

## Review Article

**BIOMARKERS, DRUG TREATMENT AND  
EXPERIMENTAL MODELS OF MULTIPLE  
SCLEROSIS****Rishab V Jain\*, Dr Shivalinge Gowda KP**

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**ABSTRACT**

Multiple sclerosis (MS) is a disease of demyelinating nature affecting the central nervous system. The chronic inflammation gives rise to plaques in the white and grey matter of the spinal cord and brain regions and also leads to diffuse neurodegeneration in the majority of the brain. Molecular biomarkers for MS are derived from the branches of neurobiology and immunology owing to the causal pathomechanisms. Currently, there is no definite cure for MS and its treatment is mainly centered on the administration of immunosuppressive and immunomodulating agents. Existing management approaches are dedicated to the reduction of biological activity through DMTs, treating acute attacks, and symptomatic treatment. Experimental models are essential to evolving the complexity and pathogenetic mechanisms of the disease and in the design of specific and effective treatments. Various in vitro and in vivo models have been developed in the quest for the design of experimental models of MS.

**INTRODUCTION**

Multiple sclerosis (MS) is a disease of demyelinating nature affecting the central nervous system (CNS). The chronic inflammation gives rise to plaques (or lesions) in the white and grey matter of the spinal cord and brain regions and also leads to diffuse neuro degeneration in the majority of the brain. These lesions are caused due to the infiltration of immune cells through the blood-brain barrier (BBB) and they promote demyelination, inflammation, neuro axonal degeneration, and gliosis, thus leading to disruption of neuronal signaling. These features are considered a hallmark of multiple sclerosis. The appearance of T cells occurs early in lesion formation, and therefore the disease is considered to be autoimmune, initiated by auto-reactive lymphocytes that mount aberrant responses against CNS auto-antigens, the meticulous mechanism of which, however, remains elusive. Aside from demyelination, the other sign of the disease is inflammation. Inflammation which is predominant in various phases of MS is largely

prominent in acute phases when compared to chronic phases. In early lesions, the infiltration of immune cells from the periphery and changes in the vascularity of BBB is observed. The infiltrate is dominated by macrophages followed closely by CD8+ T cells, whereas the numbers of other cells like B cells, CD4+ T cells, and plasma cells are low. As the disease progresses, inflammatory T cell and B cell infiltration, astrocyte and microglial activation, and extensive axonal injury and myelin reduction are evident. This leads to more pronounced atrophy of the grey and white matter. Although the number of T cells does not differ as the disease progresses, the relative number of plasma cells and B cells are found to be increased. Microglia and macrophages remain in a chronic state of activation throughout the disease. It was recently proposed that the innate synthesis of reactive oxygen species (ROS) and oxidative damage are key mechanisms driving demyelination and neurodegeneration, particularly in the

progressive disease stage. Additionally, the mitochondrial injury may further propagate ROS production and amplify neurodegeneration and demyelination through histologic hypoxia caused by energy deficiency.

Currently, there are four sub-types of MS as described by the US National MS Society (NMSS) and the MS international federation (revised in 2013): (a) Clinically isolated syndrome (CIS) –It is the first clinical presentation of MS presented by the characteristics of inflammation and demyelination that can be categorized as MS, but has yet to fulfill the criteria to be characterized as MS at that time. (b) Relapsing-remitting MS (RRMS) –It is the most frequently appearing MS phenotype found in MS patients (85%). RRMS is categorized by discontinuous periods of neurological dysfunction-relapses and phases of virtual clinical stability which are free of any neurological symptoms-remissions. (c) Secondary progressive MS (SPMS) – SPMS is characterized by the steady progression of the disease which may occur with or without the relapses after the RRMS stage. Phenotypically, the course of SPMS is non-uniform and consists of periods of disease progression with possible superimposition of relapse activity but also periods of relatively stable disability. (d) Primary progressive MS (PPMS) – It is categorized by the steadily worsening neurologic function from the commencement of symptoms without initial relapses or remission. [1-5]

## **BIOMARKERS OF MS**

A biomarker is a well-defined character that may be quantitatively measured and evaluated. It serves as a sign of normal biological, pathological, and pharmacological processes. Molecular biomarkers for MS are derived from the branches of neurobiology and immunology owing to the causal patho mechanisms. The significance of biomarkers for MS has been greatly documented in recent years. But their authentication is a cumbersome process so that very few biomarkers have thus far been routinely utilized in clinical practice.

### **Oligoclonal bands**

Oligoclonal bands are immunoglobulin bands observed when the patient's blood serum and CSF are analyzed in parallel. Oligoclonal bands (OCB) appear in the CSF analysis by isoelectric focusing electrophoresis in MS patients. They appear due to the presence of IgG and M IgM created by plasma cells within the CNS. These bands are present in almost every MS patient with a clinically distinguishable course and their existence is a strong affirmative of intrathecal antibody synthesis as they are found within the CSF, but not in the serum.

