Characterization of Rab32 and HMGB1 involved in the disease course of Experimental Autoimmune Encephalomyelitis, An animal model of Multiple Sclerosis.

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THESIS ACCEPTANCE CERTIFICATE

Certified that final contents and form of MS/MPhil thesis entitled **"Characterization of Rab32 and HMGP1 involved in the disease course of Multiple Autoimmune Encephalomyelitis, An animal model of Multiple Sclerosis" written by Tayyaba Shafique, (Registration No. 00000273698)**, of SMME, has been vetted by undersigned, found complete in all respects as per NUST Status/Regulations, is free of plagiarism, errors, and mistakes and is accepted as partial fulfillment for the award of MS/MPhil degree. It is further certified that necessary amendments as pointed out by GEC members of the scholar have also been incorporated in the said thesis.

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DECLARATION

I, Tayyaba Shafique, declare that all work presented in this thesis is the result of my work. Where information has been derived from other sources, I confirm that this has been mentioned in the thesis. The work herein was carried out while I was a post-graduate student at the School of Mechanical and Manufacturing Engineering (SMME), NUST under the supervision of Dr. Saima Zafar.

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Tayyaba Shafique

00000273698

Dedicated to

My Beloved Parents

For their endless support, endearment, care & encouragement

My Supervisor

For her guidance and patience

My Friends

For standing with me in every high and low

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Abstract

Multiple Sclerosis (MS) is the most common chronic inflammatory, neurodegenerative and demyelinating disease of the central nervous system among young adults which leads to permanent neurological damage. Despite the rapid advancement of experimental and clinical research into MS, the exact molecular mechanism behind the disease progression remains elusive. Currently, available MS medications will only slow down the disease's progression and do not function for all patients therefore, more successful therapies are needed. One of the most well-studied and well-established models to investigate neuroinflammatory pathways involved in MS is Experimental Autoimmune Encephalomyelitis (EAE). EAE can be induced in different animal models but mice are the most widely used species for this model. High Mobility box 1 group (HMGB1) has emerged as a possible candidate because of its role in EAE/MS pathogenesis. The objective of this study was to determine whether HMGB1 can be a therapeutic target for EAE. To achieve this objective, we created an Animal model of MS by administrating the MOG35-55 peptide. This study will allow a wide range of possible therapeutic interventions to be developed and preclinically tested.

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Chapter 1: Introduction

Multiple Sclerosis (MS) is the most common chronic inflammatory, neurodegenerative, and demyelinating ailment of the central nervous system among young adults which leads to permanent neurological damage. It is mainly caused by the interaction between genes and the environment. In MS, the myelin sheath which covers the brain and spinal cord is damaged which results in distorted axons to a different degree. The symptoms of MS vary depending on the location of the plaque within the CNS. The pathological hallmarks of the disease are denoted by the loss of Myelin sheath due to the focal demyelination in the white and grey matter of the brain and spinal cord. (Fred D. Lublin, May,2014) It is an autoimmune disease with its initiation and progression stages being mainly dependent on the autoimmune response to myelin antigens. It has been reported that about 2.3 million people are being affected by MS worldwide with two times higher risk for women than men.MS has increased expanded consideration due to its high recurrence in individuals between 30-40 years influencing their reproductive age and adding to a negative social and monetary effect. There are various kinds of the clinical course of MS, including the relapsing/remitting MS(RRMS) represented by single attacks which can be repaired completely or mostly, present at the 80-85% of the MS patients. There is an initial course of RRMS disease during secondary progressive MS (SPMS), leading to serial degradation with or without occasional relapses. Whereas primary progressive MS (PPMS) is characterized by progressive accumulation of disability since the onset of the disease and occurs in approximately 15-20 percentSensory disruptions in the limbs, optic nerve dysfunction, pyramidal tract, bowel dysregulation, ataxia, and diplopia are the most commonly observed symptoms. (Marvin M. Goldenberg, 2012)

Currently, available MS medications will only slow down the disease's progression and do not function for all patients. Therefore, more successful therapies are needed, but a poor understanding of MS pathogenesis has disrupted their growth. Twin and adoption research demonstrated a major genetic dimension in MS predisposition, and thus it could be more understandable to detect underlying disease processes to determine genetic factors involved. The genetic study is also perhaps more critical in MS than in complex disorders, in which factors of controllable lifestyles, such as diet, are considered to play an important role, considering the unknown environmental factors contributing to Ms. However, like other complex diseases, the

detection of MS which are prone to genetic variants is difficult and the Major Histocompatibility Complex (MHC) locus has remained the only well known genetic finding for almost four decades. (Kemppinen, 2011)

