transport of excess cholesterol from the CNS to the blood involves its conversion by metabolically active neurons to 24S-hydroxycholesterol (24S-OHC), which can cross the BBB. The production of 24S-OHC is unique to the CNS and its concentration in circulation is dependent on the rate of production in the CNS and elimination by the liver [114]. The levels of 24S-OHC in the bloodstream had been proposed as a direct measure of the number of metabolically active neurons [110,112,114]. Surprisingly, increased plasma levels of 24S-OHC were identified in MS [110]. However these increased levels were not significant compared with OND and healthy subjects [110]. Subsequently, Leoni *et al.* showed that CSF and plasma 24S-OHC levels were decreased in older RRMS, SPMS and PPMS patients, whereas its levels were increased in younger patients [121]. In support of this observation, two different reports have also shown that serum 24SOHC levels were decreased in older RRMS and PPMS patients [122,123]. Additionally, Teunissen *et al.* showed that 24S-OHC levels

significantly increased in serum during early stages of EAE (days 9 to 17) [124]. Taken together, the difference in the number of functioning neurons between recently diagnosed and longer-term patients (and similarly in early stages versus later stages of EAE) may be the reason for the differences in levels of 24S-OHC between older and younger patients. Importantly, Teunissen and colleagues showed a significant increase in serum 24S-OHC levels prior to clinical onset of EAE (day 9). Thus, 24S-OHC might be a potential biomarker to predict clinical onset for recently-diagnosed CDMS or CIS patients. Furthermore, outside of the CNS, cells produce 27S-hydroxycholesterol (27S-OHC) for removal of cholesterol. This compound is not normally found in the CNS and its presence in the CSF has been correlated with disruption of the BBB [125]. The ratio of 24S-OHC to 27S-OHC is being studied as a marker for the state of neuronal death and the disruption of the BBB [125]. Thus serum 24S-OHC and the CSF ratio of 24S-OHC to 27-OHC may be potential biomarkers of neuronal damage and BBB permeability.

Other oxidation products of cholesterol can be formed via auto-oxidation upon exposure to reactive oxygen species (ROS) [125]. Increased oxidative stress in the CNS during MS can promote the formation of oxysterols from cholesterol during destruction of the myelin sheath [126]. Some of these oxidized derivatives of cholesterol have been shown to be neurotoxic [127-129] and are studied as potential sources of ongoing neuronal damage in progressive forms of MS [130]. One of the identified oxysterols, 7-ketocholesterol (7-KC), was reported to be highly increased in the CSF of RRMS patients and EAE mice [130]. Further studies have also reported increased levels of CSF 7-KC, albeit not to same degree as reported initially [131]. The differences among these studies may be due in part to auto-oxidation occurring during sample preparation and handling, which suggests limitations for the potential of 7-KC as laboratory test [131]. Furthermore, two species of 15-oxysterols (15-OS) were increased in serum from SPMS patients as well as in serum from NOD EAE mice during the secondary progressive phase [132]. Importantly, the increased levels correlated with the transition of RRMS to SPMS, whereas a different 15-OS compound (15-HC) was elevated only in the progressive phase of SPMS patients. Thus, 15-OS compounds might be promising biomarkers for disease progression and conversion of RRMS to SPMS.

III. Oxidized Phospholipids—ROS can also oxidize cellular phospholipids and the resulting compounds have been observed in CNS lesions of MS patients [133]. In particular, isoprostanes (isoP), prostaglandin-type compounds generated from the peroxidation of arachidonic acid in phospholipids, have been studied as potential biomarkers in MS [134-139]. For instance, Sbardella *et al.* showed increased levels of CSF isoP in CIS patients, and increased levels were associated with higher risk of relapse [134]. Furthermore, Teunissen *et al.* showed that isoP serum levels were decreased in PPMS compared with CIS, whereas the levels were comparable between RRMS and CIS [135]. Importantly, Miller *et al.* showed that increased isoP levels can be detected in urine of SPMS patients, further supporting the potential use of isoP as biomarker of disease progression [136]. Additional reports have shown an increase of oxidized phospholipids in plasma of MS patients [137,138]. Although these levels were increased in all MS patients, no differences among MS subtypes were observed. Taken together, oxidative phospholipids might be more valuable biomarkers for diagnosis rather than prognosis of MS [138].

Proteins

Various proteins are measured in clinical laboratories for diagnosis and prognosis of many diseases including cancer [140], cardiovascular disease [141], autoimmune diseases [142,143], and infections [144-146]. Immunoassays are the primary tool for the identification of protein biomarkers; however, other tools are also available including western blot and mass spectrometry. Ideally, protein biomarkers of neuroinflammatory diseases, such as MS, can be detected in blood, which allows for minimally invasive testing. Proteomics-based discovery of biomarkers in blood is challenging due to the dynamic range of protein concentrations in plasma spanning across 12 orders of magnitude and the fact that approximately over 95% of protein weight in plasma is composed of several high-abundant proteins [104,147]. However, proteomics methods also have certain advantages for MS biomarker studies. First, leakage of CNS-specific proteins across the BBB into the serum is expected at appreciable levels only in patients but not in healthy individuals [148]. Second, proteomics methods allow for global investigation of changes in serum protein profiles and identification of novel molecules. A summary of recent proteomics-based biomarker studies is shown in Tables 2 and 3, and supplementary Tables 1 - 3.

