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REVIEW ARTICLE

Biomarkers of Multiple Sclerosis

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Abstract: The search for an ideal multiple sclerosis biomarker with good diagnostic value, prognostic reference and an impact on clinical outcome has yet to be realized and is still ongoing. The aim of this review is to establish an overview of the frequent biomarkers for multiple sclerosis that exist to date. The review summarizes the results obtained from electronic databases, as well as thorough manual searches. In this review the sources and methods of biomarkers extraction are described; in addition to the description of each biomarker, determination of the prognostic, diagnostic, disease monitoring and treatment response values besides clinical impact they might possess. We divided the biomarkers into three categories according to the achievement method: laboratory markers, genetic-immunogenetic markers and imaging markers. We have found two biomarkers at the time being considered the gold standard for MS diagnostics. Unfortunately, there does not exist a single solitary marker being able to present reliable diagnostic value, prognostic value, high sensitivity and specificity as well as clinical impact. We need more studies to find the best biomarker for MS.

Keywords: Multiple sclerosis, Imaging, Genetic-immunogenetic, Biomarkers, Magnetic resonance, Laboratory.

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1. INTRODUCTION

Multiple Sclerosis (MS) is one of the most common causes of neurological diseases among adolescents and young adults [1]. MS is an immune-mediated inflammatory disease, which assaults myelinated axons in the Central Nervous System (CNS), thereby breaking the myelin sheaths and the axons in variable degrees [2]. The cause of MS is still, after a century of studies, unknown; however, it is likely that many factors act in concurrence to trigger the disease. The general consensus is that MS manifests when an environmental agent (*e.g.*, viral, bacterial infections, smoking, diet, vitamin-D deficiency or an exposure to chemicals) acts together with a genetic susceptibility to immune dysfunction [3].

While the etiology of MS is yet to be entirely uncovered, it is generally accepted that the first step in the disease progression is the breakdown of the blood-brain barrier (BBB). This breakdown of the BBB then allows autoreactive autoimmune and a cohort of inflammatory cells to enter the CNS leading to a cascade of myriad neurodegenerative events eventually manifesting as typical plaque lesions as well as clinical symptoms seen in MS [4].

The clinical course can vary from patient to patient in many diversifying manners, which demonstrates the complexity of the pathophysiology. The clinical picture is ever-elusive as the mechanisms of axonal neurodegeneration, inflammatory demyelination, gliosis and re-myelination repair fuse to form different clinical pictures for each patient [5]. The disease onset and progression are influenced by idiosyncratic factors. Identifying the biomarkers of these factors serves as an essential first step in determining the clinical impact they might have on the disease prognosis and treatment [2]. We will review the materials from which the biomarkers are extracted focusing mainly on the serum, cerebrospinal fluid, imaging and genetic-immunogenetic biomarkers. The focus will be on describing the biomarkers and identifying the benefits as well as the clinical relevance of each biomarker.

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2. MATERIALS AND METHODS

This review is based on the scientific articles found in validated sources such as PubMed, The National Centre for Biotechnology Information (NCBI) and the published books. It is comprised of publications dating from 2000 up to the present day, which deal with MS and biomarkers in general.

3. RESULTS AND ANALYSIS

3.1. Definition of a Biomarker

The Biomarkers, EndpointS and other Tools (BEST) from

the National Institutes of Health (NIH) defines a biomarker as a "defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions." The types of biomarkers are molecular, histologic, radiographic, or physiologic characteristics. "A biomarker is not an assessment of how an individual feels, functions, or survives." BEST offers seven biomarker categories - susceptibility/risk, diagnostic, monitoring, prognostic, predictive, pharmacodynamic/response, and safety [6]. It is slightly different from the World Health Organization definition who defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [7].

3.2. Materials of the Biomarkers Extraction

The biomarkers can be extracted from blood, tears, urine, saliva, Cerebrospinal Fluid (CSF), genes, immunity factors and additionally interpreted *via* imaging techniques [2]. The methods used vary depending on the substance or object of measurement focus. Blood is the easiest and most available source in terms of infection control, excluding saliva, tears and urine. The limitations of samples gathered from blood come from concomitant conditions affecting serums substrate levels such as kidney functions, liver functions and infections [2]. Additionally, circadian rhythm has been shown to cause fluctuations in serum biomarker level [8, 9]. Cerebrospinal Fluid (CSF) is unbeatable in providing biomarkers, as its connection to the CNS is indisputable. Neither CSF is affected by liver or kidney functions [2]. The downside in the CSF is the invasiveness of the sample collection procedure and the reported fluctuations in the circadian rhythm. It has been hypothesized that a sample collection during morning hours after night fasting is the most optimal time [10, 11].

To extract biomarkers, excretory sources - urine, saliva and tears are analysed. Urine is the easiest to collect, usually 24-hour urine collection is taken. Only bacterial infections may distort a sample biomarker levels [2]. Saliva is used mainly for soluble Human Leukocyte Antigen (HLA) type II [12]. Oligoclonal Bands (OCBs) have been measured from tears, with similar results to those of CSF [13]. Within imaging techniques, magnetic resonance imaging (MRI) is still considered as the golden standard when it comes to biomarkers in MS [2]. However, there are more novel and advanced imaging techniques being developed that will be dealt with further below.

3.3. Methods and Techniques of the Biomarkers Extraction

3.3.1. Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISA) as a solid-phase technique may be classified into two main types: (1) competitive assays using either an antigen-enzyme conjugate or an antibody-enzyme conjugate, and (2) non-competitive assays using a double antibody "sandwich" technique where the second antibody has an indicator enzyme conjugated to it [14]. ELISA can be performed with every substance that yields antibodies or antigens.