After research, three different types of animal models are further investigated for the exploration of the exact mechanism of MS. Viral models such as the Theiler Murine Encephalomyelitis Virus (TMEV), models triggered by toxic agents such as cuprizone, and finally, different variants of experimental encephalomyelitis autoimmune. Among all these models, EAE is the most well studied and well-established model to investigate neuroinflammatory pathways involved in MS and for treatment strategies. EAE can be induced in different animal models but mice are the most widely used species for this model. In the EAE model of mice, the pathophysiological reaction of the immune system against brain-specific antigens can be seen. This reaction causes inflammation and degradation of carrier structures of the antigen and produces comparable neurological and pathological features in MS patients. (Stefan Bittner, April, 2014)

Study Objectives

The current study primarily focuses on the most common risk factors involved in Multiple Sclerosis. The findings of this study can be useful in the prevention and cause-directed treatment of MS with the help of novel therapeutic techniques.

Objectives of the study are :

1- To detect the disease in the early stages helps to slow down the progression of MS and damage to nerve cells.
2- To detect the role of different proteins involved in MS through Bioinformatics.
3- Characterization of Rab 32 and HMGP1 proteins involved in the disease course of EAE model of MS.

Chapter 2: Literature Review

2.1 Epidemiology

In the United States and about 2.5 million worldwide, MS affects around 400,000 individuals. Every week in the United States, more than 200 people are diagnosed with MS.(Fig. 1) The major cause of non-traumatic injury in young adults is usually MS between the ages of 20 and 40. Multiple Sclerosis is predominantly found in the region of Europe and it is less common in Asian countries and as well in Native Americans. Estimates of prevalence in Asia range in western countries from 2 to 100,000 to around 1 to 1,000, while in some countries the prevalence of 1 to 400 people was observed. (Rosati, 2001) Since the 1950s the prevalence of MS has risen, particularly among women; it may be an increase in the burden of disease, but also improved access to the improved healthcare center, better treatment and diagnostic precision, and improved life expectancy. But the predominance of the female can not justify these causes. This disease is double in females as compared to males and this ratio grew to about 3:1 in the last 10 years from 2:1 ratio in the 1950s in some areas. Symptoms with MS are the main direct death factor, in greater than 50 percent of patients even though the overall population rises dramatically in infections and suicide. (Sarah-Michelle Orton, october,2006)

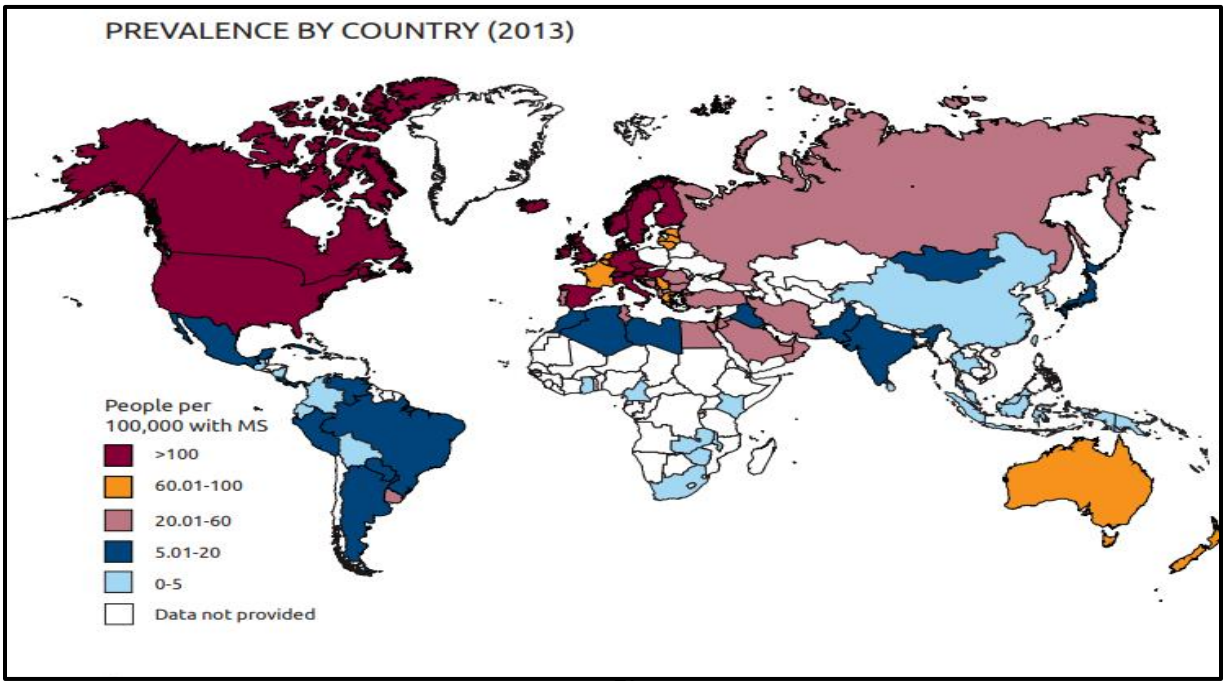

Figure 2.1: This figure shows the percentage of MS around the globe. It shows that the rate of MS usually varies between the countries. The percentage of this disease is higher in those countries with higher Latitude.