OCBs are detectable in the CSF of greater than 95% of MS patients.

### **IgG index**

The IgG index describes the ratio of the CSF/serum quotient of IgG to the CSF/serum quotient of the reference protein albumin. The albumin quotient, which describes the ratio of albumin in CSF/albumin in serum, serves to indicate the extent of blood-CSF barrier integrity in MS. IgG index is indicative of intra-thecal synthesis of immunoglobulin. An IgG index value > 0.7 indicates an increase in the intra-thecal B cell proliferation and thus, the presence of MS. An increase in the IgG index is observed in approximately 70% of MS patients.

### **Chitinase-3-like-1**

The protein chitinase-3-like-1 (CHI3L1) is a glycosidase secreted by activated astrocytes, monocytes, and microglial cells. Its exact physiological role in the CNS is still not known. Although, its presence in the inflammatory lesions implies its importance in the inflammation of CNS further modulated by the astrocytes. It is usually quantified in the CSF. High CHI3L1 levels were associated with faster disability progression.

### **Neuro filaments (NF)**

NFs are cytoskeletal structural proteins of axons and consist of a light (NFL), an intermediate (NFM), and a heavy (NFH) chain. They are helpful in the establishment of axonal diameter and are implicated in axonal transport. If axonal or neuronal damage occurs, NFs are released and their levels can be determined in the CSF and blood. NFL levels can be correlated with acute axonal damage, while NFH levels may reflect chronic axonal damage and may be more strongly associated with disability progression. Patients with MS have higher levels of NFL when compared to the control, with a robust relationship of values measured concurrently in the CSF and serum. Serum NFL levels can also be correlated with the rate of brain atrophy, degree of disability, and activity in MRI scanning. Furthermore, the NFL is additionally appropriate as a predictive biomarker for the transformation of CIS to MS. Lately; a technique called single-molecule arrays (SIMOA) has been devised and is ultra-sensitive, facilitating its detection in the serum.

### **Glial fibrillary acidic protein (GFAP)**

GFAP is a component of astrocytes filaments. Its increased levels in CSF are correlated to astrocyte damage. Increased levels of GFAP have been observed in patients compared to healthy controls. MS patients

with greater disease severity have higher levels of GFAP compared to patients with lesser disabilities and healthy controls.

### **Cytokines and chemokine**

Many cytokines and chemokine in CSF have been studied as potential biomarkers of MS. They contribute to the inflammatory response as they are responsible for the modulation of recruitment and migration of cells to the inflammatory sites. An elevation of pro-inflammatory and a down-regulation of anti-inflammatory cytokines and chemokine are observed during MS exacerbations. Interleukin 6 (IL-6), a pro-inflammatory cytokine shows elevated levels in the CSF during MS relapses. Chemokine ligand 13 (CXCL13), a B cell chemo attractant actively contributes to the B-cell follicular formation, important in the pathophysiology of MS. The CSF levels of CXCL13 are elevated in several subtypes of MS and can be correlated with disease activity. Osteopontin is another pro-inflammatory cytokine produced by both immune cells and non-immune cells. Its candidacy as a biomarker came about upon the discovery of increased Osteopontin gene expression in demyelinated lesions and normal-appearing white matter in MS. Higher levels of Plasma Osteopontin were subsequently found in the active form of RRMS, in SPMS but not in PPMS.

### **Fetuin-A**

Fetuin-A ( $\alpha$ 2-Heremans-Schmid glycoprotein) is a glycoprotein determined by proteomic analysis of CSF. It is elaborated in a vast range of biologic functions that may affect BBB integrity and immune functions including its activity as a TGF $\beta$ -1 antagonist. It is linked with a greater risk of disease progression from CIS to RRMS. Fetuin-A is also found to be elevated in SPMS and RRMS, thus suggesting that elevated fetuin-A is a potential biomarker of MS activity.

### **N-Acetyl aspartic acid (NAA)**

NAA is an amino acid derivative of unknown function, found in abundance in neurons and neuronal processes, with some expression in oligodendrocytes. NAA levels provide a measure of neuronal metabolism, which correlates well with the degree of axonal injury. Studies examining NAA by immunohistochemistry of MS brains showed a high correlation between NAA levels and axonal number and volume. NAA is abundant in the CNS and it can be quantitatively detected in vivo by magnetic resonance spectroscopy (MRS), used in conjunction with conventional magnetic resonance imaging (MRI). Studies have shown a significant decrease in NAA levels in MS lesions that can be

correlated with clinical disability and progression of brain atrophy.