3.3.2. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) provides the researcher with the ability to detect genetic information. MS has a significant genetic component, which can be detected using PCR (such as HLA-DRB1). Basic components of the method are primers (short DNA fragments sequences complementary to the target area), as well as a DNA polymerase, which enhances the replication. Quantitative PCR methods include competitive, non-competitive, and real-time PCR [2].

3.3.3. Immunofluorescence

Immunofluorescence is a technique that uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets within a cell, and therefore allows visualization of the target molecule distribution through the sample. The specific region an antibody recognizes on an antigen is called an epitope. There are two types of immunofluorescence direct and indirect, which refers to the use of one or two antibodies respectively. This is a very specific method that enables the possibility to detect singular haptens, antibodies and proteins of different sizes *in vivo* [2].

3.3.4. Flow Cytometry

This is a laser- or impedance-based method where target cells are obtained *via* cell culture or tissue sample and then suspended in the tubes or microtiter plates. The samples are then dyed by fluorescent-labelled antibodies detecting biomarkers in the form of surface antigens, DNA and RNA variations, protein expression, enzymes, intracellular antigens and then passed through a focused light source (laser) where sensors detect the types of light that are refracted or emitted from the cells. The data is then compiled to build a comprehensive picture of the sample [2].

3.3.5. Western Blotting

The term refers to a protein detection method, where samples are taken from tissues or cells and then undergo cell lysis to extract proteins. The mixture of proteins is then separated based on molecular weight and then by type through gel electrophoresis. There are also colorimetric and fluorescent detection techniques in conjunction with electrophoresis that have been developed [2, 15].

3.3.6. Isoelectric Focusing

Isoelectric Focusing (IEF) is a technique of impeccable resolution and high sensitivity, which gives it greater discrimination between different biomarkers, immunoglobulins and proteins. As with western blotting, the basic principle behind IEF is gel electrophoresis, however in IEF the proteins move towards their isoelectric PH points [2, 16].

3.3.7. Omics Technologies

"Omics" refers to new arising technologies that make it possible to do empirical analysis and identification of biomarkers in several planes of cell biology, such as DNA, RNA, lipids, proteins, metabolites and epigenetic modifications [2]. The omics can be categorized further in: epigenomics: investigates the possible effect that changes in chromatin structure can have in MS prevalence [17]; proteomics: delves

into protein distribution.; genomics: investigates the entire DNA sequence; transcriptomics: investigates RNA sequences. There are two main types in use, next generation sequencing and microarrays [18]; lipodomics: investigates specific CNS lipid epitopes and cellular lipid pathways [19]; metabolomics: investigates the significance of metabolic pathways in MS pathogenesis.

3.4. Classification of MS Biomarkers

Various articles divide the biomarkers into different groupings, for example, Paul *et al*. recommends a three-group classification of MS biomarkers - diagnostic, disease activity, and treatment response biomarker [20]. In this paper, we classify the biomarkers in three distinct categories: laboratory markers - this encompasses bodily fluids; imaging markers - biomarkers achieved by imaging technologies; genetic-immunogenetic markers - biomarkers related to genetics and immunogenetic. These three categories include risk, diagnostic, monitoring, prognostic, predictive, response and safety biomarkers. Further we will provide a more detailed review of each biomarker.

3.4.1. Laboratory biomarkers

3.4.1.1. Biomarkers of Immunological Activation

These biomarkers can be extracted from all bodily fluids. However, the best results are usually achieved from the serum or CSF samples depending on the biomarker. Due to the nature of immunological biomarkers, immunofluorescence or ELISA is the golden standard method for the evaluation.

3.4.1.2. Oligoclonal Band Immunoglobulin G and Immunoglobulin M in the Cerebrospinal Fluid

Oligoclonal bands are IgG class antibodies that are synthesized intrathecally and are evidence of inflammatory events in the central nervous system [21, 22]. Advancement risk to Clinically Definite Multiple Sclerosis (CDMS) was discovered to double when Oligoclonal Band (OCB) immunoglobulin G (IgG) was affirmed in the CSF of patients with Clinically Isolated Syndrome (CIS) [23]. Other studies also found consistency with positive OCB IgG finding relation into CDMS progression [2, 24]. Karussis' [25] study reveals that more than 2 ”OCBs in the CSF have a positive predictive value of 97%, a negative predictive value of 84%, a sensitivity of 91%, and a specificity of 94% for developing relapsing remitting MS after a CIS.” They talk about that presence of OCBs within 3 months of CIS nearly doubled the risk of a second clinical attack over 50 months. The diagnostic value of OCB IgG is undisputed in its sensitivity (>90%), however what it gains in sensitivity it lacks in specificity and OCB IgG can be found in other inflammatory disorders of the CNS (~35%) [2]. In the revised McDonald criterions OCB seems to Disseminate In Time (DIT) and allows for patients with clinical isolated syndrome making a diagnosis of multiple sclerosis. Mantero *et al*, in his study reported that “OCBs reassume now a more relevant role in the MS workup and OCBs can be viewed as substitution for the DIT requirement. Its prognostic value remains undiscussed.” [26]. OCBs are the best biological markers to predict conversion to MS, the above-mentioned study says.

OCB Immunoglobulin M (IgM) in the CSF has not gained the same consideration by researchers as IgG, as it has been found to correlate badly with MS progression [27]. Another study revealed that the data offered no support for the concept that the presence of OCB IgM in the CSF might predict an unfavourable course in MS [28]. One study however has found data which successfully corroborate that OCB IgM against myelin lipids can predict an aggressive MS course and demonstrate that OCB IgM that do not recognize myelin lipids represent a more benign disease course with a transient immune response [29].

