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EXPERT REVIEWS

Neuroproteomics and microRNAs studies in multiple sclerosis: transforming research and clinical knowledge in biomarker research

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Multiple sclerosis (MS) is a complex disease characterized by extensive phenotypic variability. Biomarkers to capture the different aspects of MS heterogeneity, and to help make a diagnosis and monitor disease progression, while providing insights into etiopathogenesis and response to treatment, are urgently needed. Omics technologies and research efforts with microRNAs have provide unparalleled opportunities for exploring altered protein profiles associated with molecular mechanisms of disease, substantially expanding the list of candidate biomarkers for MS. This review presents evidence from proteomic studies that have focused on identification of biomarkers released in biofluids as a result of the different pathophysiological processes of MS. Also discussed is the emerging role of miRNAs as complementary biomarkers related to cellular processes occurring in MS patients. Also provided is an overview of candidate biomarkers that have been proposed for elucidating pathophysiological processes and disease activity and for guiding clinical diagnosis and/or therapeutic interventions in MS.

Keywords: multiple sclerosis • proteomics • miRNA • biomarkers • diagnostics • prognostics

Introduction

Multiple sclerosis (MS) is a chronic autoimmune disorder of the central nervous system (CNS) mostly impacting women between 20 and 40 years of age.[1] It represents the leading cause of non-traumatic neurological disability in young adults that decrease life expectancy by approximately 5–10 years.[2,3] According to the World Health Organization, the disease affects around 2–2.5 million people around the world. In 2010, approximately 18,000 people died from MS [4] rising to 20,000 in 2013 .[5]

The pathology of MS is characterized by demyelination of the axons in the CNS as a result of autoimmune processes.[6,7] Initially, the myelin loss is reversed by the oligodendrocytes (ODs).[2] However, repetitive loss and repair of the myelin, microglial activation, leukocyte infiltration due to increased permeability of the blood–brain barrier (BBB), along with decreased ODs' efficiency result in long-term axonal degeneration, neuronal death, and plaque formation. The subsequent accumulation of neurological damage and progression of neurodegenerative processes ultimately leads to irreversible neurological disability.[7,8]

MS is classically classified into four categories: relapsing remitting multiple sclerosis (RRMS), which represents around 85% of the cases, secondary progressive multiple sclerosis (SPMS), primary progressive multiple sclerosis (PPMS) and progressive relapsing multiple sclerosis (PrMS).[9] Yet, an extensive phenotypic variability coupled with

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unpredictable clinical relapses and remissions make MS treatment and prognosis more challenging. Thus, new tools such as biofluid-based biomarkers to distinguish between the different clinical phenotypes of MS, predict progression and inform clinical management of the disease are of great importance and urgently needed.

A biomarker is defined as an objectively measurable indicator of physiological processes, pathogenic processes or pharmacological responses to a therapeutic intervention.[10] Identification of reliable biomarkers for MS is under intensive investigation. Researchers have reported that biomarkers for MS can be used to assess, confirm or revise MS phenotype descriptions; to determine disease activity or to predict disease course and progression.[11,12] In addition, biomarkers that reflect pathogenic mechanisms of MS have the potential to guide individualized targeted therapies and predict response to treatments.[1,13] Recently, recognizing the importance of biofluid biomarkers for enhancing our understanding of MS, it has been discussed their inclusion in the McDonald diagnostic criteria improves the sensitivity and specificity of diagnosis.[14] However, the insufficient evidence of a reliable link between MS clinical course and biological markers, as well as considerable population and interlaboratory variability, and a lack of standardized procedures did not allow their inclusion at this time. Further advances in technology, development of sensitive biomarkers assays and clinical validation studies are necessary .[15]

This review aims to describe the recent proteomic and microRNA (miRNA) findings and their utilization to better understand the disease mechanisms in MS. Current promising candidate biomarkers that are under evaluation and validation as diagnostic, disease activity or prognostic tools for MS will also be discussed.

Pipeline of biomarker discovery in MS is presented in Figure 1.

Neuroproteomic studies

Proteomics can be defined as a branch of biotechnology concerned with applying molecular biology, biochemistry and genetics techniques to the analysis of proteins structure, function and interactions. Proteomics organizes the information generated on proteins in databases and provides an applications framework for these data. Proteomics should not be confused with genomics. In genomics, inferences are made with the gene as the starting product, while proteomics starts with functionally modified protein and works back to the gene. Moreover, unlike the genome, which is relatively static, the proteome varies with health or disease, tissue type, stage of cell development and effects of medications. Tens of thousands of intracellular and extracellular environmental signals and conditions result in continuous proteomic changes.[16] As a result, the targeted application of proteomics becomes an essential approach for understanding the disease mechanisms, facilitating early diagnosis, predicting disease progression and, ultimately, finding treatment targets.[17]

Over the last decade, the number of studies employing proteomic analysis to investigate MS has increased significantly. As cerebrospinal fluid (CSF) is in direct contact with the brain and