Non-antibody immune-related proteins

I. Cell surface markers: Given the clear association between T cells and MS, several studies focused on T cell surface markers as biomarkers, particularly for measuring the treatment efficacy of immunomodulatory drugs. Shown in Table 2 is a summary of current research on T lymphocyte cell surface markers as putative biomarkers in MS. For instance, Muraro *et al.* showed that the expression of VLA-4 was decreased on CD8+ and CD4+ memory (CD45RO+) T cells following IFN- β treatment [149]. Additionally there was an increase of CD27 expression on these cells post-therapy [149], indicating an increase in “resting” T lymphocytes [150]. Along this line, Zafranskaya *et al.* reported an increase of myelin oligodendrocyte glycoprotein (MOG)-reactive CD4+ and CD8+ memory (CD45RO+) T cells in MS patients as compared with healthy individuals [151]. Furthermore, the proportion of memory T cells and their proliferation capacity returned to normal values upon therapy with IFN- β [151]. A report by Chatzimanolis *et al.* showed an increase in the ratio of CD45RA+ ICAM-3+ T lymphocytes (resting/naïve lymphocytes) in peripheral blood and CSF of RRMS patients [152]. This increase positively correlated with EDSS scores, but was not altered as a result of IFN- β 1 treatment. Thus, this subpopulation of lymphocytes may be used to monitor progression (disease activity) but not IFN- β 1 drug efficacy. Interestingly, however, a significant increase in CD45RA+ ICAM-3+ lymphocytes in peripheral blood was seen after high-dose glucocorticoid (methylprednisolone) treatment during acute MS relapses [152]. Moreover, several reports showed that high-dose methylprednisolone treatment increased the number of CD8+ CXCR3+ T cells and decreased the number of CD4+ T cell subsets expressing CD25, CD29, and CCR4 in the CSF, and CD4+ CXCR3+ T cells in peripheral blood [153]. In summary, cell surface marker expression on T lymphocytes might be predictive of drug responses in MS. However, future studies are needed to unravel how the drugs, often with immunosuppressive and immunomodulatory properties, affect the expression of these markers.

II. Cytokines/chemokines: In addition to cell surface markers on lymphocyte, other molecules produced by immune cells, such as cytokines and chemokines, are studied as biomarkers for MS and other autoimmune diseases (Table 2). Because cytokines and chemokines are directly involved in the pathogenesis of these diseases, it is conceivable that differential expression of these proteins corresponds to disease status and treatment responses. Consequently, numerous studies have tried to identify differences in the expression of inflammatory mediators in MS and to evaluate them as biomarkers. Here we focused on tumor necrosis factor-alpha (TNF), interleukin (IL)-6 and IL-17 as potential biomarkers, as these cytokines have been extensively studied in MS.

TNF is one of the most extensively studied cytokines as a biomarker. Franciotta *et al.* reported that the levels of TNF in CSF and serum of RRMS, CPMS, OND patients and healthy individuals were comparable between the groups [154]. In contrast, Sharief and Hentges reported that TNF was increased in the CSF and serum of patients with CPMS, compared with stable MS and OND [155]. Furthermore, the level of CSF TNF correlated with the severity and progression of the disease. Another report showed no significant difference in CSF TNF levels between MS subjects versus controls, but found an association between CSF TNF levels and active MS [156]. Vladic *et al.* could not detect TNF in the CSF of RRMS patients, but found a 20% increase in serum samples [157].

Investigation of IL-6, another cytokine implicated in the pathogenesis of MS, also generated conflicting results. Initially, IL-6 levels were reported increased in the CSF and serum of RRMS and SPMS patients as compared with healthy controls [158]. Additionally, its levels correlated to disease severity [158]. However, subsequently it was reported that IL-6 was measurable in only 10% of all serum samples and in 5% of CSF samples. Moreover, no significant differences in IL-6 levels were observed between RRMS and SPMS patients. These results led the authors to question the utility of IL-6 as a biomarker for MS [157]. Several more reports showed increased IL-6 in CSF but not serum [159]. However, increased IL-6 concentration in the serum of RRMS patients was positively correlated with the number of relapses in female but not male patients, with no correlation to EDSS. Additionally the age of the patients influenced the results [160].

IL-17A and IL-17F are two signature cytokines produced by Th17 cells and have long been implicated in the pathogenesis of MS and demyelinating diseases [161]. Several different reports have attempted to use these cytokines as predictive biomarkers to measure drug response in MS, particularly for IFN- β treatment. Axtell and colleagues reported that IFN- β treatment was effective in reducing EAE symptoms induced by Th1 cells but exacerbated disease induced by Th17 cells [162]. Interestingly, they reported that in Th17-induced disease, IFN- β treatment still reduced IL-17A production, but without benefit. In addition, IFN- β non-responders showed higher IL-17F concentrations in serum compared with responders. Later, they showed that serum levels of IL-7 may be indicative of Th1-mediated disease, and thus they suggested the measurement of IL-7 together with IL-17F as a marker for IFN- β response [163]. Balasa *et al.* reported that RRMS patients with high serum IL-17A levels did not respond well to IFN- β therapy and had shorter intervals between relapses as compared with patients with low IL-17A levels [164]. However, Hartung *et al.* showed that increased levels of IL-17F before, and shortly after treatment with IFN- β were not associated

with poor responses, suggesting that IL-17F might not be a predictive biomarker for IFN- β treatment [165]. Taken together, further research is needed to elucidate the exact impact and influence of IFN- β treatment in relation to IL-17A/IL-17F production, as well as the use of IL-17A and IL-17F as predictive biomarkers for IFN- β treatment in MS. Nonetheless, rather than a single cytokine, the ratio between IL-17A and IL-17F, and or between other cytokines and IL-17A/F could be useful as a potential measurement for IFN- β treatment response.

Many additional cytokines have also been investigated for their potential utility as biomarkers for MS, including: IFN- γ , IL-1, IL-2, IL-4, IL-10, IL-12, etc. [166-170]. However, overall, the results remain inconclusive and contradictory, and no single cytokine has so far emerged as an undisputed biomarker candidate.

In addition to cytokines, differential expression of certain chemokines has been suggested as biomarkers for MS, including expression of CXCL12 and CXCL13 (supplementary Table 1) [171]. Since chemokines and their receptors guide the migration of inflammatory cells into the CNS during attacks of MS, this could suggest utility of these molecules as biomarkers for disease activity, severity, and treatment responses. Along these lines, Bielekova *et al.* suggested that the combinatorial measurement of CSF CXCL13, IL-8 and IL-12p40 might be a potential biomarker panel for active CNS inflammation in MS compared with other non-inflammatory neurological diseases [172]. Additionally, CXCL13 was reported increased in the CSF of RRMS and SPMS patients [171,173] but not in PPMS patients [174]. CXCL13 levels were also shown to correlate with axonal damage [174]. Furthermore, serum levels of CXCL13 were increased in patients with active MS and were not affected by IFN- β or GA treatment [175]. Overall, CXCL13 and other chemokines may be promising biomarkers, but more work is needed to determine specificity and sensitivity for MS vs. other neuroinflammatory conditions [176,177].