OCBs are extracted from the CSF then evaluated by immunofluorescence and cross-referenced with imaging techniques to determine correlation disease activity.

3.4.1.3. Measles-Rubella-Zoster Endothecal Reaction

Compared to OCB IgG the Measles-Rubella-Zoster (MRZ) IgG reaction showed improved specificity for MS diagnosis as well as prognostic value for progression from CIS to CDMS [30]. Brettschneider *et al*. in their study found that MRZR and MRZS could be used as a predictive marker to patients with CIS together with lesions in MRI [31]. They indicate that CIS patients having two or more T2-hyperintense lesions in MRI and a positive MRZS are at highest risk to develop MS and should therefore be candidates for an early beginning of an immunomodulatory therapy. Positive predictive value (PPV) could be increased to 91% by combination of MRZS with MRI (>2 lesions) or by combination of MRZS with MRI (>2 lesions) and OCBs. However, this finding was rarely evaluated systematically because of lack of CSF approved assays that would allow a routine application, which is not restricted to special laboratories. In the studies, positive MRZ reaction patients with MS and negative OCBs have been found [32]. In the recent study Hottenrott *et al*. found MRZ reaction -2 specificity to MS and suggests that this could be a useful diagnostic biomarker for distinguishing from other inflammatory neurological diseases [33]. They found that MRZR is less frequent in other inflammatory CNS diseases than in MS. Additionally, the MRZ reaction implies a immune response that is mostly B-cell mediated [34]. This can guide the therapeutically choice towards a suitable immunomodulating agent [2]. The IgG for MRZ reaction were isolated from the CSF of the patients and then evaluated *via* ELISA.

3.4.1.4. Epstein-Barr Virus Reaction

Epstein-Barr Virus (EBV) causes infectious mononucleosis and increases the risk of developing MS. EBV may also contribute to MS pathogenesis indirectly by activating silent human endogenous retrovirus-W [35]. High level of IgG antibodies against the Epstein-Barr Viral (EBV) protein epitopes BRRF2 and EBNA-1 in the CSF and serum samples of MS patients has been reported [36]. Another study also managed to isolate exceedingly specific T-cells for epitope EBNA-1 from MS patients [37]. Furthermore one study showed breach of the Blood-brain Barrier (BBB) endothelial cells with subsequent entry of autoreactive T-cells leading to immune cell adherence which is a decisive step in MS pathogenesis [38]. Thus EBV antibodies may be associated with increased inflammatory activity in MS patients [39] and

its potential interaction with both genetic and other environmental factors to increase susceptibility and disease severity of MS [35]. In studies, we don`t find that EBV could be used as a biomarker in MS, it is more like a risk factor. Anti-EBV IgG, IgM and Anti-EBNA-1 IgG are extracted from the blood and CSF by ELISA, in turn EBV DNA by PCR.

3.4.1.5. Kappa Free and Lambda Free Light Chains in Cerebrospinal Fluid

Kappa free and Lambda free light chains are B lymphocytes produced proteins during the process of antibody synthesis [40]. Those can be detected in the blood and CSF. Increased amounts of Kappa Free Light Chains (kFLC) in the CSF of MS patients has been frequently reported [2]. When compared with OCBs IgG, kFLC showed better sensitivity (96%) while the specificity was lower (86%) for MS patients [41]. Villar *et al*., found that high kFLC in the CSF accurately predicts CIS conversion to MS [42]. Nazarov *et al*. showed an association between kFLC and the degree of irreversible disability in MS patients. The authors showed that MS patients with high level of kFLC reached disability faster than patients who had low kFLC level, suggesting that it can be a good prognostic marker in MS [43]. Lambda Free Light Chain (λFLC) was found to be sensitive in detection of intrathecal immunoglobulin synthesis for inflammatory CNS disorders [44]. At present, Kappa free and Lambda free light chains clinical utilization is limited by analytical factors, the absence of reference values and clinically-validated cut-off [43]. Kappa free and Lambda free light chains extracted by ELISA.

3.4.1.6. Antibodies Against Myelin Basic Protein and Myelin Oligodendrocyte Glycoprotein

Myelin Oligodendrocyte Glycoproteins (MOG) and Myelin Basic Proteins (MBP) are glycoproteins expressed on the outer membrane of myelin, involved in maintaining myelin structure, and found within the central nervous system. A meta-study found that MOG and MBP prognostic and diagnostic relevance in MS is decidedly controversial. Much of the controversy can be associated with the methodical variation between studies [2]. Berger *et al*., 2003 found that MOG and MBP antibodies would serve as good predictive values for CIS conversion in to CDMS [25, 45]. Controversially, Kuhle *et al*., 2007 found that there was no correlation between anti-MOG and anti-MBP IgM or IgG antibodies and progression to CDMS [46]. In the latest studies, it has been found that Anti-MOG positive patients with MS may have a higher risk of progressive disease, high relapses rates and need for escalated therapy. MS patients with positive Anti-MOG observed severe spinal cord and brainstem involvement. Anti-MOG has its own disease phenotype and more related studies are needed. In studies, it is recommended to determine Anti-MOG at the disease onset and in the later stages of the disease because it can fluctuate and indicate on the disease course [47]. At the time, Anti-MOG is a biomarker of MOG-associated encephalomyelitis [43]. Paul *et al*. not found utility measuring MBP level in the CSF [20]. We need more studies about Anti-MOG and MS; thus maybe in the future it could be looked upon as the disease predictive biomarker. Anti-MOG is extracted by flow cytometry.