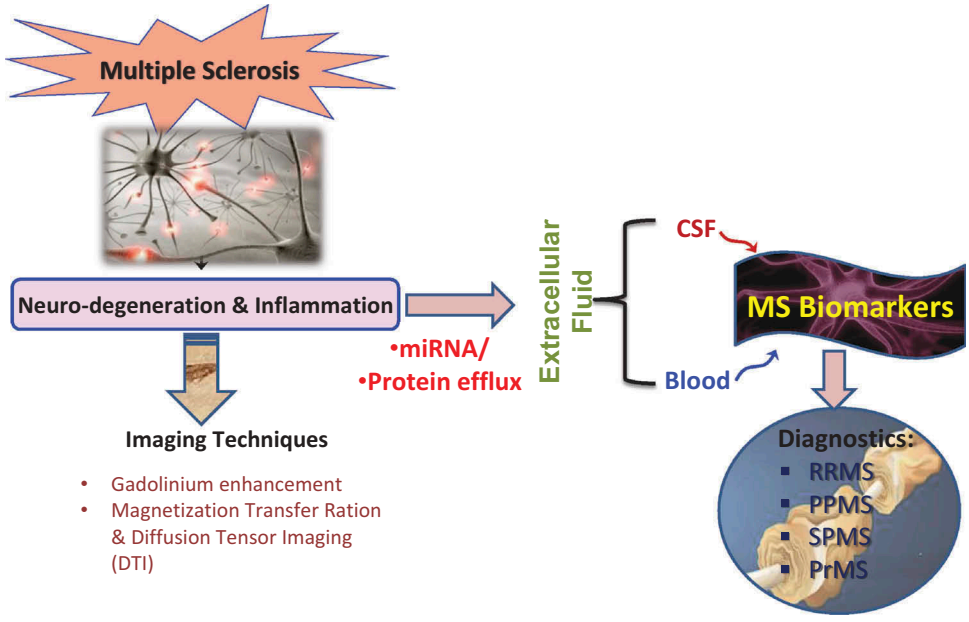

Figure 1. Pipeline discovery of biomarkers for multiple sclerosis. MicroRNA analysis and altered proteins that can be used for different multiple sclerosis phenotype classifications: relapsing remitting multiple sclerosis (RRMS), primary progressive multiple sclerosis (PPMS), secondary progressive multiple sclerosis (SPMS), progressive relapsing multiple sclerosis (PrMS)

more accurately reflect cerebral pathological changes, most proteomic studies have used CSF as an optimal source for MS biomarkers in living patients. However, several studies evaluated release patterns of biomarkers also in blood samples.[18] Still, there is no consensus on the biofluid to use and the results remain too broad and complex. In addition, the various proteomic studies in MS differ in terms of biofluid analyzed (CSF or blood), techniques employed, criteria for assessment, study design and even the organisms used. This variability, inconsistent standards and lack of a standardized approach significantly limit the clinical implication and translation ability of their findings.

The Matrix-Assisted Laser Desorption Ionization Time of Flight mass spectrometer in linear-mode geometry and in delayed extraction mode (m/z range: 1000–25000 Da) was recently used to investigate the existence of peculiar profiles in CSF characterizing different MS phenotypes at different phases of their clinical course.[19] In this study, the peak intensity of the signals of fibrinogen and Fibrinopeptide A were significantly down-regulated in clinically isolated syndrome (CIS) compared to PrMS, while those corresponding to Secretogranin II and Protein 7B2 were significantly up-regulated in RRMS patients compared to PrMS. Moreover, only the Tymosin b4 peak was found to be significantly different between the CIS and RRMS patients.[19]

Another study by Füvesi *et al.* (2012) aimed to identify potential protein biomarkers in CSF related to rapid progression in MS showing for the first time proteomic analysis of CSF of a fulminant MS. This study used a differential shotgun-proteomic approach based on isobaric tag labeling and nano-LC-MALDI TOF/TOF MS.[20] Seven proteins, namely, Ig kappa and gamma-1 chain C region, Complement C4-A, Fibrinogen beta chain, Serum amyloid A protein, Neural cell adhesion molecule 1 and Beta-2-glycoprotein 1, that are involved in the immune response, blood coagulation, cell proliferation and cell adhesion, were found substantially increased in fulminant MS compared to RRMS patients and controls. This observation, thus, indicates potential differences in the CSF proteome of a rapidly progressing MS patient compared to more typical clinical forms of MS and control subjects.[20]

Jia *et al.* (2012) compared levels of 26 selected potential protein biomarkers among CSF samples of patients with SPMS, patients with SPMS treated with lamotrigine, patients with non-inflammatory neurological disorders (NIND) and healthy controls (HC). A highly specific and sensitive selected reaction monitoring assay was obtained using high performance liquid chromatography-coupled mass spectrometry.[21] Only MY15B significantly differed between SPMS and HC groups, while three proteins (A4, APOE and KLK6) were found to significantly differ between SPMS and NIND. Furthermore, MY15B and PEDF discriminated between NINC and HC, while 11 biomarkers (1433F, 1433G, AACT, CAD13, TAU, NFH, NFL, NFM, NRCAM,OSTP and SAMP) showed significant difference between a lamotrigine-treated and untreated SPMS group. Taken together, this study demonstrated that the different cohorts significantly differed by 5 proteins and 11 proteins were associated with therapeutic intervention.[21]

Two recently published studies analyzed blood protein profiles in different phenotypes of MS. Raphael *et al.* used a methodologically novel approach that combines rapid microwave and magnetic (M2) sample preparation and quantitative MS/MS-based proteomics at multiple time points in an experimental autoimmune encephalomyelitis (EAE) animal model of MS.[22] Four proteins (14-3-3 ε, GPI, PLP1 and PRX1) were found to strongly correlate with disease progression demonstrating that M2 proteomics is a potential rapid method to quantify putative prognostic/predictive protein biomarkers and therapeutic targets of disease progression.[22] More recently, in a seminal study of Tremlett *et al.*, isobaric tagging, absolute protein quantification methodology and advanced multivariate statistical analysis were used to quantify and identify potential serum biomarker proteins of MS progression in two groups of patients (benign versus aggressive MS).[18] Eleven proteins involved in inflammation, opsonization and complement activation differed between the two groups. In particular, seven of these proteins, platelet glycoprotein V (GP5), ficolin-2 (FCN2), thrombospondin-1 (THBS1), protein S100-A9 (S100A9), platelet basic protein (PPBP), insulin-like growth factor-binding protein 3 (IGFBP3) and butyrylcholinesterase (BCHE), were found to be more abundant in the aggressive group compared to the benign one. Conversely, the other four proteins, lipopolysaccharide-binding protein (LBP), leucine-rich alpha-2-glycoprotein (LRG1), corticosteroid-binding globulin (CBG) and inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), appeared less abundant in the aggressive MS group as compared to patients with benign MS. These findings are remarkable as they could be used to design a targeted multiple reaction monitoring mass spectrometry-based assay to demonstrate the reproducibility of these results.[18] However, candidate markers require a rigorous clinical validation across different patient populations in order to be employed clinically in management of the disease.