### **Oxidative biomarkers**

Oxidative damage by reactive oxygen and nitrogen species to DNA, protein, and lipids, is a chief hallmark of MS neuropathology. It occurs in RRMS and progressive stages of the disease. Elevated levels of nitric oxide (NO) and Malondialdehyde (MDA) in the plasma, serum, and CSF of patients with MS have been strongly supported by evidence. It serves as a diagnostic marker in patients with an acute relapsing form of the disease.<sup>[6-10]</sup>

### **TREATMENT OF MS**

Currently, there is no definite cure for MS and its treatment is mainly centered on the administration of immunosuppressive and immunomodulation agents. However, many disease-modifying treatments (DMTs) have been identified that can diminish the rate of attacks and defer progression and mainly target inflammatory response in these patients. Existing management approaches are dedicated to the reduction of biological activity through DMTs, treating acute attacks, and symptomatic treatment.

### **Disease-modifying treatments**

These drugs modify the disease course through suppression or modulation of immune function. They wield anti-inflammatory effects predominantly in RRMS; they reduce the relapse rates, reduce the build-up of MRI lesions and alleviate, defer, and in certain cases humbly recover disability.

### **Interferons**

interferon beta-1a and beta-1b, Interferon-beta (IFN- $\beta$ ) is a class I interferon whose mechanism of action likely involves immunomodulation through down regulating the expression of MHC molecules on antigen-presenting cells, decreasing pro-inflammatory and increasing anti-inflammatory cytokines, inhibiting T-cell proliferation, and blocking trafficking of inflammatory cells to the CNS. IFN- $\beta$  modestly reduces the relapse rate and MRI disease measures and slows the accumulation of disability. Common adverse events include flu-like symptoms and minor anomalies in routine laboratory evaluation, as well as injection-site reactions with subcutaneous administration. Side effects can usually be managed with non-steroidal anti-inflammatory medications.

### **Monoclonal antibodies**

Natalizumab, alemtuzumab, ocrelizumab, daclizumab, and ofatumumab. Natalizumab, a humanized

monoclonal antibody, is an  $\alpha 4\beta 1$  integrin inhibitor which is an adhesion molecule expressed on the exterior of lymphocytes and is elaborated in transmigration across the endothelium into the CNS. Natalizumab is administered as an I.V (intravenous) infusion once in a month. It can reduce relapses and slow the disease progression in RRMS patients quite effectively. It is largely well tolerated; however, the long-term treatment carries the threat of progressive multifocal leukoencephalopathy (PML) [an opportunistic infection in the brain caused by the John Cunningham (JC) virus], occurring in ~0.4% of natalizumab-treated patients. It is extremely efficacious in RRMS (2nd line).

Alemtuzumab binds to CD52 protein on the exterior of developed lymphocytes. It is a non-specific immune depleter. The adverse reactions include infusion reactions, infections, opportunistic infections, leukopenia, and secondary autoimmunity (thyroid, immune thrombocytopenic purpura, renal, etc.). It is given as IV infusion once daily for RRMS (1st line).

Ocrelizumab is a humanized monoclonal antibody against the CD20 molecule existing on the exterior of developed B lymphocytes and has been widely used since its approval in 2017. Ocrelizumab is highly efficacious against relapses and quiet advancement in patients with RRMS and has dramatic benefits in halting the expansion of new lesions. It causes the selective depletion of CD20-expressing B lymphocytes thus preserving pre-existing humoral immunity and the capability for B-cell re-formation. B lymphocyte exhaustion is accompanied by a potent interruption in B-cell trafficking from the periphery to the CNS, reduced B-cell antigen demonstration to T-cells, and modulation of pro-inflammatory cytokine discharge by B cells, and reduced activation and differentiation to immunoglobulin secreting plasma blasts.

Ocrelizumab is administered as an I.V infusion every 24 weeks. Initial findings from the phase 3 trials indicated a possible low risk of increased malignancies including breast cancer, although longer follow-up revealed cancer rates identical to epidemiologic expectations. It is mainly prescribed for use in RRMS and PPMS (1st line).

Daclizumab is a humanized monoclonal antibody that is administered once every 30 days subcutaneously. This drug was approved for MS treatment in May 2016 and concerns regarding its safety led to its withdrawal in March 2018. Daclizumab modulates IL-2 signaling by binding to the IL-2 receptor subunit- $\alpha$  (also known as CD25). This binding causes the induction of immune tolerance through the expansion of immuno regulatory

CD56 bright natural killer cells and the reduction of early T cell proliferation. In a phase III trial, daclizumab significantly decreased the acute RR by 45% compared with IFN $\beta$ -1a. The untoward effects of daclizumab include liver damage, skin abnormalities, lymphadenopathy, and opportunistic infections.