3.4.1.7. Cytokines

Active demyelination causes an inflammatory response which releases a multitude of cytokines that can serve as the biomarkers of MS disease activity [2]. Proinflammatory cytokines in the periphery mainly come from T- and B-cells. While in Relapsing Remitting MS (RRMS) the B-cells appear to be primarily accountable for intrathecal generation of cytokines, monocytes adapt a more immunoregulatory aspect in the CSF [48]. IL-6 acts as a connecting link between T-cell and B-cell immune response along with the Th-17 response triggering factor. The IL-6 levels in the serum correlated notably with relapse frequency [49].

Recent research has indicated that a Single Nucleotide Polymorphism (SNP) at -592 position of the main anti-inflammatory cytokine IL-10, produced by several immune cells, affects the regulation of the CNS autoimmunity in MS patients among others [50]. Additionally, two studies have shown MS patients to overexpress IL-15 in the CSF and serum [51, 52]. Dimisianos *et al*. [53] in their study about "Cytokines as biomarkers of treatment response to IFNb in relapsing-remitting multiple sclerosis" found that IL-17A could be a treatment response biomarker. According to the above-mentioned study, high serum baseline level of IL-17A has a good treatment response to IFNb. They express the view that it is associated with IFNb effect on the IL-17A reduction. In their study, they found that a patient with EDSS < 3 and disease duration of 10 years without treatment, has low level of all serum proinflammatory cytokines and few gadolinium enhancement lesion on MRI and low level of inflammatory cells in the CSF.

Mouzaki *et al*. [54] in their study investigated signature cytokines in MS patient that can distinguish from other inflammatory CNS diseases. They found the parameter distinguishing multiple sclerosis patient from other CNS inflammatory diseases - IgG intrathecal synthesis, IgG index and IL-4 level in the CSF. They did not find statistically significant differences between age and sex in MS and other inflammatory diseases. Cytokines level are higher in the serum than in the CSF, with one exception of CSF IL-6 levels. The cytokines could be extracted by ELISA.

3.4.1.8. Chemokines

CXCL13 is a chemokine that activates B-cells and T-helper cells through interaction with CXCR5 receptors towards demyelination lesions [55, 56]. Increased level of CXCL13 has been found in both CIS and CDMS patients [56] while Khademi *et al*. came to the conclusion that CXCL13 at higher level is predicted CIS conversation to MS [57]. Karussis reveals that CXCL13 could be the best positive predictive value to CIS conversion to CDMS combining it with MRI [25]. Ferraro *et al*. in their study found correlation between CXCL13 level in the CSF and cell count, total protein, IgG index, and OCBs patient with CIS and they are at level that could be a good positive predictive value and specificity for MS diagnosis [58]. Another chemokine, CXCL12 has shown to possess a protective aspect versus CNS inflammation in Experimental Autoimmune Encephalomyelitis (EAE), however the data are

still in experimental stages [59]. CXCL13 from the CSF extracted by ELISA.

3.4.1.9. Adhesion Molecules and Osteopontin

The elevated level of soluble intercellular adhesion molecules (sICAMs) in the CSF are caused by proinflammatory cytokines. Increased MS activity has been reported in conjunction with elevated ICAM-1 molecule levels [60]. Osteopontin is a phosphoprotein derived from macrophages which amplifies IL-12 and INF-γ levels while diminishing the levels of neuroprotective IL-10 [2]. Osteopontin levels in the serum and CSF have been reportedly elevated in an active MS relapse episode [61]. ELISA is used as an extraction method.

3.4.1.10. Neurofilament Light Chain

The Neurofilament Light Chain (NfL) is a major structural protein that occurs exclusively in neurons, myelinated axons. NfL chains level increases in axonal damage. It shows axonal damage in MS patients in the early stage of MS. It may be increased in patients with other neurodegenerative diseases too, like Alzheimer`s disease. It can be determined in the blood and in the cerebrospinal fluid, last studies have shown that the blood NfL measured with a highly Sensitive Single Molecule Array (SIMOA) are strongly correlated with NfL in the CSF of patients with MS [62, 63]. That allows to take a blood sample and detect NfL in an easier way. NfL chains level increases in all stages of MS and correlates with clinical expression and MRI findings. Moreover, it is a promising marker in the future as the disease activity, predictive marker and treatment response marker. It is confirmed also by Karussis [25] in his study. Where it says that NfL are potential CSF biomarkers for disease progression, as their presence at high levels may reflect acute axonal damage and imply a prognostic value for conversion from CIS to CDMS. But we need more long-term cohorts to understand how it works in long term [64 - 66].

3.4.1.11. Vascular Endothelial Growth Factor-A

The Vascular Endothelial Growth Factor-A (VEGF-A) possesses neuroprotective properties [2], VEGF-A is extracted by flow cytometry. Reduced m-RNA expression of VEGF-A in monocytes of the serum from patients with Secondary Progressive MS (SPMS) in comparison to RRMS patients were found to have, thus setting VEGF-A up as possible biomarker for RRMS progression to SPMS [67].