Considerations

The field of proteomics in MS has greatly progressed over the few years and several candidate protein biomarkers have been proposed, even within different phenotypes of MS. Importantly, some studies assessing altered protein profiles in patients with and without treatment have shown that these markers might be valuable in monitoring the effect of a drug as well as in guiding treatment.[19] A summary of the recent MS proteomic studies is shown in Table 1.

However, only a small part of the proteome has been examined so far [15]; thus, there is still a large potential for exploration in this field. More studies and novel approaches are needed to assess the rest of the proteome, to identify new biomarkers and to reproduce and reliably validate the available markers.

Table 1. CSF and blood proteomics studies on clinical multiple sclerosis subjects.

Alternated proteins	Clinical sample	Condition	Results	Reference
THBS1, PPBP, IGFBP3, BCHE, LRG1, CBG, ITIH4, S100A9, GP5, FCN2,LBP	Serum	• Aggressive MS • Benign MS	• Higher concentrations of the proteins were measured in these two groups compared to other groups. • Four (LRG1,CBG,ITIH4 and LBP) proteins were significantly elevated in those with benign multiple sclerosis, while the remaining 7 were significantly enriched in the serum of those with aggressive multiple sclerosis	Tremlett *et al.* [18]
Secretogranin II, Protein 7B2, Fibrinogen, Fibrinopeptide A and Tymosin	CSF	• CIS patients • (RR)MS, • (Pr) MS patients	• Secretogranin II and Protein 7B2 were significantly increased in (RR) MS patients compared to PrMS • Fibrinogen and Fibrinopeptide A were significantly down-regulated in CIS compared to PrMS patients • Only Tymosin was significantly discriminated between the CIS and RRMS	Liguori *et al.* [19]
Serum amyloid P-component, Coagulation factor X, Fibronectin Immunoglobulin lambda-like polypeptide 5, Biotinidase, Histidine-rich glycoprotein, Plasma protease C1 inhibitor, Apolipoprotein B-100, Inter-alpha-trypsin inhibitor heavy chain H3, Complement factor I, Ig kappa chain V-III region SIE, Coagulation factor XIII B chain, Keratin, type I cytoskeletal 10, Attractin, Keratin, type II cytoskeletal 2 epidermal, Complement C4-A, Transferrin, Ig kappa chain V-I region EU, Prothrombin, Complement C3, Plasminogen, Phosphatidylinositol-glycan-specific phospholipase D, Leucine-rich alpha-2-glycoprotein, Zinc-alpha-2-glycoprotein, Alpha-1-antichymotrypsin, Alpha-1-acid glycoprotein 2 (ORM2), CXCL7, Ig heavy chain V-III region TIL, Ig gamma-1 chain C region, Apolipoprotein C-II, Ig heavy chain V-III region VH26, Protein AMBP, Apolipoprotein D, Thyroxin-binding globulin Beta-2-glycoprotein 1	Serum	• HD • DC • Pre-symptomatic MS • Post-symptomatic	• Proteins elevated in MS patients compared to control were those involved in the complement and coagulation pathways and lipid transport • Pre-symptomatic proteins from the MS group were differentially regulated compared with both control groups	M. T. Wallin *et al.* [24]
Ig kappa chain C region (IgK), Osteopontin (OSTP) (FM2), Serum amyloid A protein, Basement membrane-specific heparan sulfate proteoglycan core protein Ig gamma-1 chain C region, Complement C4-A, Fibrinogen beta chain, Neural cell adhesion molecule 1 and Beta-2-glycoprotein 1	CSF	• Fulminant MS • (RR) MS patient • – Headache patient, whose CSF analysis was normal	• Some proteins of the seventy eight found were seen to be increased in at least one MS sample compared to the control sample • Seven proteins were found to be up-regulated in both fulminant MS samples but not in the relapsing-remitting case compared to the control.	Füvesi *et al.* [20]
1433F, NFL, A2MG, 1433B, RTN4, CNTN1, AMD, 1433G, CAD13, AACT, NRCAM, KLK6, VGF, MOG, PEDF, A4, APOE, SODC, OSTP, SAMP, TAU, NFH, NFM, KLKB1, AGRIN, MY15B	CSF	• SPMS • patients with SPMS treated with lamotrigine • NIND patients • HC	• MY15B protein showed significant difference between SPMS and HC • A4,APOE and KLK6 proteins differed between SPMS and NIND while MY15B and PEDF proteins between NIND and HC • Protein biomarkers differed between a lamotrigine-treated and untreated SPMS group	Jia *et al.* [21]

miRNA in MS studies

Another class of biomarkers that has just started to being investigated is miRNAs. miRNAs play critical roles in immune system regulation.[23] A series of studies provided evidence that autoimmune diseases are linked to dysregulations in the expression of various miRNAs.[25] The expression of miR-27b, miR-128 and miR-340 has been shown to be up-regulated significantly in CD4+ T cells of MS patients.[26] Other studies have

Table 2. Dysregulated miRNAs in MS patients.