Ofatumumab is a fully-humanized anti-CD20 monoclonal antibody which is administered by monthly subcutaneous (S.C) injection. It has a similar efficacy profile to ocrelizumab with a high degree of safety in phase 3 trials for RRMS. It is given as an SC injection every 4 weeks for RRMS (1st line).

### **Immunomodulators**

Glatiramer acetate, mitoxantrone, teriflunomide, and dimethyl fumarate. Glatiramer acetate is the acetate salt of a blend of random polypeptides composed of four amino acids. Its mechanism of action may involve favorably altering the equilibrium between pro-inflammatory and regulatory cytokines. It modestly reduces relapse rates and some disease severity measures and can be reflected as an equally effective alternative to IFN- $\beta$  in RMS. Common adverse events include injection-site reactions, flushing, chest tightness, dyspnea, palpitations, anxiety after injection, and less commonly, lipoatrophy that can rarely be disfiguring and require treatment cessation. It is given subcutaneously once daily or 3 times weekly in RRMS.

Mitoxantrone functions through inhibition of type II topoisomerase and disruption of DNA synthesis. Mitoxantrone diffusion occurs through the disrupted BBB and can encourage microglial loss. It was permitted by the FDA for treating SPMS and RRMS after several clinical trials. Mitoxantrone is administered at a dose of 12 mg/m<sup>2</sup> through infusions at monthly intervals. The cumulative dose is restricted because of the manifestation of hematologic and cardiologic side effects.

Teriflunomide, the active metabolite of leflunomide is an immune suppressant medication used in rheumatoid arthritis. Teriflunomide acts through the inhibition of the enzyme dihydroorotate dehydrogenase involved in pyrimidine synthesis. It inhibits the proliferation of auto reactive activated lymphocytes. The most severe side effects include the threat of liver toxicity and teratogenesis. Communal side effects include nausea, diarrhea, alopecia, headache, and an elevation in hepatic alanine transferase.

Dimethyl fumarate acts through activation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway and

Nrf2-independent pathways and thus exerts its anti-inflammatory and cytoprotective effects in this manner. It is usually well-tolerated, but treatment has been linked with the risk of developing PML. Most of these cases were lymphopenic, thus monitoring for lymphopenia every 6–12 months is recommended. Diroximel fumarate was recently approved and is metabolized to active metabolite monomethyl fumarate just like dimethyl fumarate.

### **Sphingosine-1-receptor modulators**

fingolimod, siponimod and ozanimod. Fingolimod (FTY720), approved by FDA in 2010, was the first line of oral treatment for relapsing forms of MS. The drug is given as a capsule at a dosage of 0.5 mg once daily. Fingolimod acts as an antagonist to the sphingosine-1-phosphate (S1P) receptor. It non-selectively degrades the S1P1 receptor on a lymphocyte. The drug causes the culmination of inflammation in MS by the capture of T-cells in secondary lymphatic tissues.

The most dominant adverse effects of fingolimod are diarrhea, cough, back pain, headache, and infections of the upper respiratory tract. It is advisable to perform an electrocardiogram (ECG) monitoring steadily for 6h after the first dose of fingolimod due to the possibility of bradycardia and atrioventricular (AV) block upon first administration.

Siponimod, also called BAF312, is a regulator of the sphingosine pathway and is available for oral administrations. Its action is more selective in comparison to fingolimod by the virtue of targeting S1P-1 and S1P-5. Amendment of brain MRI plaques and exacerbation rate was observed upon fingolimod treatment when evaluated in a phase II trial for the treatment of RRMS.

Ozanimod is another oral medication that selectively modulates the S1P receptor. Ozanimod showed promising ameliorative effects in a phase II trial as quantified through MRI appearances.

### **Cladribine**

Cladribine is an adenosine deaminase-resistant purine nucleoside and is usually prescribed as a chemotherapeutic agent primarily for the management of hairy cell leukemia as well as other forms of cancer. Cladribine preferentially acts on monocytes, lymphocytes, and intercalates within the DNA of dividing cells leading to apoptosis. Although cladribine selectively causes exhaustion of circulating T- and B-lymphocyte numbers, it shows little effect on NK cell count<sup>[11-14]</sup>

### **TREATING ACUTE ATTACKS**

The terms 'acute attack', 'acute exacerbations', and 'relapses' refer to the beginning of deteriorating neurologic discrepancies that last either a day or more in the absence of infection or any fever. The word "pseudo exacerbation" is used when relapses occur due to the presence of an infection or fever. The first-line treatment choices for acute attacks include glucocorticoids as they provide short-term clinical benefit by reducing the severity and shortening the duration of attacks. Typically IV methylprednisolone is administered at a dose of 1 g/day for 3– 5 days. Later, prednisone is given orally, commenced at 60–80 mg/day dose followed by tapering over 2 weeks.