3.4.1.12. Vitamin D

Vitamin D is derived from cholesterol, and has an immunomodulatory function controlling the transcription of a lot of genes relating to immunity. Vitamin D levels measured by a high-performance liquid chromatography. Many epidemiological studies have found correlation with latitude from the equator and level of sun exposure and higher relative MS occurrence risk. Vitamin D can be considered a neuroprotective agent as it activates many neurotrophic factors and suppresses Th-1 immune response in many ways [2]. Another study found that vitamin D intake has a protective effect on MS risk [68]. Ramagopalan *et al* [69] found a direct link between vitamin D and HLA-DRB1*15 gene expression. They found a VDRE on the HLA-DRB1*15 gene promoter and showed VDR binding *in vitro* (EMSA) and *ex-vivo* (ChiP). The authors proposed that a lack of vitamin D in utero or early childhood can affect the expression of HLA-DRB1 in the thymus, impacting on central deletion. For MS, in HLA-DRB1*15 bearing individuals, a lack of vitamin D during early life could allow auto reactive T cells to escape thyme deletion and thus increase autoimmune disease risk. To note, antigen presentation in the thymus of VDR knock-out mice is impaired. As we know natural killer T cells have an important role in immune regulation and Vitamin D regulates T cell response but not T cell development. Yu *et al*. [70] in their study found that VDR has an important role on V14 invariant NKT (iNKT) cells development and if VDR are absent it is results an iNKT cells diminished in the thymus and the periphery. Mowry *et al*. in their study found that low D vitamin level has association with MS activity in the MRI - new lesions and gadolinium-enhancing lesions on the brain MRI [71]. In another study, Mowry *et al*. found that vitamin D could reduce neurodegeneration after CIS and could reduce long-term disability in MS patients [72]. Vitamin D with its neuroprotective feature can be used as a part of the treatment and maybe in the future it could be as a predictive biomarker. In this case, we need more studies.

3.4.1.13. B-cells

A study investigated the difference in inflammatory response between CIS and different stage MS patients and found that mature B-cell as well as plasma-blast levels were elevated in the CSF of both CIS and RRMS patients, correlating positively with increased disease activity [73]. Intrathecal production of centroblasts, a B-cell subset found exclusively in secondary lymphoid organs, in the CSF of MS patients has also been reported [74]. B cells have a significant role in MS pathogenesis and the latest studies are focused with the B cells role in the treatment [75].

3.4.1.14. T-cells

T-cells use the CXCR3 cytokine receptor to enter the CNS. CXCR3 however has a weak specificity value for MS because of its prominence in other inflammatory disorders [76]. The T-cells and specifically CD4(+) CD28(-) cells were shown to migrate and accumulate in the CNS lesions of MS. The migration occurred in response to a chemotactic gradient of fractalkine, where they then demonstrated cytotoxic effects in the target tissue contributing to the inflammatory process of MS [77]. T cells have a phenotypic and transcription signature of myelin-reactive T cells in MS patients and may act as MS progression and pathogenesis [78], but to use it as a biomarker is still debatable.

3.4.1.15. Natural Killer Cells

Natural Killer (NK) cell surface antigen CD-56 was found to exist in high levels in the RRMS patients at remission. A recent study has successfully linked NK cell-mediated negative immunoregulation of activated T cells in MS [79].

3.4.1.16. T-cell Receptor Excision Circles

They are intracellular side products of T-cell receptor remodelling. The presence of T-cell Receptor Excision Circles (TRECs) inside a T-cell has been found to be a good indicator of T-cell naivety. This comes in useful when measuring the functional state of the thyroid gland, which can be evaluated by the percentage of naive T-cells circulating in peripheral blood. Decreased levels of TRECs in MS patients have been observed indicating deteriorated thyroid function in the disease. Naive T-cells were found to be increasingly decreased in patients with Primary Progressive Multiple Sclerosis (PPMS), opposed to RRMS [2].

3.4.1.17. Lipocalin 2

Lipocalins are proteins which transport tiny hydrophobic molecules and are thereby involved in many processes of the immune system. The gene encoding Lipocalin 2 was found upregulated in relapses of Experimental Autoimmune Encephalomyelitis (EAE) mouse model of MS, primarily arising from neutrophils infiltrating the choroid plexus, as well as astrocytes in affected regions. Additionally, increased levels of lipocalin 2 from the CSF were found in two separate MS cohorts. Marques *et al*. in their study found that lipocalin 2 levels in the cerebrospinal fluid coincided with the active phases of the disease. The increase of lipocalin 2 in the cerebrospinal fluid was reverted by immunomodulatory therapy. They mention that lipocalin 2 could be a diagnostic or monitoring biomarker [80].

3.4.1.18. Matrix Metalloproteinase Proteins

In primary progressive MS serum and CSF Matrix Metalloproteinase Protein (MMP) levels are constantly increased, specifically MMP-9 which were elevated even in patients with RRMS [81]. Autoimmune CCR2(+) CCR5(+) CCR6(-) Th1 cells play a crucial role in the pathogenesis of MS. The CCR2(+) CCR5(+) T cells constitute a unique population selectively enriched in the cerebrospinal fluid of MS patients during relapse. CCR2(+) CCR5(+) T cells exhibited a distinct ability to produce matrix metalloproteinase-9 and osteopontin, which are involved in the CNS pathology of MS [2, 82].

3.4.1.19. Ninjurin - 1

Ninjurin - 1 expression by endothelial cells of the blood brain barrier (BBB) and myeloid antigen-presenting cells (APCs) has an essential role in the transmigration and localization of the APCs inside the CNS, as proven by proteomic screening of human BBB cells. These APCs along with activated microglia are thought to be pivotal in the initiation of the Central Nervous System (CNS) - targeted immune response in MS and EAE. Ninjurin - 1 levels were found to be increased in active demyelinating lesions, while Ninjurin - 1 neutralization was shown to decrease migration of APCs across the BBB. Finally, complete blockade of Ninjurin - 1 reduced clinical MS activity and histopathological indices of EAE as well as decreased infiltration of macrophages, dendritic cells and APCs into the central nervous system [2, 83].

3.4.2. Imaging Biomarkers

3.4.2.1. Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) is an essential tool for MS diagnostics, as well as for monitoring treatment and disease activity. MRI gives the clinician a vast array of neuroinflammatory biomarkers to work with. However, typical MRI approach methods lack in proper correlation with MS disability advancement and signs of neurodegeneration [2]. MRI has great opportunities with different sequences.