Technological advancements such as novel multiplexed immunoassays might accelerate this area of research by facilitating rapid measurement of multiple “signature” cytokine patterns at high sensitivity in low specimen volumes [178]. For instance, Hagman *et al.* analyzed serum samples from different subtypes of MS (CDMS, RRMS, SPMS, PPMS and CIS) for 14 different proteins, including cytokines, chemokines and pro-apoptotic molecules in blood and compared the patient expression patterns to EDSS and MRI activity [179]. They found that the levels of Fas were increased in all MS subtypes with a worsening EDSS score and accumulation of hypointense lesions by MRI. Importantly, levels of Fas and MIF were higher in progressive MS. Additionally, increased serum levels of TNF and CCL2 were observed, particularly in PPMS [179].

III. Other inflammatory markers: A number of additional proteins involved in inflammation and/or downstream events have been proposed as biomarkers for MS. This includes proteins involved in maintaining the integrity of the BBB and other tissues, and molecules regulating inflammation such as oxidative stress. Importantly, some of these molecules have been reported as differentially expressed in neuroinflammatory diseases such as neuromyelitis optica (NMO) and can be used to distinguish between NMO and MS [180]. For example, matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, were found increased in the CSF and/or serum during MS relapses and correlated with MS

activity (supplementary table 1) [181-183]. The concentration of MMP-9 was found to be higher in patients carrying the MS-associated HLA-DRB1*15:01 molecule [182]. Importantly, serum MMP-9 levels were shown decreased following IFN- β treatment and predicted new active lesions in patients with SPMS and RRMS [184,185]. Thus, MMPs could potentially be used as biomarkers to measure drug responses, as well as disease activity.

Antibodies

I. OCB in CSF: OCB in CSF, as detected by isoelectric focusing and Western blotting (immunoblot), are a distinct set of two or more bands of immunoglobulin (Ig) derived from a restricted set of B cell clones as compared with one or no such band in healthy individuals [186]. Many patients with autoimmune CNS inflammation, including MS, are often positive for CSF intrathecal IgG OCB [187]. The occurrence of OCB in the CSF is used as a diagnostic tool in support of an inflammatory condition of the CNS, in particular MS [188]. A variety of experimental techniques have been used to determine the presence of OCB in MS patients [186]. Although OCB are not specific to MS, and approximately 10% of MS patients never show them, they are used in conjunction with other clinical tools to support the diagnosis of “possible-MS” [188,189]. Modern laboratory techniques have improved the accuracy of their detection and show a high correlation between OCB and the diagnosis of MS in most studies [186,187]. Less favorable results observed in some studies may be attributed to differences in methods used, or potentially be due to intrinsic differences in the MS patient populations tested, in particular when tested across different ethnicities [186]. Interestingly, the antigen specificity of CSF OCB has not been fully elucidated and IgG reactivity has been observed to antigens unrelated to the CNS, such as pathogens (e.g. EBV) has come into focus [190,191]. Detection of IgM production in CSF has implicated intrathecal B cell and plasma cell differentiation in the pathogenesis of this disease [192-195]. IgM OCB have been suggested as an indicator for disease outcome [195] and the conversion from CIS to either CDMS [194,196] or optical neuropathy (ON) [197]. Lastly, CSF IgM OCB levels were shown to correlate to drug response, suggesting use as a predictive biomarker to measure efficacy of treatment [195,198]. Further investigations are required to independently verify these results and resolve some contradictory findings.

II. Autoantibodies: The view that an autoimmune attack against the myelin sheath underlies MS has suggested that autoantibody production against its components, such as proteins and lipids, may aid in the diagnosis and prognosis of MS (supplementary Table 2) [199,200]. Indeed, anti-myelin antibodies were found in the circulation and CSF of MS patients [201]. However, anti-myelin antibodies were also found in patients with OND, and not all MS patients are positive for these antibodies [201,202]. Berger *et al.* showed that MS patients with serum anti-MOG and anti-MBP antibodies had more frequent and shorter intervals between relapses than patients without [203]. In their studies, an increase in anti-MOG and anti-MBP autoantibodies in serum of CIS patients was predictive of conversion to CDMS [203]. However, these results could not be independently confirmed [204]. The differences in findings might be due to differences in study populations and designs [205]. Additionally, antibodies against MBP peptides 43-68 and 146-170 distinguished MS patients from OND patients, and the autoantibody-mediated cleavage of epitope 81-103, as well as

higher levels of anti-MBP 48-70 and 85-170 antibodies, could distinguish between MS patients and healthy individuals [202]. Along these lines, Quintana and colleagues performed antigen microarray analysis to characterize patterns of antibody reactivity in MS serum against a panel of CNS protein and lipid autoantigens [206]. Interestingly, they found unique autoantibody patterns that distinguished RRMS, SPMS and PPMS from both healthy controls and OND patients [206]. More recently, Srivastava *et al.* identified the glial inward rectifying potassium channel KIR4.1 as a target of serum IgG antibodies in MS [207]. Importantly, approximately 47% of MS patients were positive for serum autoantibodies to KIR4.1, whereas less than 1% of OND patients and no healthy individuals were positive for this IgG. However, these results could not be confirmed by others and await further clarification [208-210]. The evaluation of autoantibody function may be more useful for the diagnosis of MS, rather than simply their presence or absence, as demonstrated by Hedegaard *et al.* They showed that anti-MBP autoantibodies from MS patient sera, but not healthy individuals, facilitated the production of IFN- γ and IL-5 [211]. Taken together, it may be beneficial to combine the presence of autoantibodies with epitope specificity and functional properties to obtain a higher specificity biomarker for MS.