Dexamethasone and oral prednisone at a high-dose are other alternative glucocorticosteroids that have been proved to be correspondingly effective. For patients who are impervious to glucocorticoid treatment, alternative treatment options need to be considered. Some second-line treatment options include plasma pheresis, IV immunoglobulin (IVIG), and adrenocorticotrophic hormone (ACTH) administration. Plasma pheresis (plasma exchange) is usually reserved for severe symptomatic cases non-compliant to glucocorticoid therapy and usually involves five to seven exchanges (40–60 mL/kg per exchange) for 14 days every alternate day.

IVIG usually finds its merit as off-label therapy in patients unresponsive to corticosteroid treatment as second- or third-line treatment; notably, this is the preferred treatment for postpartum patients. ACTH is another FDA-approved option but is seldom used because it is highly expensive. It is usually administered at doses equivalent to 80-120 units intramuscularly for 2-3 week duration and tapered<sup>[15-16]</sup>

### **Symptomatic treatments**

Symptomatic therapies are usually aimed at treating CNS damage associated symptoms through the use of physical and pharmacological therapies. It is advisable to promote attention to a vigorous lifestyle, together with maintaining an optimistic outlook, a healthy diet, and regular exercise as tolerated for all patients These are summarized in (table no: 1):[17-18]

### **EXPERIMENTAL MODELS OF MS**

Experimental models are essential to evolving the complexity and patho genetic mechanisms of the disease and in the design of specific and effective treatments. Various in vitro and in vivo models have been developed in the quest for the design of experimental models of MS.

MS symptom	Approx. frequency in MS	Non-pharmacological treatments: Key recommendations	Pharmacological treatments: Key recommendations
Spasticity and spasms	90%	<ul style="list-style-type: none"> <li>• Physiotherapy (including stretching)</li> <li>• Occupational therapy</li> </ul>	<ul style="list-style-type: none"> <li>• Baclofen (10–120mg/day)</li> <li>• Tizanidine (2–36mg/day)</li> <li>• Gabapentin (300–3600mg/day)</li> <li>• Clonazepam (0.25–2mg/day)</li> <li>• Diazepam (6-15 mg/day)</li> <li>• Cannabinoids</li> <li>• Botulinum toxin injection</li> </ul>
Impaired gait	80%	<ul style="list-style-type: none"> <li>• Adaptive devices</li> <li>• Physiotherapy</li> <li>• Functional electrical stimulation</li> </ul>	<ul style="list-style-type: none"> <li>• 4-Aminopyridine (Dalfampridine)(20mg/day)</li> </ul>
Pain	Up to 86%	<ul style="list-style-type: none"> <li>• Pain management</li> </ul>	<ul style="list-style-type: none"> <li>• Gabapentin (300–2400mg/day)</li> <li>• Pregabalin (150–600mg/day)</li> <li>• Duloxetine (20-120mg/day)</li> <li>• Amitriptyline (25–150mg/day)</li> <li>• Carbamazepine (100–1600mg/day)</li> <li>• Lamotrigine (200–400mg/day)</li> <li>• Topiramate (200–300mg/day)</li> </ul>
Ataxia/tremor	80%	<ul style="list-style-type: none"> <li>• Physiotherapy</li> <li>• Occupational therapy</li> <li>• Wrist weights</li> <li>• Thalamotomy</li> <li>• Deep-brain stimulation</li> </ul>	<ul style="list-style-type: none"> <li>• Carbamazepine (400–600mg/day)</li> <li>• Propranolol (40–240mg/day)</li> <li>• Topiramate (100–333mg/day)</li> <li>• Cannabinoids</li> <li>• Primidone (up to 750mg/day)</li> <li>• Clonazepam (3–6mg/day)</li> <li>• The response to these agents is generally poor.</li> </ul>
Bladder dysfunction	70-80%	Assessments: Urodynamic testing Treatments: <ul style="list-style-type: none"> <li>• Pelvic floor exercises</li> <li>• Electrical stimulation</li> <li>• Fluid-intake management</li> <li>• Urinary aids</li> </ul>	<ul style="list-style-type: none"> <li>• Mirabegron</li> <li>• Oxybutynin</li> <li>• Tolterodine</li> <li>• Solifenacin</li> <li>• Trospium chloride (40–60 mg/day)</li> <li>• Desmopressin (up to 20µg)</li> </ul>
Depression	50%	<ul style="list-style-type: none"> <li>• Psychotherapy</li> <li>• Counseling</li> </ul>	<ul style="list-style-type: none"> <li>• Fluoxetine</li> <li>• Sertraline</li> <li>• Escitalopram</li> <li>• Bupropion</li> <li>• Venlafaxine</li> </ul>
Fatigue	75%	<ul style="list-style-type: none"> <li>• Cooling</li> <li>• Regular exercise</li> <li>• Physiotherapy</li> <li>• Sleep hygiene</li> </ul>	<ul style="list-style-type: none"> <li>• Modafinil (200–400mg/day)</li> <li>• Armodafinil</li> <li>• Methylphenidate</li> </ul>
Cognitive dysfunction	40-70%	<ul style="list-style-type: none"> <li>• Attention testing</li> <li>• Memory training</li> <li>• Cognitive rehabilitation</li> </ul>	<ul style="list-style-type: none"> <li>• Lisdexamfetamine</li> </ul>
Paroxysmal symptoms	10-20%	<ul style="list-style-type: none"> <li>• Thermocoagulation</li> <li>• radiotherapy</li> </ul>	<ul style="list-style-type: none"> <li>• Carbamazepine (100–300 mg/ day)</li> <li>• Oxcarbazepine</li> <li>• Lamotrigine (up to 400mg/day)</li> <li>• Gabapentin (up to 1600mg/day)</li> <li>• Topiramate (up to 300mg/day)</li> </ul>