T1 lesions with contrast enhancement: biomarkers of acute neuroinflammation. T1 lesions are thought as the gold standard for the blood brain barrier disruption imaging. Several recent studies have indicated that the same diagnosis could be reached in several instances without contrast enhancement, but with combinations of T1, T2 and T2-weighted FLAIR imaging characteristics alone [84].

MS lesions typically appear in areas of high signal on T2 weighted MRI. However, a proportion of these lesions, when viewed on T1 weighted MRI, appear hypointense compared with the surrounding white matter. Hyperintense T2-weighted lesions, which reflect a multitude of mechanisms such as inflammation, demyelination, axonal damage and edema. The diagnostic significance is high, but the correlation with disability is moderate [85]. Hypointense T1-weighted lesions (also known as black holes), while they are considered as moderate biomarkers for axonal damage, their correlation with MS disability is debatable [86, 87]. Karussis' [25] study revealed that the presence of at least one cortical lesion in CIS may help identify a high-risk patient with conversion to clinically definite MS.

The most broadly used whole brain atrophy biomarker is the brain parenchymal fraction. When untreated MS patients were compared with a healthy control group the brain atrophy rates were found to be much higher among the former [88]. The worsening rate at initial diagnosis has been proposed as a prognostic biomarker for disability progression [89]. Sormani et el. found that the brain atrophy correlated with the disease disability and treatment efficacy [90].

Recent longitudinal study found that the grey matter atrophy rates could serve as reliable biomarkers in all forms of MS. Double inversion recovery techniques displayed the grey matter demyelination, axonal damage and atrophy, with increased decline rates in SPMS [91]. Another study found that increased decline rates of the grey matter atrophy in clinically isolated syndrome patients correlated well with aggressive conversion to RRMS, thus validating the grey matter as prospective biomarker [92]. The studies disclose the correlation between the grey matter atrophy and clinical disability, cognitive dysfunction that may be useful predictive marker for severity of the disease [91, 93, 94].

The spinal cord atrophy biomarkers focusing on upper cervical cord area imaging techniques have shown apparent atrophy in progressive forms of MS, which have correlated well with disability progression. Upper cervical cord area atrophy in the early stages of RRMS has been regarded as bad prognostic biomarker of future disability [95].

3.4.2.2. Contrast Magnetization Transfer Ratio

Magnetization Transfer Ratio (MTR) is a sensitive parameter to quantify the integrity of myelinated white matter in patients with multiple sclerosis. This is a novel MRI method that is based on the interaction between protons, free water and macromolecules. In the absence of axonal loss, acute MRI lesions, which display improvement, similarly increase in MTR. MTR therefore might prove a viable outcome measure to assess the effect of remyelinating agents [96]. Another study suggests that MTR should be used mainly as an axonal damage biomarker. As seen by their results, regarding optic nerve MTR reduction after optic neuritis showed good correlation with Retinal Nerve Fibre Layer (RNFL) thickness [97]. Yet, decisive assessment of treatment outcomes on re-myelination has been shown [98]. MTR has been an effective monitoring tool for pathology in MS.

3.4.2.3. Diffusion Weighted Imaging and Diffusion Tensor Imaging Techniques

Diffusion Weighted Imaging (DWI) method is grounded on the mobility and spatial distribution of water molecules, while Diffusion Tensor Imaging (DTI) measures movement in different directions in the space. DTI method gives four measures, Axial Diffusivity (AD), Radial Diffusivity (RD), Mean Diffusivity (MD) and Fractional Anisotropy (FA) [2]. Axial diffusivity indicates loss of axons, radial diffusivity is related to demyelination, mean diffusivity is average diffusion and fractional anisotropy integrate AD and RD [99, 100].

In hyperintense T2-weighted lesions we can observe an increase in MD and decrease in FA. This type of phenomena can be seen in Normal Appearing White Matter (NAWM) areas in regular MRI, likewise for Normal Appearing Grey Matter (NAGM) areas, more notably on progressive forms of MS [101]. Early MS stages show *corpus callosum* changes in DTI, while regular MRI lesions are still not even present [102]. MD changes could be used as a dependable biomarker for MS relapse as they precede typical MRI blood brain barrier damage by a minimum of 5 months [103]. *Corpus callosum* DTI changes in secondary progressive MS have been reported as a bad prognostic biomarker of eventual disability [104].

3.4.2.4. Magnetic Resonance Spectroscopy

In Magnetic Resonance Spectroscopy (MRS) it is possible to measure cellular metabolism in the CNS, which is important in MS and can`t be detected with standard MRI. In MRS usually detect N-acetyl aspartate, choline, creatine, myoinositol, glutamate, glutamine and lactate, which are in the brain and the spinal cord, in small amounts. Neuronal dysfunction is related to a low level of N-acetyl aspartate. Different studies show different points of view - NAA deficits do not correlate significantly with EDSS others found NAA correlation with EDSS. Reduction of N-acetyl aspartate level indicates reduction of oedema at active lessons in the CNS. During active demyelination, the level of choline is elevated; it could be detected up to 12 months prior to the white matter lesion formation. Glutamate converted to glutamine and Gamma-Amino Butyric Acid (GABA). In studies, it was found that the GABA levels are reduced in the hippocampus and sensorimotor cortex for patients with SPMS. MRS takes a longer time to do compared to routine MRI, and MRS has a reduced spatial resolution. Due to the mentioned characteristics, it is not suitable for the clinical practice [105].