In addition to anti-myelin autoantibodies as biomarkers for MS, anti-cytokine autoantibodies have been observed in CSF and serum from MS patients [212]. However, these autoantibodies seem to be present in many autoimmune, inflammatory and non-inflammatory diseases, as well as in healthy individuals [213]. Nonetheless, patients positive for neutralizing antibodies (NAb) to injectable IFN- β showed abrogated responses to the treatment. Additionally, high titers of IFN- β NAb were associated with increased MRI activity. Thus, measurement of IFN- β NAb in MS patients might be useful as a marker to determine responders vs. non-responders and the necessity to initiate a change of treatment regimen [99].

Overall, additional studies are needed to elucidate the specificity of autoantibodies for MS, as compared with healthy individuals, patients with meningitis or with stroke [212]. The presence or absence of anti-myelin autoantibodies may need to be further evaluated in conjunction with epitope specificity and functional properties to increase their utility as biomarkers for MS. Similarly, further studies are needed to establish the effect/utilization of IFN- β NAb on the efficacy of IFN- β treatment in MS [99]. Importantly, Monson and colleagues identified unique antibody gene specific signatures in the CSF of MS patient [214]. Moreover, they showed that this pattern was predictive of conversion from CIS to CDMS, and thus can be used as a biomarker for early diagnosis of MS [214,215].

III. Antibodies against microbial antigens: Studies as far back as 1973 showed the presence of high intrathecal antibody production against neurotropic viruses such as measles, rubella and varicella zoster, also known as MRZ reaction in MS patients [216]. Shown in supplementary Table 3 is a summary of the current research on anti-microbial antibodies as potential biomarkers in MS. The MRZ-reaction was found to be less sensitive but more specific than detection of IgG OCB in MS patients [217]. Importantly, an increased MRZ reaction was found to predict and to correlate with the progression of CIS to clinically definite RRMS [218]. Additionally, an intrathecal MRZ reaction correlated with disease activity measured by MRI, thus it was suggested as an additional prognostic tool for

MS [216]. In addition to MRZ, CSF antibodies against Epstein-Barr virus (EBV) have also been reported to be a feature of MS [219]. Early reports showed that 100% of MS patients are EBV-seropositive and that their blood contains higher antibody titers than in healthy individuals [220]. While a recent report showed that EBV infection is not a characteristic feature of multiple sclerosis brain tissue and that less than 20% of MS patients are CNS-positive for EBV [221], Bray *et al.* reported that 85% of MS patients have antibodies against EBV in the CSF as compared with less than 15% of EBV-seropositive controls [220]. The same authors also showed that the EBV protein EBNA1 shares two pentapeptide moieties with MBP and that CD4+ T lymphocytes in MS patients recognize these pentapeptides and activate B cells, while in healthy controls this was not the case. They and others concluded that this phenomenon is dependent on the MS-associated HLA-DR alleles [220,222]. Along these lines, Cepok *et al.* showed that the most frequent IgG antibody in the CSF of MS patients was specific for EBNA1, and that these antibodies were also increased in patient serum as compared with healthy individuals [223]. It is interesting to note that the association between anti-EBNA1 antibody titers and MS risk was similar in HLA-DRB1*15:01 -positive and -DRB1*15:01 -negative individuals; however carriers of the DRB1*15:01 allele with elevated anti-EBNA1 antibody titers showed an increased risk for MS [224]. Furthermore, increased intrathecal synthesis of antibodies against several other viruses has been reported, including herpes simplex virus (HSV) [225] and JC virus [226]. Taken together, an increased intrathecal antibody synthesis against several different viruses seems to be a feature of MS which may contribute to the presence of OCB. The specificity of these antibodies for microbial antigens, e.g. viruses, or against self-antigens requires further investigation. Nonetheless, combining the analysis for increased viral antibody titers with other features, such as HLA-DR alleles or antibody function, may improve their utility as clinical tool for MS diagnosis [227].

CNS-specific proteins (CSPs) (Table 3)

I. Myelin breakdown: As the myelin sheath is the main target of the autoimmune attack in MS, it is not surprising to find an increase in myelin breakdown products, which are often observed in the CSF of MS patients [228]. These breakdown products were already observed over 30 years ago and have been suggested as potential biomarkers to determine the extent of myelin injury in MS [229,230]. An increase of CSF MBP and its breakdown product MBP₄₅₋₈₉ peptide was reported to correlate with RRMS disease activity and severity as measured by MRI and the EDSS score [231]. Treatment with methylprednisolone during acute relapses decreased the levels of CSF MBP and correlated with improvement of clinical disability, reduction in EDSS score, and reduction of MRI activity [231,232]. Along this line, our group has also measured an increase of several MBP breakdown products in the CNS of EAE mice which correlated to disease progression [233,234] and glucocorticosteroid treatment efficacy (unpublished). However, OND are also associated with increased myelin or its breakdown products in the CSF [235,236]. Taken together, the specificity of MBP or its breakdown products as biomarkers in MS remains an open and intriguing question. Nonetheless, it shows high sensitivity to discriminate healthy versus MS, and to correlate with clinical disability. Furthermore, it may be a potential biomarker for glucocorticosteroid efficacy.

II. Axonal death and gliosis: One of the hallmarks of MS is axonal death [237]. AlphaB-crystallin is a small heat-shock protein found to be increased in brains with demyelination, and elevated levels of antibodies against this protein were detected in serum and CSF of MS patients [238]. Although autoantibodies against AlphaB-crystallin were also observed in patients with OND, approximately 60% of MS patients are positive [238]. In addition, AlphaB-crystallin was increased in the CSF in 100% of MS patients and in approximately 90% of OND patients [239]. Taken together, the sensitive and specificity of this marker (as autoantibodies and/or protein) is low and may need to be combined with other laboratory measurements [240].

GFAP is the main intermediate filament protein in mature astrocytes [241]. It is elevated as an astrocyte response to CNS injury, de/re-myelination, and neuronal damage during aging and neurological diseases, a function known as astrogliosis [241]. Increased levels of GFAP in the CSF are associated with many neurological diseases, including MS [241,242]. CSF GFAP levels were reportedly increased in SPMS but not in RRMS patients [243]. In SPMS and RRMS patients, CSF GFAP levels correlated with neurological disability (EDSS) and disease progression as measured by multiple sclerosis severity score (MSSS) [243]. Interestingly, unlike neurofilament proteins and MBP, CSF levels of GFAP are much higher in NMO as compared with MS and, thus, may be a useful biomarker to distinguish between the two diseases [244].