**In vitro models**

They are usually helpful in facilitating the research of cell-cell interactions by the isolation of mammalian neuronal cells and cultures of immortalized cell lines. These cell cultures offer the advantages of being altered genetically, high stability, and ease of subjection to a wide variety of experimental conditions. The cell cultures which are usually utilized include neurons, oligodendrocytes, oligodendrocytes precursor cells (OPCs) (ectoderm), astrocytes, and microglial cells (mesoderm)

The culture of oligodendrocytes allows us to study the chain of events triggered by demyelination, activation, and signal transduction pathways involved in oligodendrocytes replacement, the support of astrocytes metabolism in demyelinating lesions, and remyelination of demyelinated axons. It is possible to study the proliferation of inflammatory factors along with their effects, testing of new therapies, and strategies that can promote oligodendrocytes generation and remyelination by the use of OPC cultures or neural stem cells. Primary astrocyte cultures are also useful since these cells are involved in the local environment, which is essential in remyelination. The growth factors, like insulin-like growth factor 1 (IGF-1) and platelet-derived growth factors (PDGF) secreted by astrocytes stimulate remyelination. The role of microglial cells in the disease progression can be extrapolated by the use of microglial cell cultures. They help analyze the response of these cells to biochemical signals and to study the mechanism of activation of these cells. The inability to extrapolate the response of individual cell types to tissues serves as the major drawback of these models.<sup>(19-22)</sup>

Slice cultures offer a reliable alternative to cell cultures by providing an accurate simulation of the in vivo environment and thus facilitating the study of the interaction between different cells. Slice cultures have some limitations, particularly the slicing procedure: slices must be 200-500µm thick, obtained from young or even neonatal animals, and manipulated under special conditions with carbogen gas.<sup>[23]</sup>

**In vivo models/ Animal models**

Various animal models have been developed to understand a variety of aspects of human MS over the last few decades. The various types of animal models of MS are (1) the experimental autoimmune encephalomyelitis (EAE) model, (2) viral-induced models (3) neurotoxin-induced models, and (4) transgenic models.

**Experimental autoimmune encephalomyelitis (EAE)**

This model has directly contributed to the advancement of many first-line treatments that ameliorate the inflammatory stage of the disease. EAE refers to the CNS inflammation caused by the stimulation of the immune system cells against antigens which are specific to CNS. Methods of inducing EAE include (a) active EAE through CNS peptide immunization and (b) passive EAE through the adoptive transmission of encephalitogenic T-cells.<sup>(24)</sup>

**a) EAE induction through active immunization**

This is achieved by the use of CNS specific antigens like spinal cord homogenate (SCH), myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocytes glycoprotein (MOG) and is usually emulsified in incomplete Freund's adjuvant (IFA). The adjuvants are used to increase the efficiency of EAE induction by mimicking the pathway of immune system activation. The triggering of specific T-cells directed against the myelin antigen occurs due to immunization and they subsequently proliferate and mature into effector T-cells. These effector T cells express integrins on their surface enabling their entry across the BBB.

The resident myelin antigen-presenting cells (APCs) re-activate these effector T-cells inside the CNS. This causes the expression of pro-inflammatory cytokines like interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-17 (IL-17), tissue necrosis factor- $\alpha$  (TNF- $\alpha$ ), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Furthermore, it also causes the recruitment of  $\gamma\delta$  T-cells, macrophages, neutrophils, and monocytes into the CNS. All these processes lead to the destruction of the myelin sheath, the pathology of which usually resembles pattern I or II lesions of MS.<sup>(25-27)</sup>

**b) EAE induction through adoptive transmission**

The second method for EAE induction involves the transmission of encephalitogenic T cells into naïve animals. One of the first studies to demonstrate the adoptive transfer method showed that the transfer of lymphocytes from immunized rats into naïve rats induced significant neuro-inflammatory disease. This method has helped in the deduction of mechanisms involved in the pathogenesis of EAE by T-cells. The migration pattern of T-cells into the CNS can be tracked by labeling them before transfer and study their subsequent behavior during EAE development.