3.4.2.5. Optical Coherence Tomography

Optical Coherence Tomography (OCT) is a non-invasive method of RNFL thickness estimation. It is done by means of infrared light emission through the pupil and detecting the reflection from retina. RNFL thickness reduction, aka thinning, can be used as a biomarker for axonal loss, which correlates well with the brain atrophy levels as was discussed above [106]. Another study has suggested the use of RNFL thickness as a biomarker for MS disease progression when used in conjunction with MRI techniques [107]. In the last studies, was found thicker RNFL associated with the disease activity in MS, and increased risk of disability. OCT could be used as predictor biomarker of the disease progression [108, 109].

3.4.3. Genetic - Immunogenetic Biomarkers

3.4.3.1. Human Leukocyte Antigen

Genetic risk in human leukocyte antigen (HLA) class II antigen polymorphisms seem to be a decisive factor in attributing the genetic burden for MS. Initial studies have found positive interaction between DRB1*1501-DRB5*0101-DQA1*0102-DQB1*0602 haplotypes and disease occurrence. Numerous late researches done in MS cohorts have come to the conclusion that HLA-DRB1*1501 is the allele primarily responsible for the genetic risk in MS [110 - 112]. Furthemore, HLA-DRB1*1501 expression is regulated up to a degree by vitamin D through interaction at a genomic level, which explains the known connection in latitude and MS prevalence. Moreover, the coexistence of some alleles likely precipitates augmentation of the comprehensive risk by epistatic mechanisms [113]. Additionally, there has been documentation of association with different HLA loci in other studies, like DR3 and DR4 haplotypes [114] and DRB1*04 Hutterite families [115], which further demonstrate the diversity of MS disease. Haplotype DRB1*1303-DQA1*05-DQB1*030 was found to have a positive association with MS among some Jewish sub-populations.

Confirmatory correlation of HLA-DRB1*1501 allele with OCB in the CSF of MS patients has been found by observations in Asian cohort and was confirmed by successive research efforts [116].

Clinical and imaging correlations of HLA-DRB1*15 alleles were found with early onset MS [117]. One study observed MS patients and found those with positive HLA-DRB1*1501 to have larger T1 lesion burden in MRI and lowest brain atrophy scores [118]. Another study reported HLA-DRB1*15 positive MS patients to have larger white matter lesions, lower brain atrophy scores as well as declined cognitive function and decreased N-acetyl-aspartate (NAA) levels alongside with NAWM [119]. Correlation with RRMS and SPMS is still to be fully confirmed [120, 121].

3.4.3.2. Transducer of ERBB2-1 and Apolipoprotein E

Transducer of ERBB2-1 (TOB-1) gene plays an important role versus T-cell replication by holding autoreactive cells in an inert state. Decreased TOB-1 expression gives rise to a more intensified immune response, with increased Th1 and Th17 cells and further decreased T-regulatory cells. TOB-1 polymorphisms express an autonomous factor affecting the advancement from CIS to CDMS [122]. Apolipoprotein E (ApoE) is a protein found mainly in astrocytes, with the function of regulating lipid homeostasis. MS patients found carrying the $\varepsilon 4$ allele of ApoE have been attributed on having higher risk of developing psychic disorders. ApoE role as a biomarker for MS showed promising future prospect for a high specificity and sensitivity in animal models of EAE, further study is however required [123].

4. DISCUSSION

Multiple sclerosis is a diverse disease with many different forms manifested in a wide variety of ways from patient to patient. Though it is the most common neurodegenerative disorder among adolescents and young adults, there still are many unanswered questions revolving around it, despite extensive research spanning multiple decades. From the etiology to pathogenesis to effective diagnostic methods MS continues to elude humanity's best efforts in unveiling its secrets. This leads inevitably to a great amount of potential theories and findings in each area, as seen in the above analysis. The biomarkers described above represent the most existing biomarkers for MS. We divided the biomarkers into three subgroups - laboratorial, imaging and genetic-immunogenetic biomarkers, which include risk, diagnostic, monitoring, prognostic, predictive, response and safety biomarkers. Very important task is to find the best biomarker for MS.

The laboratorial biomarkers are by far the most diverse and extensive subgroup of all available biomarkers. They could be affected by circadian fluctuation but, unfortunately, there are not enough studies where circadian fluctuation would have proven its essential role. There are some recommendations on when certain samples should be collected from the CSF or serum. Additionally, the research findings on the effects of circadian rhythm on biomarker levels were hesitantly inconclusive at times due to lack of proper guidelines. Therefore, it is only logical to deem the creation of a standard "time" which should be upheld by future measurements. This would create solid reliable results, which would not be hampered by diversity or fluctuations of circadian rhythm, thus removing the problem. Further studies are required.