Neurofilament (NF) is a major structural component in neurons and, as such, its breakdown products are indicative of neuronal death. NF has three major subunits: NF-heavy (NF-H), NF-medium (NF-M) and NF-light (NF-L). Several studies have investigated NF-L and NF-H as markers for neuronal death in patients with MS and illustrated their potential usage as biomarkers [245]. NF-M levels have not yet been extensively investigated in MS, although there is a report on elevated intrathecal antibodies against NFM in the CSF [246]. Wekerle and colleagues showed that NF-M is an important autoantigen in EAE [247]. Indeed, during axonal damage, neurofilament subunits are released into the extracellular space and can be found elevated in different body fluids, in particular the CSF. However, this phenomenon is not limited to MS patients and is also observed in OND patients as well as elderly healthy individuals, however to a lesser degree [245].

Increased levels of CSF NF-L were found in RRMS patients positive for OCB [248], and CSF NF-L levels increased during relapses and correlated with the formation of new lesions detected by MRI [248]. Additionally, increased CSF NF-L levels were indicative for conversion from CIS to MS [249] and distinguished MS patients from healthy individuals [250]. Importantly, treatment with natalizumab led to a 3-fold decrease in CSF NF-L levels, but did not affect CSF GFAP levels [251]. NF-L levels did not seem to correlate with EDSS scores and disease progression in RRMS and SPMS [243].

CSF NF-H levels were reported to correlate with disease progression and disability [252,253]. Additionally, the levels were found to frequently increase in PPMS and SPMS patients as compared with RRMS patients, indicating disease progression [252]. CSF NF-H levels correlated with new lesion formation and MRI activity [254]. Increased levels of CSF NF-H (including tau) were reported to be more sensitive than MRI in predicting conversion

from CIS to MS [255]. Importantly, NF-H was identified in patient serum and levels were altered following acute treatment (indicating axonal damage) [256]. In addition to the presence of NF proteins, autoantibodies against NF are also associated with MS, mainly in chronic disease [245]. In summary, NF subunits can potentially be used as markers for axonal damage in MS. However, each protein may have a different underlying biological mechanism in conjunction with MS, as their levels do not correlate [249]. Taken together, NF-H seems to be a better candidate biomarker for progression, while NF-L may be a better indicator for relapses and treatment efficacy. Indeed, a recent report comparing NF-H and NF-L as therapeutic biomarkers supported this view [257]. Combined measurement of NF-H and NF-L may be a useful tool with higher specificity and sensitivity than MRI to predict the conversion from CIS to MS. Additionally, GFAP also seems to be a potential biomarker to measure progression.

III. Other CSPs: Several additional CSPs have been suggested as biomarkers for MS. These include, but are not limited to, the 14-3-3 and tau protein families [258]. Several different reports have shown an association between elevation of 14-3-3 in CSF with MS [258]. 14-3-3 proteins were found to correlate with disease severity and IgG index in RRMS [259,260]. Although an increase of this protein in the CSF is associated with ONDs, it is more frequently observed in MS patients [258]. Further investigations are needed to elucidate the mechanisms by which the different 14-3-3 protein isoforms are involved in MS and to increase the sensitive and specificity of this protein family as biomarkers, perhaps in conjunction with other markers. The tau protein family has also been associated with MS. Several different reports showed that CSF tau levels are increased in MS patients [261,262]. Patients with RRMS had higher CSF tau levels than SPMS patients and levels of tau were found to negatively correlate with EDSS score [263]. Importantly, tau can also be detected in patient serum [263]. The increase of CSF tau has been suggested to reflect axonal injury [261]. Additionally, it was found to correlate with CSF 14-3-3 [260]. In contradiction to these reports, two studies did not find differences between CSF tau in patients with early MS versus healthy controls [264,265]. This could be due to differences in isoform specificity (e.g., ttau versus p-tau) or due to the selection of patients with different criteria (CIS versus MS). Furthermore, an increase in CSF tau is strongly associated with Alzheimer's disease [266] and Creutzfeldt-Jacob (CJ) disease [267]. Nonetheless, the difference in CSF tau levels may be useful to discriminate between MS and OND. In summary, the usefulness of the tau protein family as biomarker in MS and its prognostic and diagnostic values will need to be further investigated.

Part III: Expert commentary & five-year view

Here, we reviewed the present literature pertaining to MS biomarker discovery. MS is a heterogeneous disease, as indicated by its various clinical and pathological features, as well as in response to available treatments [268]. Currently, the major diagnostic and prognostic tools that are being routinely used in the diagnosis of MS are MRI, CSF OCB index, and clinical features, such as the EDSS score [44]. However, a comprehensive MS outcome prediction model is still unavailable, leaving most MS patients with many unknowns regarding their prognosis. Likewise, many of the drugs used in MS treatment show variable outcomes, and some have serious side effects. Patients with more severe disease are often

given aggressive treatment that poses significant adverse effects and risks for the patient. Thus, biomarkers that predict disease trajectory and drug efficacy will benefit patients, in particular during the early stages of MS. Consistent with this view, clinical features in the first few years of the MS disease course correlate with, and to an extent, predict later outcomes [269,270]. However, it is apparent that additional sensitive and specific laboratory biomarkers are urgently needed since clinical responses to treatment may take months to become apparent [95].

MS biomarker discovery studies have focused primarily on either the identification of new potential biomarkers or the validation of several known targets, such as autoantibodies or changes in T cell phenotypes. Identification of new biomarkers remains challenging due to the nature of the disease, its unknown etiology, and yet to be discovered pathophysiological mechanisms. However, even once a new biomarker candidate has been discovered, it is often not sufficiently validated, partially because of the lack of good strategies to prioritize which candidate will be worth the effort, time and money spent on such “high risk” markers. In recent years, numerous studies have exploited “omics” techniques, including genomics, transcriptomics, lipidomics and proteomics leading to a large number of potential new biomarker candidates [271-274]. Thus, the issue of how to prioritize and validate potential biomarkers has become an even greater priority for the field.