Author(s)	miRNA	Expression	Target	Biomarker	Effect
Keller et al. [31]	miR-7-1-3p	Down-regulation	?	Blood	?
Fenoglio *et al*. [32]	miR-15b	Down-regulation	?	Serum	?
Lindberg *et al*. [43]	miR17-5p	Up-regulation	PIK3R1, PTEN, Bim	PBMC	?
Keller et al. [31]	miR-20a-5p	Down-regulation	?	Blood	?
Du et al. [33]	miR-21	Up-regulation	?	?	Th1 and Th17 increase
Fenoglio et al. [34]					
Fenoglio *et al*. [32]	miR-23a	Down-regulation	?	Serum	?
Zhu *et al*. [35]	miR-23b	Up-regulation	?	?	Th17 increase
Guerau-de-Arellano *et al*. [26]	miR-27b	Up-regulation	?	?	Th1 increase
Guerau-de-Arellano *et al*. [26]	miR-128	Up-regulation	IL-13	?	Th1 increase
Gandhi *et al*. [36]	miR-145	Up-regulation	?	Plasma, PBMC	?
Sondergaard *et al*. [37]					
Waschbisch *et al*. [38]	miR-146a	Up-regulation	?	?	Th17 increase
Zhang *et al*. [60]	miR-155	Up-regulation	CD47	Plasma, serum, PBMC, brain	Th1 and Th17 increase
Waschbisch *et al*. [38]					
Junker *et al*. [23]					
Fenoglio et al., [32]	miR-223	Down-regulation	?	Serum	?
Waschbisch et al. [38]	miR-326	Up-regulation	Ets-1, CD47	?	Th17 increase
Fenoglio et al., [34]					
Junker et al., [23]					
Guerau-de-Arellano et al. [26]	miR-340	Up-regulation	BMI1, IL-4	?	Th1 increase
Gandhi et al. [36]	let-7e	Up-regulation	IL-10	?	Th1 and Th17 increase

Note: The symbol "?" stands for "unknown".

revealed an increase in the expression of miR-34a, miR-155 and miR-326 at the active MS lesions leading to a decrease in CD47 expression and thus to a macrophage release from inhibitory control and phagocytosis of myelin.[27] Recent work has also demonstrated the multiple roles of miRNAs in several cellular processes such as proliferation, differentiation and apoptosis in the context of MS.[28,29] Moreover, the dysfunction of the BBB in MS was reported to be associated with the down-regulation of miR-125a-5p which is involved in the regulation of immune cell efflux and tightness of endothelial cells of the BBB.[30]

miRNAs for MS can be classified into three categories: miRNAs in the CNS lesions and in cells of CNS, miRNAs of the immune cells and circulating miRNA.

A summary of the major miRNAs studies in MS is shown in Table 2.

miRNA in brain lesions and in cells of CNS

Junker *et al*. showed that 20 different miRNAs were up-regulated in the active white matter lesions in the brain of MS patients.[27] Conversely, eight different miRNAs including miR-23b, miR-139, miR-181c, miR-184, miR-328, miR-340, miR-487b and miR-656 were down-regulated in comparison to brain white matter tissues of normal humans.[27] Neuropathological studies in the brains of MS patients have demonstrated increased miR-124 expression in the *hippocampi*.[39,40] Increased expression of miR-155, miR-338 and miR-491 has also been demonstrated in the cerebral white matter of MS patients compared to subjects without MS.[41]

In MS, CNS demyelination results in severe motor, sensory and cognitive impairment [42] and is mainly due to OD damage. Several miRNAs coordinate the rapid transition of gene expression during OD differentiation (e.g., miR-338, miR-219, miR-23 and miR-9) and proliferation (e.g., miR-17-5p and miR-19b).[25] Furthermore, miR-17-5p and miR-19b control the proliferation of ODs by targeting PTEN (the inhibitor of PI3K signaling) which is a negative regulator of cell proliferation in RRMS.[42]

Microglia activation is a feature of various neuro-inflammatory and neurodegenerative diseases.[43] Studies in the EAE model showed that both microglia and macrophages of the murine CNS have lower levels of miR-124 compared to control brain. Treatment with pro-inflammatory cytokines, such as interferon (IFN)-γ and GM-CSF, resulted in a miR-124 up-regulation.[44,45] Upon miR-124 up-regulation, microglial cells switch from an activated state into a quiescent state with the potential to inhibit the onset of EAE.[46,47]

Astrocytes have a critical role in lesions' formation in MS. The 10 mostly abundant miRNAs in active MS lesions were expressed by astrocytes.[27] These miRNAs included miR-21, miR-34a, miR-142-5p, miR-155, miR-193a, miR-214, miR-320 and miR-326 that target CD47, which is known to be down-regulated in active lesions.[25] Astrocyte stimulation *in vitro* with inflammatory cytokines such as interleukin (IL)-1β, TNF-α, IFN-γ and TGF-β results in over-expression of miR-23a, miR-146a and miR-155.[48]

Research conducted in EAE models have shown by using real-time quantitative PCR analysis that miRNA-21, miRNA-142-3p, miRNA-146a, miRNA-146b and miRNA-155 were up-regulated in the spinal cords of EAE mice and marmoset EAE brains,[49] whereas miR-23b expression decreased significantly in the spinal cords of EAE mice.[35]

miRNAs of the immune cells – miRNA in CD4$^+$ and CD8$^+$ T cells

CD4$^+$ T cells orchestrate the full cascades of immune responses. They induce B cells to produce antibodies and macrophages to develop an enhanced microbicidal activity. They recruit neutrophils, eosinophils and basophils to sites of inflammation and produce cytokines and chemokines.[50] Microarray analysis revealed that the expression of 21 miRNAs were down-regulated in T cells from peripheral blood samples of MS patients.[51] Furthermore, both miR-15a and miR-16-1 were down-regulated in CD4$^+$ T cells from peripheral blood samples of RRMS patients.[26] However, miR-27b, miR-128 and miR-340 were up-regulated in CD4$^+$ T cells taken from MS patients.[42]