## **Correlation between EAE and MS studies –successes and failures**

The importance of this model in preclinical research is signified by the fact that several drugs approved for MS treatment have been devised through this model. However, despite these successes, there have been numerous examples where translation has not been successful. TNF- $\alpha$  manifestation correlates with MS severity and its subsequent inhibition advances the course of disease in the EAE model. However, clinical trials of TNF- $\alpha$  blocker infliximab worsen MS patient symptoms. The greatest criticism of the EAE model is that it fails to mimic some important features of multiple sclerosis, especially those concerning the immune system activation: EAE is mainly mediated by CD4+ T-cells, whereas, in multiple sclerosis, the CD8+ T-cells play a predominant role.

Besides, EAE is usually characterized by spinal cord demyelination, and in contrast to human pathology, cortical lesions are nearly absent. Cortical demyelination is a prominent marker of chronic multiple sclerosis. Another critical point is the enormous variability of EAE pathology, due to the different activities of the available antigenic peptides, and to the variable immune responses by the different animal species and strains. For these reasons, the choice of the peptide and the animal species/strain is critical for study design and data interpretation. These drawbacks have quizzed the legitimacy of this model in effectively replicating the human condition. However, these limitations are seen throughout animal research and the exact human condition is very difficult to achieve in animals. <sup>[28-30]</sup>

### **Virus-induced inflammatory demyelination models**

Certain virus infections in animals can give rise to a condition characterized by inflammation and demyelination with striking similarities to MS. The basic mechanism involved in demyelination, inflammation, and neurodegeneration can be deduced by the study of this model. The mechanism of disease induction or propagation in the brain and spinal cord can also be studied. The two currently available viral models are a) Theiler's murine encephalomyelitis virus (TMEV) model and (b) mouse hepatitis (corona) virus model.

#### **a) Theiler's murine encephalomyelitis virus (TMEV)**

In mice, the pathology is induced via an intracerebral injection of Picornaviridae, which is a family of single-stranded RNA viruses belonging to the Cardio virus genus. Two main types of TMEV are known, one highly

aggressive that causes extremely severe neuropathology leading to death within 1 week (induced by GDVII and FA strains of TV), and the other, less aggressive and not fatal (induced by DA and Be An strains). The latter can induce either a monophasic or a biphasic disease, depending on the mouse strain. The monophasic disease is inducible in most of the murine strains, whereas the biphasic form is inducible only in specific susceptible strains. These phases are characterized by acute neuronal apoptosis in gray and white matter, appearing 1 week after the injection of the virus. The monophasic disorder clears out within three weeks and the biphasic disease (usually from 1-month post-injection) sets the stage for chronic and progressive inflammation, and demyelination begins and is characterized by the activation of macrophages and glial cells, apoptosis of oligodendrocytes, demyelination, and axonal damage, mostly in the spinal cord.

The peak demyelination is reached from the third month of virus injection. In parallel with the worsening of the pathology, motor disabilities are observed. The neurological effects of TMEV are believed to be arbitrated by the activation of T-lymphocytes, such as the CD8+ T-cells, rather than by direct interaction of the virus with the myelin proteins; moreover, the permanence of this virus in the brain seems to depend on the astrocyte activity that supports viral replication. In summary, TMEV is useful to reproduce acute or chronic/progressive phases of the disease.

#### **b) Chronic inflammatory demyelinating disease induced by the Mouse Hepatitis (corona) Virus (MHV)**

MHV is a large coronavirus, which may induce hepatic, enteric, respiratory, or neurological disease, depending upon the virus strain. The disease can be induced by intracranial or nasal infection with a virulent strain of the virus. The development of disease occurs in two stages: (a) the first phase characterized by the observation of panencephalitis within a few days of infection. After recovery from the first phase, the development of the second phase occurs after 4 weeks. Neuro-paralytic disease, inflammation, and demyelination are the main characteristics of this phase. Inflammatory infiltrates consist of T-lymphocytes and activated macrophages or microglia. B-cells and plasma cells also play a major role in the inflammatory process as indicated by the intrathecal production of immunoglobulin. Demyelination occurs due to antigen-independent (bystander) destruction of oligodendrocytes by CD8+ T-cells and  $\gamma\delta$  T-cells.