Maybe not surprising, biomarker discovery has primarily focused on clinical studies, and studies using animal models such as EAE are underrepresented, most likely because of a perception that molecules discovered in preclinical models may not apply to human MS [271,275]. However, models such as EAE, have been extensively used to unravel disease mechanisms underlying the human disease and to develop drugs, particularly for MS [276]. Many disease-related molecules that were discovered in the EAE model were found to correlate with human studies, thus emphasizing the potential of this platform for MS biomarker discovery [271,275]. Animal models such as EAE allow investigations under controlled conditions with much less biological variation than humans, including large-scale discovery and evaluation of potential biomarkers and a better understanding of their involvement in disease mechanisms. Along these lines, using a novel proteomic approach (M² Proteomics), our laboratory has demonstrated expression changes of several different CNS-specific proteins in EAE which can be detected in mouse serum [233,277]. A number of these proteins, such as 14-3-3, GFAP and MBP, have also been reported to be differentially expressed in MS patients [233,234]. Our studies have revealed additional putative CNS-specific biomarkers including Synapsin-1 and Alpha-II-spectrin (neuronal), which were not previously reported in MS. Thus, developing biomarkers in well-defined autoimmune animal models, including progressive and relapsing-remitting EAE models, may provide important insights that cannot be as easily obtained in human studies.

Lastly, specificity and sensitivity of biomarkers remains a major challenge in MS, potentially due to disease mechanisms in common with other diseases, such as neuroinflammatory/neurodegenerative diseases. We propose that biomarker profiles or “fingerprints” may be required to increase the specificity and sensitivity of potential clinical markers, which could include proteins, lipids, and other molecules and/or their modifications or metabolites. Improvements in technology may facilitate the investigation of

lipids, glycosylation, or metabolic intermediates as biomarkers for different aspects of MS. Since MS is considered a neuroinflammatory disease, markers that reflect CNS-specific changes, including axonal and myelin damage, may be most useful if detected in body fluids such as serum or CSF [105,277].

In summary, biomarker discovery still faces many obstacles in MS. Improvements over current methodologies in the coming years will help to enable more sensitive and specific measurements, and with the immense amount of data being generated, improvements in bioinformatics analysis and prioritization of potential markers will be critical. Biomarker discovery in animal models of MS, i.e. EAE, can provide important insights to guide the development of markers in human MS patients, which should preferably be studied in the context of clinical trials to establish clinical correlations. Clearly, finding better biomarkers for MS is of the utmost importance to improve diagnosis, provide better prognoses, and to find new treatments.

Supplementary Material

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Key issues

- MS is a clinically heterogeneous disease of unknown etiology and potentially different pathophysiologic mechanisms.
- Prognosis and response to treatment is variable between individual patients and current diagnostic measures cannot accurately predict individual patient outcomes and treatment responses.
- Despite decades of attempting to identify sensitive and specific biomarkers for diagnosis, prognosis, and treatment efficacy of MS, no generally accepted markers or laboratory tests have emerged as of yet.
- Biomarkers are particularly needed to test the efficacy of novel treatments in progressive MS.
- Promising research in the areas of microRNA, messenger RNA, lipids, and proteins suggests that useful biomarkers for MS may be available in the near future.

Table 1

microRNA, mRNA and lipids as candidate biomarkers in MS

microRNA	Author	Top ranked marker(s) (level) [comments/add. info]	Disease subtype	Tissue	Ref
	Otaegui <i>et al.</i>	miR-193a (↑) and miR-328 (↑) [remission vs. healthy] miR-18b (↑) and miR-599 (↑) [relapse vs. healthy] miR-328 (↑), miR-18b (↑) and miR-96 (↑) [relapse vs. remission]	RRMS	PBMC	[60]
	Lindberg <i>et al.</i>	miR-17-5p (↑) [CD4+ cells] miR-17-5p (↑) and miR-193a (↑) [in CD4+ cells upon stimulation] miR-497(↓) miR-1 (↓) and miR-126 (↓) [in CD4+ cells upon stimulation]	RRMS	lymphocytes	[61]
	Keller <i>et al.</i>	miR-422a (↑), miR-223 (↑), miR-145 (↑), miR-186 (↑), miR-664(↑), miR-422a (↑), miR-142-3p (↑), miR-584 (↑), miR-1275 (↑), miR-491-5p (↑) and miR-20b (↓)	RRMS	Peripheral blood cells	[62]
	Siegel <i>et al.</i>	miR-422a (↑), miR-614 (↑), miR-572 (↑), miR-648 (↑), miR-1826 (↑), miR-22 (↑) and miR-1979 (↓)	N/A	Plasma	[63]
	Fenoglio <i>et al.</i>	miR-15b (↓), miR-23a (↓) and miR-223 (↓)	RRMS and PPMS	Serum	[64]
	Ridolfi <i>et al.</i>	miR-223 (↑) and miR-23a (↑) [PBMC] miR-15b (↓), miR-23a (↓) and miR-223 (↓) [serum]	RRMS in remission and PPMS	PBMC and serum	[65]
	Du <i>et al.</i>	miR-326 (↑)	RRMS and EAE	Peripheral blood leukocytes	[66]
	Junker <i>et al.</i>	miR-155(↑), miR-34a (↑) and miR-326 (↑)	N/A	Active lesions	[67]
	Cox <i>et al.</i>	miR-17 (↓) and miR-20a (↓) [a part of cluster miR-17-92]	RRMS, SPMS and PPMS	Peripheral blood cells	[68]
	Sievers <i>et al.</i>	miR-17-92 cluster (↓) and miR-106b-25 cluster (↓) [upon natalizumab treatment]	RRMS	B lymphocytes	[69]
	Haghikia <i>et al.</i>	miR-922 (↓), miR-181c (↑) and miR-633 (↑) [MS] miR-181c (↓) and miR-633 (↓) [SPMS vs. RRMS]	RRMS, PPMS and SPMS	CSF	[70]
	Guerau-de-Arellano <i>et al.</i>	miR-128 (↑) and miR-27b (↑) [naïve CD4+ cells] miR-340 (↑) [memory CD4+ cells]	N/A	Peripheral blood CD4+ lymphocytes	[71]
mRNA	Bomprezzi <i>et al.</i>	112 mRNA identified; notable ones include: PAFAH1B1(↑), TNFRSF7(↑), ZAP70(↑), ZNF148(↑), IL7R (↑), HSPA1A(↓), CKS2(↓), JUN(↓), TIMP1(↓), SERPINE1(↓), HIF2 (↓)	RRMS & SPMS	PBMC	[80]
	Achiron <i>et al.</i>	1578 mRNA identified; notable ones include: TOSO(↑), BCL2(↑), CD3E(↑), AKT1(↑), RELA(↑), TAX1BP(↑), BAX(↓), APAF1(↓), CASP1(↓), CASP2(↓), CASP8(↓), CASP10(↓)	RRMS	PBMC	[82]
	Bryndal <i>et al.</i>	939 mRNA identified MS CSF compared with controls; controls; 266 genes identified comparing PBMC of relapsing MS patients with patients in remission; notable ones include: IFIT1(↓), JAK2(↑), STAG1(↑), PERP(↓), PTEN(↑), AKAP10(↑), PRKAG2(↑), SLC8A1(↑), CSNK1A1(↑), BCL6(↑), MAP3K3(↑), UBE2D3(↑), ABCB1(↓)	RRMS	CSF & PBMC	[83]