Santis *et al.* have shown that that nine miRNAs including miR-1, miR-17-5p, miR-126, miR-193a, miR-200b, miR-376a, miR-485-3p, miR-486 and miR-497 are increased, whereas miR-34a are decreased in CD4$^+$ T cells from peripheral blood mononuclear cells of RRMS patients. T regulatory cells (Tregs), a subset of T-helper cells with immune suppressive abilities, collected from peripheral blood samples of MS patients showed up-regulation of 17 miRNAs and down-regulation of 7 miRNAs including miR-138-2, miR-324-3p, miR-338-5p, miR-489, miR-512-3p, miR-564 and miR-886-3p.[52] TGF-β is involved in the differentiation and the maturation of Tregs. In particular, in MS patients, Tregs lose their suppressive ability due to the disruption of TGF-β signaling pathway that may result from the up-regulation of miR-106b and miR-25.[33]

Th17 cells, another subset of T-helper lymphocytes, are the main effector cells in the pathogenesis of MS. It was shown that the severity of MS in human patients and EAE in mice was highly correlated with the expression of miR-326 in Th17 cells.[33] Furthermore, the *in vivo* silencing of miR-326 resulted in fewer Th17 cells and mild EAE, whereas its over-expression led to a higher number of Th17 cells and a more severe EAE.[52] In addition to the pathogenic role of Th17 cells, MS patients are thought to have a chronic Th1 inflammation due to the elevated levels of IFN-γ, the major cytokine produced by Th1-helper lymphocytes. MiR-29b regulates the secretion of IFN-γ, but upon T-cell activation in MS patients, miR-29b level decreases significantly and dysregulation in the secretion of IFN-γ may contribute to such chronic inflammation.[26] Moreover, miR-27b and miR-128 levels increased in naïve CD4$^+$ T cells, and miR-340 was up-regulated in memory CD4$^+$ T cells. These miRNAs act to suppress Th2 differentiation and set the stage for pro-inflammatory Th1 immune responses in mouse and human cells.[25]

CNS and CFS of MS patients are enriched with CD8$^+$ T cells with an activated/memory phenotype suggesting that such CD8$^+$ T cells may be pathogenic.[25] Studies have shown that miR-629 is up-regulated, whereas miR-30a-3p, miR-149 and miR-497 are down-regulated in CD8$^+$ T cells from peripheral blood samples of RRMS patients.[42]

miRNAs of the immune cells – miRNA in B cells and monocytes

Sievers *et al.* demonstrated that the expression of 49 miRNAs were down-regulated in B cells taken from peripheral blood samples of RRMS patients.[53] MiR-106b-25 and miR-17-92 were seen to be up-regulated in B cells taken from MS patients in comparison to normal controls.[25] miRNA/RNA interaction analysis revealed that the affected pathways by the above up-regulated miRNAs in B cells were PI3-kinase and PTEN, which are two B-cell receptor key signals.[25] In a single study, miR-155 expression was significantly increased in peripheral circulating CD14$^+$ monocytes in MS patients compared to normal donors.[54]

Circulating miRNAs

Several miRNAs were detected in extracellular body fluids such as plasma, urine and saliva. Studies have suggested that circulating miRNAs are either actively released from cells, or they are the by-products of necrotic or apoptotic cells.[55] It has been shown that miR-768-3p was up-regulated, whereas 25 different miRNAs were significantly down-regulated in peripheral blood of patients with all MS subtypes including PPMS, SPMS and RRMS.[56]

In a study by Gandhi and colleagues, up-regulation of 29 different miRNAs was observed in plasma of RRMS patients.[39] On the other hand, miR-22, miR-422a, miR-572, miR-614, miR-648 and miR-1826 were up-regulated significantly in the plasma of MS patients, but expression of miR-1979 was decreased significantly.[57] Levels of miR-155 were found increased in serum samples from MS patients.[58] Another study showed that free-circulating miR-15b, miR-23a and

miR-223 levels were lower in serum samples from MS patients compared to normal controls.[32]

MS treatments and miRNA expression

As stated above, miRNAs were highly dysregulated in MS patients and EAE mouse model. It is clear that numerous miRNAs are either up-regulated or down-regulated in PBMCs, blood cells, plasma and brain white matter tissues from MS patients, suggesting that such dysregulated miRNAs play a role in MS pathogenesis and may be used as a signature for MS. In addition, several treatments of MS showed an effect on the expression of multiple miRNAs in MS patients. For instance, seven miRNAs were up-regulated, whereas 13 others were down-regulated in PBMCs of MS patients treated with IFN-β.[59] MiR-23b down-regulates IL-17 [35]; however, it was shown that IL-17 suppressed miR-23b expression in the spinal cords of EAE mice.[35] Such results suggest that cytokines may regulate miRNA expression in MS. Glatiramer acetate (GA) is an immunomodulator currently used in the treatment of MS.[60] MiR-146a and miR-142-3p expression in PBMCs of MS patients have decreased significantly upon GA treatment, after it was shown that their expression is up-regulated in PBMCs and brain white matter lesions of MS patients and EAE mice.[34] Furthermore, it was demonstrated that 10 miRNAs were up-regulated in B lymphocytes of RRMS patients treated with a monoclonal antibody against α4-integrin called Natalizumab, when compared with untreated RRMS patients.[53] In addition, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin, an activator for aryl hydrocarbon receptor, increases the expression of miR-132 and induces anti-inflammatory response in EAE mice.[61]

MiRNA as therapeutic targets in MS

Several lines of evidence have shown that the suppression of miR-155 inhibit the development of Th1 and Th17 cells. Supporting these observations, anti-miR-155 treatment was reported to inhibit EAE development in mice.[62] Consequently, some researchers have postulated that miR-155 might be a potential therapeutic target for MS.[63]

Furthermore, miR-326 expression has been confirmed to increase significantly in active MS lesions,[23] while *in vivo* gene silencing of miR-326 resulted in Th17 inhibition and decreased significantly EAE.[33] Taken together, these studies indicate that miR-326 may represent a therapeutic target for MS.