We have lot of biomarkers in MS but the highest diagnostic value in laboratorial biomarkers was seen in OCBs, CSF MRZ reaction, NfL and kFLC. The OCBs was found by multiple studies to be a reliable biomarker for transition from CIS to CDMS and is associated with aggressive disease course, with an undisputed sensitivity of above 90 percent. However, it was found to lack specificity (~35%). On the other hand, OCBs are the gold standard in MS diagnosis and it is used as a diagnostic biomarker in MS. In the revised McDonald Diagnostic Criteria of 2017 OCBs are recognized as a dissemination of lesions in time [26]. We should reconsider MS diagnosis if OCBs are negative; therefore we should be careful and must exclude MS mimics. OCB is detected in the CSF and blood by immunofluorescence. In the CSF sample, should be two or more OCB detected, but in the blood sample - should not. It is simple to use in daily clinical practice. MRZ reaction boasted higher overall specificity as well as predictive value in conversion from CIS to CDMS. MRZ reaction could be used as diagnostic biomarker in MS and as a measure for distinguishing other inflammatory neurological diseases from MS [32, 33, 124]. MRZ reaction is detected in the CSF by ELISA and it could be used in the clinical practice. But we should keep in mind that only positive MRZ reaction does not prove an MS diagnosis. MRZ reaction could be helpful in situations when OCBs are negative. NfL is a recently founded promising MS biomarker. NfL could be used as the disease activity and treatment response biomarker [125, 126]. In the future, we need more studies about NfL, their commitment to co-morbidities and standardized measurement. NfL level is also high in other neurodegenerative diseases; that is the reason why we need more studies about NfL in the future. At the time in the clinical practice, it`s not used as routine measurement, it needs special technique (SIMONA) to detect the NfL in the blood. The kFLC has a 96 per cent sensitivity and an 86 per cent specificity for MS. It has also been found to be a reliable predictive biomarker for CIS conversion into MS and as a prognostic biomarker to MS, to prevent faster disability and escalate therapy. In clinical practice, it is not used, at the time, as a routine test, this is because of its limited analytical factors. More studies on this are needed. These are all biomarkers of immunological activation and while many of the biomarkers in this subgroup possess some predictive value as well as specificity or sensitivity; they are not adequate on their own. Therefore, for a reliable and accurate diagnosis multiple biomarkers should be evaluated simultaneously. At present, we don`t have one single biomarker to detect an MS diagnosis; OCBs are very promising. We should be careful with MS mimics and other inflammatory neurological diseases.

Vitamin D works as a neuroprotector such as Th-1 immune response suppression and a genetic aspect with inhibitory function near the notorious HLA-DRB1*1501 through VDRE. Its levels have shown MS occurrence risk to increase with a distance from the equator by epidemiological correlation studies. Vitamin D level is associated with the MRI activity - higher vitamin D level is associate with lower risk of new lesions in the MRI, while low vitamin D level is strongly associated with new T2 lesions in the brain MRI [71]. We don't have a specific level of vitamin D supplementation to reduce the risk for MS and MS clinical activity [127]. We need more studies researching vitamin D and maybe in the future we could use vitamin D as predictive biomarker and could predict disease clinical activity and reduce long-term disability in MS patients. Now we use vitamin D in routine clinical practice as dietary supplement. It is easy to be used and detected in the blood.

Cellular subpopulations are extremely diverse, widely disseminated and play an essential role in the pathogenesis, they are present in all stages of MS disease. This also applies to many other inflammatory disorders. Therefore, their specificity

tends to be lacking. The B-cell, T-cell and TRECs can all be considered good biomarkers for disease activity, progression between different MS stages and thereby also prognosis up to a degree. Lipocalin 2 has shown similar promise in EAE models, but further study is warranted. Before the mystery around the pathogenesis of multiple sclerosis becomes clearer and the revelations that will be brought forth with it, these biomarkers will likely remain as measurements of MS disease activity. Ninjurin-1 has a major role in the development of neuroinflammatory lesions in the brain. It was found in active lesions in the brain compared with healthy population. Blocking Ninjurin-1 reduces the disease clinical activity and decreases inflammatory cells in the brain [83]. This could be used as a therapeutic target in the future but not as a biomarker.

Imaging biomarkers present a unique variety of new novel techniques and time proven standardized methods. The MRI is rightfully considered the golden standard for diagnostics of neuroinflammation and neurodegeneration in MS. Contrast enhanced MRI T1 and T2 lesions are undisputable findings indicating MS disease. An MRI could detect new lesions, active lesions, number of lesions and brain atrophy. MRI gives us great options with many sequences and different programs like T1, T2 sequences, DWI, MRS but in the routine clinical practice T1 and T2 sequences are used. While DTI and MRS are not used in the routine clinical practice, those are used in the researches. Standard MRI protocol is recommended for MS. MRI is an essential tool for MS diagnostics and monitoring and it is used as one of the main monitoring and diagnostic biomarkers. OCT has been proven to correlate brain atrophy rates well and thereby with disease progression. Thickness of the retinal inner nuclear layer associated with progression of the disease activity and disability. OCT could be used as predictive biomarker of the disease progression. Thus, use of MRI techniques together with OCT would create a solid biomarker for disease diagnosis, progression and treatment response.

Genetic-immunogenetic biomarkers have been proven by multiple studies to have an unequivocal role in the genetic risk for MS disease presentation. While there are many haplotypes that correlate with disease occurrence the HLA-DRB1*1501 stands above the rest. This was further validated by the discovery of vitamin D inhibitory effect through VDRE on the HLA-DRB1*1501 coding zone. As well as the presence of OCBs in the CSF of HLA-DRB1*1501 allele positive patients. Furthermore HLA-DRB1*1501 was also reported to correlate with clinical and imaging findings. Therefore, its place as a diagnostic marker has been established. However, screening for the HLA-DRB1*15 in CIS patients or those under suspicion might be indicated.

CONCLUSION

In this review, we have seen that there are many biomarkers available with clinical relevance, and it is enough when they are used together. Nevertheless, there does not exist a single solitary biomarker being able to present a reliable diagnostic value, prognostic value, high sensitivity and specificity as well as clinical impact on its own. At the time, a biomarker plays a crucial role in the clinical practice to diagnose MS is MRI and OCBs. Recent studies have shown good results as a biomarker of the disease diagnosis can be MRZ reaction and OCT. OCT could also be used as the disease progression and treatment response biomarker. In the future, hopefully, the disease monitoring and treatment responsible biomarker could be NfL. The above mentioned, biomarkers are used together, complementing each other. In case of some negative biomarker, one should be very careful with making MS diagnosis. We need more studies about MS biomarkers in the future, to provide better disease monitoring and treatment response.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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