	Booth <i>et al.</i>	8 mRNA identified comparing CPMS to controls; Notably: MMP17(↑), QPCT(↑),IGHG1(↓),ITGB2(↓),CDCA7L(↓), PIPOX(↓), APOC3(↓) 25 mRNA identified comparing PPMS to SPMS; Notably: IL17R (↓)	PPMS & SPMS	WB	[84]
	Hecker <i>et al.</i>	5 mRNA identified comparing “poor” to “good” outcome MS patients: CA11(↑), GPR3(↓), IL1RN(↓),PPFIA1(↑),YEATS2(↑) 12 mRNA identified comparing “very poor” to “good” outcome MS patients: CA2(↓), CLCN4(↓), DNM1(↓), FPR2(↓), IL7(↑), NAMPT(↓), RRN3(↑), IL17RC (↓), IL17RA (↓), TUBB2B(↓), GPR3(↓), IL1RN(↓)	RRMS	PBMC	[85]
	Satoh <i>et al.</i>	30 mRNA identified; Notable ones include: NR4A2(↑), RIPK2(↑), SODD(↑), TRAIL(↓), BCL2(↓) DAXX(↓)	RRMS & SPMS	PBMC	[86]
	Romme Christianson <i>et al.</i>	In whole blood (WB): IFNG(↑), IL1B(↑), IL7(↑), IL10(↑), IL12A(↑),IL15(↑), IL23(↑), L27(↑), LTA(↑), LTB(↑) In CSF: IFNG(↑), CD19(↑),IL10(↓), CD14(↓)	RRMS	WB & CSF	[87]
	Hecker <i>et al.</i>	121 mRNA identified in IFN-β-1a treated patients varying over treatment course	RRMS	PBMC	[89]
	Goertsches <i>et al.</i>	Up to 175 mRNA identified in IFN-β-1b treated patients varying over treatment course	RRMS	PBMC	[90]
	Serrano-Fernandez <i>et al.</i>	14 mRNA identified for various times points after IFN-β-1b treatment: EIF2AK2(↑), IFI6(↑), IFI44(↑), IFI44L(↑), IFIH1(↑), IFIT1 (↑), IFIT2(↑), IFIT3(↑), ISG15(↑), MX1 (↑), OASL(↑), RSAD2(↑), SN(↑), XAF1(↑)	MS	PBMC	[91]
	Sturzebecher <i>et al.</i>	125 mRNA identified between IFN-β-1b treatment responders and non-responders; Notably: IL-8(↓) and ft-3 ligand(↓), GKLF4(↓)	RRMS	PBMC	[93]
	Weinstock-Guttman <i>et al.</i>	Several mRNA identified immediately after IFN-β-1a treatment; Including: Mx1 (↑), Mx2(↑), GBP-1(↑), GBP-2(↑), IFNAR2(↑), Stat1(↑), β2m(↑)	RRMS	PBMC (monocyte depleted)	[94]
	Bertolotto <i>et al.</i>	MxA (↑) in IFN-β treated patients	RRMS	PMBC	[100, 278]
	Hong <i>et al.</i>	18 mRNA identified in IFN-β treated patients: TNFα(↓), MMP-9(↓), NF-κB(↓), ICAM-1(↑), MxA(↑), IL-12R B2(↑), IL-12 p40(↑), VLA-4(↓), IL-1b(↓), iNos(↑) 12 mRNA identified in glatiramer treated patients: Fas(↓), CXCR3(↓), IL-12 p40(↑), P-selectin(↓), ApoE(↓), CCR5(↓)	RRMS & SPMS	PBMC	[95]
	Khademi <i>et al.</i>	mRNA identified in PBMC of natalizumab treated patients: IFN-γ(↑), TNF(↑) mRNA identified in CSF of natalizumab treated patients: IFN-γ (↓), IL-23(↓),IL-10(↑)	RRMS	CSF & PBMC	[97]
Lipids	Nicholas and Taylor	Cholesterol (↑)	EAE	Urine	[116]