MiR-23b down-regulates IL-17, inhibits TNFα and IL-1-β-induced nuclear factor kappa-B activation as well as suppresses TGF-β-activated kinase 1/MAP3K7 binding protein 2 (Table 2) in EAE mice.[35] This spectrum of pleiotropic effects supports the idea that miR-23b could be important in the treatment of MS.

Emerging role of vitamin D in MS

A series of studies from multiple groups provided substantial experimental and human evidence that vitamin D has a significant effect on the incidence, course and progression of MS,[64–70] low vitamin D levels increasing the risk of developing MS and contributing to worsening over time.[71]

Vitamin D has a central role in controlling the immune responses in general and particularly in MS. High serum vitamin D levels have been reported to be associated with increased anti-inflammatory effects, while vitamin D deficiency is linked to increased levels of pro-inflammatory cytokines.[72,73] Studies investigating the mechanism of action of vitamin D on the immune response in MS have shown that the active form of vitamin D, 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], diminishes the expression of IL-17A in both human and mouse T cells, both major players in the MS autoimmune activity.[67,74,75]

The epigenetic effects of vitamin D in MS and its impact on miRNAs regulation have also been explored. Gandhi *et al.* examined the use of circulating miRNAs as biomarkers of disease staging.[36] Different miRNAs were identified by disease category and progression. hsa-miR-92a-1* was identified in the largest number of comparisons and differed the most between RRMS, SPMS and PrMS as compared to HCs. miR92 and let7 family of miRNAs were the most common differentiator of disease type and were linked to T-cell activation.[36,76]

Remarkably, measurements of circulating 25-hydroxyvitamin D, as an indicator of vitamin D status, are being incorporated into clinical trials evaluating the effects of vitamin D supplementation in MS.[13] Vitamin D-binding protein has also been proposed as a candidate biomarker of disease progression.[77]

Candidate MS biomarkers

As was discussed previously, omics technologies have enabled the identification and substantially expanded the list of novel candidate biomarkers that reflect or are involved in various alterations and pathogenetic processes of MS. These include neuronal death, glial damage, inflammatory demyelination, gliotic scar formation and axonal degeneration, diffuse astroglial and microglia activation, oxidative stress and BBB disruption,[7,78–80] all potential and attractive sources of biomarkers.

Here, we review the most promising biomarkers among the proteins identified so far that have been used in clinical research or adopted in clinical practice.

The use of MS biomarkers in clinical research

Several recent studies have explored the potential use of neurofilaments as a putative MS biomarker in human biological fluids. The neurofilament family proteins are neuron-specific intermediate filaments particularly abundant in axons [81] that have been reported as well-established markers of axonal damage in several neurological diseases.[82–84] In a recent study by Gresle *et al.*, serum heavy neurofilament levels were found to be substantially higher in first demyelinating event, RRMS and SPMS patients, than in controls. Remarkably phosphorylated heavy neurofilament protein concentrations correlated with severity scores and neuroimaging findings and fluctuated over time, likely reflecting temporal dynamics of axonal injury in MS.[85] In a thorough study by Martinez *et al.*, 12 different

glial and neuronal markers were evaluated in CSF of patients with RRMS and CIS to determine their ability to predict progression in MS. Among those, CSF neurofilament light (NFL) protein levels were the only predictor of conversion from CIS to clinically definite MS.[11] However, controversy about the clinical significance of CSF and serum levels of neurofilaments remains.[86]

Other proteins highly expressed within the CNS and proposed as promising candidate biomarkers of brain injury and brain pathology were also evaluated in MS. Several studies focusing on tau, phospho-tau and amyloid beta have yielded conflicting or inconclusive results.[87–90] These inconsistencies could be due to the well-known heterogeneity of MS or differences in the study populations as well as differences in the analytical methods associated with different detection limits.

There is substantial experimental and human evidence about the major role played by the inflammation in disease onset and pathophysiology of MS, which is reflected in the concentrations of multiple pro and anti-inflammatory cytokines. In general, levels of pro-inflammatory proteins, such as IL-1β, IL-6, IL-12, IFN-γ, TNF-α; CXCL10 and CXCL13, are increased in CSF of MS patients.[91–93] TNF-α; and IL-12 concentrations are also elevated in serum.[91,95] Conversely, anti-inflammatory cytokines, such as IL-10, IL-4, CCL2 and CCL5, are down-regulated in both CSF and blood.[91,96] Importantly, cytokine levels have been correlates with the disease activity and progression. As such, panels of multiple cytokines and cytokine profiles have been proposed as biomarkers for diagnosis and disease monitoring. However, differences can be observed between different studies [91,94] likely due to the heterogeneity of MS even within the same subtype as well as other factors such as extracerebral sources, infections or stress. These confounders make difficult the interpretation of the clinical significance of cytokines in patient with MS thereby limiting their value in the clinical setting. Nonetheless, as CSF levels of various cytokines (e.g., IL-10, CXCL13) are associated with MS treatments, investigators have proposed that they may be used as a surrogate end point for treatment efficacy.[97,98] Supporting these considerations, a research study by Dimisianos monitored patients with RRMS treated with interferon-β (INF-β) over the course of 5.3 years. Responders to INF-β had significantly higher initial baseline levels of IL-17 in serum that were decreased after INF-β therapy, indicating a role of IL-17 in the pathogenesis of MS and its potential use as a biomarker of IFNβ treatment response.[99]

In a study by Colomba using an immunoproteomics approach, an autoreactive anti-transferrin antibody was identified in CSF and serum as a candidate diagnostic marker and with a potential link with the development and progression of MS.[100]

The role of MS biomarkers in clinical practice

Currently only a small proportion of the identified biomarkers for MS are used in clinical practice with an effect on clinical decision making.