One of the major benefits is that the induction of disease occurs due to an infectious process which is a more natural process as opposed to active sensitization with CNS antigens as in EAE. This model can provide evidence on the etiology of the disease progression in humans. The complex pathogenesis, antiviral immunity, direct virus-induced toxicity, and other autoimmune mechanisms are the major limitations of this model.<sup>[31]</sup>

#### **Neurotoxin-induced models of demyelination**

The feature that distinguishes MS from other diseases of the CNS is demyelination. In-depth knowledge of the pathology involved in de- and re-myelination processes is required for the development of therapies targeting these processes. Neurotoxin models of demyelination are best suited for deriving such data. There are 2 types of neurotoxin-induced demyelination: a) induction by cuprizone by b) focal injection of lysolecithin or ethidium bromide into specific white matter tracts. These models can provide a valuable understanding of the metabolic processes involved in the destruction and repair of myelin sheath thus facilitating the better development of treatments aimed to promote the demyelination process. These models are also useful for in vivo imaging of demyelinated plaques by employing an MRI scan.

#### **a) Models of focal toxin-induced demyelination**

The direct injection of lysolecithin [lysophosphatidylcholine (LPC)] into the brain is particularly toxic to oligodendrocytes while maintaining the integrity of neurons. Lysolecithin causes the fusion of myelin lamellae and their subsequent transformation into spherical vesicles. These vesicles gradually reduce in size ultimately leading to phagocytosis. Following the injection of lysolecithin (1%) into the white matter of the spinal cord, macrophage/ microglial infiltration, reactive astrogliosis, axonal injury, and OPC proliferation/migration is observed. This is followed by spontaneous remyelination that is complete around 23 days post-injection. This model offers the unique benefit of having spatial and temporal control over demyelination, and thus this model is used to interrogate the complex mechanisms of remyelination.

Another focal model of demyelination used the injection of ethidium bromide into white matter tracts. This leads to degeneration not only of oligodendrocytes but also of astrocytes. With this model, it was shown that oligodendrocyte remyelination requires the presence of astrocytes. When absent, the lesions are repaired by Schwann cells.

#### **b) Cuprizone induced demyelination**

The cuprizone [N, N'-bis (cyclohexylideneamino) oxamide (CPZ) model of demyelination and remyelination has been exploited to differentiate between the precise mechanisms contributing towards the death of oligodendrocytes, along with the migration, differentiation, and remyelination patterns of OPCs. Swiss mice were first used to demonstrate CPZ-induced demyelination in the late 1960s. Nowadays, protocols state the use of C57BL/6 mice; but the development of induction methods for rats, which is predominantly useful for imaging studies due to its larger brain size has been well acknowledged.

CPZ is a neurotoxin capable of copper chelation thus targeting many metallo enzymes. It is mainly responsible for impairment of the activity of the copper-dependent cytochrome oxidase, decreasing oxidative phosphorylation, and the production of degenerative changes in oligodendrocytes. This sequence of events ultimately ends in demyelination. Administration of CPZ causes the apoptosis of oligodendrocytes thus leading to widespread demyelination of the white matter in the thalamus, corpus callosum (CC), anterior commissure, internal capsule, and cerebellar peduncles. Interestingly, upon withdrawal of CPZ treatment, rapid remyelination and myelin protein re-expression are observed. The specific dose of CPZ required to induce demyelination was the center of attention in many studies done since the beginning of the 1960s. CPZ administered at a dose of 0.2% - 0.6% w/w mixed with standard rodent chow was found to produce significant changes in the myelin content of CNS.

The CPZ model is suitable for the reproduction of pattern-III MS lesions, categorized by the apoptosis of oligodendrocytes and the down-regulation of myelin-associated glycoproteins (MAG). The CPZ model also facilitates the understanding of the pathology of spontaneous remyelination, as demyelination and remyelination usually occur simultaneously and a reversal of white-matter damage after the withdrawal of CPZ is seen, thus resembling RRMS. Prolonged administration of cuprizone, for 6–7 months, impairs remyelination as in progressive multiple sclerosis. Cuprizone can also be administered in repeated doses mimicking the course of relapsing-remitting multiple sclerosis. In summary, cuprizone allows an experimental reproduction of different pathological courses, such as the acute, chronic, and relapsing-remitting forms of multiple sclerosis.<sup>[32-33]</sup>

**Transgenic animal models**

Transgenic mice are characterized by the presence of gene insertions or deletions which code for certain immune factors. These models thus allow the extrapolation of the role of such immune factors in the pathogenesis of immune-mediated CNS damage. The myelin proteins of knockout animal models can be altered similar to that observed in human MS patients. Moreover, the mechanism of myelination has been the subject of research in various transgenic models deficient in myelin sheath: 1) the shiverer mice (characterized by duplication and inversion of the gene coding for MBP), 2) rump shaker mice (containing mutation for PLP coding gene), and 3) jimpy mice (single mutation in the PLP coding gene); these mouse models all develop demyelination.<sup>[34]</sup>

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