Shore <i>et al.</i>	Cholesterol (↑), HDL (↑), LDL (↑) and VLDL (↓)	EAE	Plasma	[117]
Weinstock-Guttman <i>et al.</i>	HDL (↑) [increased only in correlation with lower lesion volume], LDL (↑), cholesterol (↑) and triglyceride (↑)	N/A	Serum	[118]
Giubilei <i>et al.</i>	Cholesterol (↑) and LDL (↑)	CIS (possible MS)	Plasma	[109]
Bretillon <i>et al.</i>	24S-OHC (↑) [NS]	N/A	Plasma	[110]
Leoni <i>et al.</i> 2002	24S-OHC (↓) [plasma of older patients], 24S-OHC (↑) [plasma of younger patients. NS]	RRMS, PPMS and SPMS	Plasma and CSF	[121]
Danylaite-Karrenbauer <i>et al.</i>	24S-OHC/Cholesterol ratio (↓) [in older patients only]	RRMS and PPMS	Plasma	[122]
Teunissen <i>et al.</i> 2003	24S-OHC (↓) [PPMS, and older RRMS patients]	RRMS and PPMS	Serum	[123]
Teunissen <i>et al.</i> 2007	24S-OHC (↑) and 27S-OHC (↑)	EAE	Serum	[124]
Distel <i>et al.</i>	7-KC (↑) [in acute MS brain and in CSF]	RRMS and EAE	Brain, CSF and serum	[130]
Leoni <i>et al.</i> 2005	7-KC (↑)	N/A	CSF	[131]
Farez <i>et al.</i>	15-KA and 15-KE (↑) [in RRMS and SPMS] 15-HC (↑) [in SPMS and secondary progressive EAE(NOD mice)]	RRMS, SPMS and EAE	Serum	[132]
Sbardella <i>et al.</i>	IsoP (↑) [higher levels were associated with higher risk of relapse]	CIS (possible MS)	CSF	[134]
Teunissen <i>et al.</i>	IsoP (↓) [in PPMS compared with CIS] IsoP (similar) [in RRMS compared with CIS]	CIS, RRMS and PPMS	Plasma	[135]
Miller <i>et al.</i>	IsoP (↑) [urine] and oxidized phospholipids (↑) [plasma]	SPMS	Urine and plasma	[136]
Koch <i>et al.</i>	Lipid peroxidation (↑) [in all MS subtypes. No difference in levels between groups]	Benign RRMS, SPMS and PPMS	plasma	[137]

** Bolded are marker that were identified by at least two separated reports

Table 2

cell surface markers and chemokines as candidate biomarkers in MS

Cell surface markers	Author	Top ranked marker(s) (level) [comments/add. info]	Disease subtype	Tissue	Ref
	Muraro <i>et al.</i>	VLA-4 (↓) and CD27 (↑) [upon IFN-β treatment]	N/A	Peripheral blood memory T lymphocytes (CD45RO+)	[149]
	Zafranskaya <i>et al.</i>	MOG-reactive CD45RO+ T lymphocytes (↑) [in MS patients], MOG-reactive CD45RO+ T lymphocytes (↓) [upon IFN-β treatment]	RRMS	Peripheral	[151]
	Chatzimanolis <i>et al.</i>	CD45RA+ ICAM-3+ T lymphocytes (↑) [upon methylprednisolone treatment]	RRMS	Peripheral blood and CSF	[152]
	Wang <i>et al.</i>	CD8+ CXCR3+ T lymphocytes (↑), CD25+, CD29+, CCR4+, CXCR3+, CD4+ T lymphocytes (↓) [upon methylprednisolone treatment]	RRMS	Peripheral blood and/or CSF	[153]
Cytokines	Franciotta <i>et al.</i>	TNF (comparable levels between MS, healthy and OND)	Chronic-progressive MS (CPMS) and RRMS	CSF and serum	[154]
	Sharief and Hentges	TNF (↑) [in CSF of CPMS patients]	CPMS and “clinically stable MS”	CSF and serum	[155]
	Drulovic <i>et al.</i>	TNF (“detectable”) [in active MS. NS]	RRMS	CSF	[156]
	Vladic <i>et al.</i>	TNF (undetectable in CSF) and (↑) [in 20% of patients serum]	RRMS	CSF and serum	[157]
	Vladic <i>et al.</i>	IL-6 (↑) [only in 10% of serum samples and 5% of CSF samples]	RRMS and SPMS	CSF and serum	[157]
	Stelmasiak <i>et al.</i>	IL-6 (↑)	RRMS and SPMS	CSF and serum	[158]
	Malmestrom <i>et al.</i>	IL-6 (↑) [in ~40% of CSF samples, but not in serum]	RRMS	CSF and serum	[159]
	Chen <i>et al.</i>	IL-6 (↑) [only in female]	RRMS	Serum	[160]
	Axtell <i>et al.</i>	IL-17F (↑) [in serum of IFN-β nonresponders]	RRMS	Serum	[162]
	Lee <i>et al.</i>	IL-7(↑) and IL-17F(↓) [in serum of IFN-β responders]	RRMS	Serum	[163]
	Balasa <i>et al.</i>	IL-17A (↑) [in serum of IFN-β nonresponders]	RRMS	Serum	[164]
	Hartung <i>et al.</i>	IL-17F (↑) [was not associated with poor response to IFN-β treatment]	RRMS	Serum	[165]

Table 3

potential CNS-specific protein biomarkers in MS

Marker	Description	Comments/add. info
Myelin breakdown products	Increased in the CSF [228,230,279] and correlate with disease activity in RRMS [231]. Increased in the CNS of EAE mice and correlate with disease progression [233]. Decreased in the CSF following immunosuppressive treatment [232,280]	A marker of demyelination and axonal death. Increase in many OND patients[236,281].
AlphaB-crystallin	Increased in CSF and serum [238-240,282] and correlates with disease activity and severity [238]	A marker of axonal death. Increase in many OND patients[282].
GFAP	Increase in CSF of RRMS and SPMS patient and correlates with disease severity and disease progression[241-243]. Higher in NMO patient compared to MS patients [244]	A marker of astrogliosis
NF-L	Increased in CSF of RRMS patient [248,250]. Highly increased during relapses [248]. An increase during CIS is predictive for conversion to CDMS [249]. Following natalizumab treatment CSF levels are decreased [250].	A marker of neuronal degradation and death[245]
NF-H	CSF levels are increased in MS patients [252,253]. CSF levels are higher in progressive MS [252].	A marker of neuronal degradation and death[245]
14-3-3	CSF levels are increased in RRMS patients[258-260]	Have a role in nerve apoptosis, astrocyte function and redox balance[233]
Tau	CSF levels are increased in RRMS and progressive MS patients [260-262]. Levels correlate with CSF IgG index [260]	Modulate the stability of axonal microtubules and play a role in neuronal cell morphogenesis and axonal maintenance [283]