Assessment of CSF-specific oligoclonal bands (OCB) is routinely used in the diagnostic work-up of patients suspected of having demyelinating disease.[101,102] Although OCB are not exclusively detected in MS, they are present in CSF of more than 95% of patients with MS providing strong support for a diagnosis of MS.[103,104] In addition, in CIS patients, CSF OCB represent a risk factor for developing a clinical definite MS.[105] However, OCB tests are based on non-quantitative techniques that are difficult to standardize. More recently, a series of studies have reported CSF free light chains (FLC) as a potential alternative. In a study by Duranti *et al.*, FLCs were measured using an automated nephelometric assay test in neurological patients with non-inflammatory diseases, in neurological patients with inflammatory diseases other than MS, and in patients with definitive MS. Nephelometric assay for Kappa free light chains (kFLCs) in CSF was found to be rapid and reliable in discriminating MS patients and able to reduce tiresome manual work and variability in interpretations of results.[101] Hassan-Smith and colleagues also assessed κ-FLC and λ-FLC both in CSF and in serum to determine their diagnostic value in MS (2014). CSF κ-FLC was extremely sensitive and specific over traditional OCB testing in discriminating patients with CIS, PPMS and RRMS from those with other neurodegenerative disease or with other neurodegenerative inflammatory disease.[106] By contrast, using an ELISA format, Senel failed to show any increased sensitivity or specificity of the κFLC assay over traditional OCB testing.[107]

In the serum of patients with neuromyelitis optica (NMO) and NMO spectrum disorders, a highly specific auto-antibody marker (NMO-IgG) targeting the astrocytic water channel aquaporin-4 (anti-aquaporin-4 antibodies) has been identified.[108] This marker has been shown to differentiate NMO spectrum disorder from MS [109] and it is also included in the diagnostic criteria of NMO.[110] Noteworthy, prognoses and treatments differ between subjects with NMO and those with MS; thus, the assessment of NMO-IgG antibodies has significant implication for clinical management.

Patients treated with IFN-β might develop binding and neutralizing antibodies (NAbs) that potentially affect therapeutic efficacy.[111,112] In light of above, measurement of NAb has been proposed to be routinely integrated into clinical practice to guide individual treatment decisions and patients with persistent high-titer NAb positivity are recommended to switch to a non-IFN-β therapy.[113] Still measurement of NAb has been uniformly adopted and controversy remains about their clinical significance.[113–116]

A group of highly promising candidate serum biomarker for MS including serum anti-MBP and anti-myelin OD glycoprotein antibodies that have been studied for several decades and employed to assess and monitor clinical efficacy in clinical trials [117,118] recently resulted negative in independent validation cohorts of MS patients.[119]

Toward the development of MS biomarkers: challenges and (potential) solutions

Over the past few decades, tremendous efforts have been made to discover novel MS biomarkers for use in clinical practice. However, a striking discrepancy exists between the number of identified markers and those that make it into clinical practice due to several factors and confounding issues.

An important factor that affect biomarker discovery is the selection of the biofluid to analyze. CSF or blood? CSF provides a more accurate and well-suited monitoring tool of CNS disease activity because of its close proximity and direct contact with the sites of disease pathology. For these reasons, it is considered an optimal source of MS biomarkers. Nevertheless, CSF sampling requires an uncomfortable, invasive lumbar puncture that makes its use difficult in clinical practice and limit repeated collection. In addition, the risk of blood contamination cannot be ignored.[120] Proteins are present in the systemic circulation in a concentration 50–100-fold higher than in CSF.[121] A series of studies have investigated the impact of blood contamination on CSF proteome analysis and shown that even 0.001% blood contamination was within the detection limit of the 2-D LC-MS/MS resulting in the identification of "artifact" protein biomarkers.[121] As such, an extensive standardization is required.[122] On the other hand, despite serum is less invasively collected and suitable to assess disease modifying agents' activity directed at peripheral immune targets, it lacks accurate evaluation of the CNS processes correlated with MS progression.

Besides the aforementioned sample collection conditions, other preanlaytical factors that can obstacle the path from bench to bedside include sample processing and storage.[123,124] It has been estimated that preanalytical variability account by itself for around 68% of errors in clinical laboratories.[125]

Furthermore, issues related to study design might significantly limit reproducibility and the generalizability of findings from MS biomarker studies.

Inadequate sample size is also an important factor that can reduce the reliability and generalizability of findings from studies aimed at discovery of candidate biomarkers in MS.[121] Performing appropriate and rigorous sample size calculations is critical to avoid underpowered studies with insufficient numbers of subjects that are unable to detect meaningful differences and provide false-negative results.[126]

Validity and reliability of MS proteomics research also heavily depends on characterization of cases and the choice of controls. Although investigators have been trying to performing MS subtype-specific studies and pool data from individuals with a comparable MS phenotype, the CSF protein content might vary or be spurious owing to the heterogeneous manifestations and unpredictable course of disease. It is also worth to mention that CSF proteins tend to fluctuate during the course of the disease even amongst the same individual. To account for such limitations, identification of candidate biomarkers for MS at a particular phase should be systemically compared to the proteome profiles of subgroups of MS patients at a well-defined disease's stages. However, this is not an easy task given the labor-intensive and costly proteomic methods.[121] On the other hand, because of bioethical reasons, healthy individuals cannot be enrolled and a number of MS studies have used different types of control populations including subjects with other neurological diseases or with headaches.[20] This resulted in a lack of uniformity in the study design making it difficult to compare different proteomic data across studies. Furthermore, since MS affects many pathways, proteins found in the CSF of MS patients could be similar to those found in CSF of subjects suffering from other diseases. Therefore, the proteins of interest should be assessed using a combination of absolute quantitative proteomics and qualitative methods.

Finally, appropriate clinical end points that capture the clinical aspects of MS and potential therapeutic effects and can be reliably correlated with candidate biomarkers should be selected.[127]

Conclusion

Due to the heterogeneous characteristics and complicated course of MS, researchers have been searching for critical objective diagnostic and monitoring tools that may help clinicians in medical decision making. With the emerging era of neuroproteomics and other high-throughput advanced platforms, altered patterns of protein, gene and miRNA profiles have been investigated to correlate with specific features of diseases. Although these breakthrough technologies and novel initiatives offered unparalleled opportunities for MS biomarker development, we still lack the ideal marker. Probably a single biomarker will not suffice to reach the necessary sensitivity and specificity, and biomarker panels comprising proteins, genes and miRNAs are likely to be the best approach for understanding pathogenesis of MS, improving diagnosis, classification and evaluation of disease activity and, ultimately, response to treatment. Finally, identified panels will have to be thoroughly validated before they can be implemented as diagnostic aids.

Expert commentary

In this review, the genomic and proteomic advances in the field of MS were described. Over the past three decades, significant progress has been made toward understanding the pathology of the disease. The T- and B-cell antigen receptors have been defined along with the associated signaling cascades that follow and the related genetic and associated epigenetic changes. Most of the discoveries seem to be at a primitive stage and the disease mechanisms are yet to be explored because of the complex nature of the immune system, plasticity and interaction with the several environmental factors.

The traditional biochemical and cellular methods do not seem to be adequate to elucidate all the factors associated with MS. The genetic and proteomics of the disease and what leads to this autoimmune responses are still not clear.

Therefore, new technologies that allow genomic and proteomic analysis are crucial and the logical next step to tackle this complex disease.

The CSF proteomics research of the different types of MS has already indicated a group of biomarkers that are implicated in the course of the disease. In addition, distinct patterns of these proteins were observed within each specific type of MS. In particular, RRMS seems to have different set of implicated proteins than progressive MS, and these proteins change within each group based on treatment regimen as well as other environmental factors. These observations suggest how complex the disease is to discern and how much the immune system is affected throughout its course.

Experimental and human data have also demonstrated characteristic patterns of miRNA in brain lesions, plaques, CSF, serum and plasma suggesting a role in MS pathogenesis. Several miRNAs have been identified and their effects on key cytokines were shown. An up-regulation or down-regulation of certain miRNA may result in an increase or decrease of Th1 or Th17 levels, key players in immunology of MS. This miRNA effect has a significant impact on the course of the disease. It is yet important to discover what can cause the miRNA to up-regulate or down-regulate and whether these changes are modifiable. The environmental factors or conditions that can alter the miRNAs and alter the disease course as well are to be explored.

To date, the role of vitamin D in MS and its association with miRNA levels and cytokine fluctuation is a highly relevant area of research that has just started to be explored. Growing body of evidence supports its significant role in disease prevention and progression. The epigenetic effects of vitamin D in different MS conditions and interaction with treatments will be an important avenue for future investigation.

In addition, little is known on the effect of the currently available disease modifying drugs on miRNA levels, and other protein markers. Determining the effects of those medications on the inflammatory markers and MS-related cytokines at a molecular level and its interaction with other environmental factors such as exercise and smoking is also crucial.

Five-year view

Identifying biomarkers for more accurate diagnosis and monitoring of disease progression is the goal of genomics and proteomics research in MS. MS is a complex disease characterized by an extensive phenotypic variability. Despite determining and characterizing all possible MS-related biomarkers and mechanisms is an extremely difficult task, the advancement of technology has made it possible. In the coming five years, valuable information about pathogenesis and regulation of disease course will come from the elucidation of biomarker release patterns as a function of specific pathogenic pathways and disease processes. This knowledge might be translated into novel opportunities for targeted therapeutic interventions to prevent or reduce progressive neurological dysfunction and disability in patients with MS. It is also likely that rigorously validated markers that provide objective criteria for separating clinical phenotypes, assessing disease activity or predicting disease progression will be integrated into routine clinical practice and will be part of revised diagnostic criteria for MS.

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Key issues

- Multiple sclerosis (MS) is a complex disease characterized by an extensive phenotypic variability that poses difficult diagnostic, prognostic and therapeutic challenges to the clinician.
- An urgent need exists for new biomarkers to capture the different aspects of disease heterogeneity, help diagnosis and monitoring of disease activity and progression, as well as provide insights into MS disease etiopathogenesis and guide treatment.
- The biomarker research field is very active in MS. Several recent studies have suggested candidate biomarkers that can distinguish among different MS phenotypes, better characterize disease course and activity, and potentially predict clinical disease progression and treatment response. Yet, there is insufficient evidence for integrating them into routine clinical practice.
- The use of biomarkers closely associated with disease pathophysiology and reflecting distinct pathogenic mechanisms could fill the gap of knowledge in understanding the biological and molecular basis of disease potentially leading to a more effective targeted therapy and allowing to bring personalized medicine to MS patients.
- Although significant progress has been made in the area of biomarkers in MS, rigorous clinical validation studies are crucial to move forward on the path from biomarker discovery to clinical application, and are a high research priority in MS.

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