2.2 Risk Factors

The causes of MS appear to be unclear, although genetic susceptibility and environmental hazard factors interplay the disease.

2.2.1 Genetic factors

For all MS phénotypes, the prevalence of family MS is ~13%. In the second half of the 19th century, discovering family aggregation sheds light on the genetic aspect of the disease for the first time. The risk of developing the disease is 10- to 20-fold higher for siblings of infected persons than for a lifetime of 0.2 percent (2-4 percent), whereas the risk of monozygotic twins (30 percent) is higher. (C J Willer 1, 2003). With the percentage of genetic sharing, the probability of recurrence among families increases. The heritability of MS is polygenic in different genes, each with a slight increase in disease risk. The > 200 genetically modified risk variants for MS have been identified in large association studies; each variant shows a small impact on diseased risk, and various variant combinations possibly lead to more risk of developing genetic mutations in patients. These variants encode different molecules of the Immune system which are linked with the increased risk of other disorders related to the systemic immune system. Risk genes for MS do not interact with other neurodegenerative disorders, however, some mutations are related to a higher risk of MS in only a few genes with clear Nervous System functions. The fact that the relative risk is not 100 percent in twins, on the other hand, indicates that additional factors outside the sequence of DNA identities need to be consistent with the formation of conditions for the causation or enable of dysregulation of the MS-associated immune response. (Mohammad Hossein Harirchian 1, 2017)

2.2.2 Environmental factors contributing to Multiple Sclerosis

There is a list of environmental factors that can result in the cause of MS and therefore increase the risk of disease within a given period. A duration of vulnerability to environmental MS risk

factors during adolescence is supported by substantial evidence. (Table 1).Epstein – Barr virus (EBV) infection in adolescence and early adulthood, active or passive tobacco exposure, sunlight exposure, low levels of vitamin D, and obesity in adolescence are among the most well-known risk factors. Other factors less known include night work, heavy intake of alcohol or caffeine, and a history of infectious mononucleosis. One of the important points is that different infections are the potential causes for disease onset because of the immune-mediated pathogenesis of MS. EBV infection is most consistently and robustly correlated with the numerous pathogens investigated.

For this reason, it is important to remember that, according to epidemiological research, 100 percent of patients having MS are EBV seropositive. The exact mechanism of action through which infection elevates the risk of MS is still unclear. But it was observed that some molecular simulation is involved in the disease which results in the initiation of Antibodies and some reactive T- cells. While data support an increased risk of MS with EBV infection, it is difficult to create a direct causal relationship. Smoking has been consistently demonstrated as a risk factor for MS. The risk of MS and smoking depends on doses: if the dose of smoking is high then it is associated with a higher risk of developing the disease. The increased risk of MS was also associated with passive smoking exposure. Moreover, smoking is also associated with the development of rapid disability and the risk of converting a less intense form of MS to a more severe form. The direct toxic effects (promoting lung irritation) of certain components of smoke and an indirect systemic (peribronchial lymphatic tissue) effect have been proposed to explain the association. Exposure to the sun is one of the major factors of Vitamin D levels, especially the exposure to ultraviolet B radiation, and tends to decrease as latitudes rise. The 'latitude effect' in MS prevalence was therefore suggested as a sub-level of vitamin D. A combination of low levels of vitamin D with elevated MS risk and enhanced disease activity indicates a preventing result of normal levels of vitamin D in the disease. Even if vitamin D action mechanisms are incompletely evident, there is some evidence that indicates the active form of vitamin D (1,25-dihydroxycholecalciferol) in the regulation of immune function. (Brian C Healy 1, 2009)

Risk factor	Odds ratio	HLA gene Interaction	Effect during Adolescence	Immune system Implied
Smoking	~1.6	Yes	No	Yes
EBV infection	~3.6	Yes	Yes	Yes
Vitamin D	~1.4	No	Probable	Yes
Night work	~1.7	No	Yes	Yes
Infectious mononucleosis	~2.0	Yes	Yes	Yes
Passive smoking	~1.3	Yes	No	Yes
Alcohol	~0.6	No	Unknown	Yes
Low sun Exposure	~2.0	No	Probable	Yes

Table 2.1 Environmental risk factors for MS (Lars Alfredsson 1, 2019)

2.3 Pathophysiology

Focal plaques (also known as lesions), typically placed around post-capillaire venules, are pathological features of all MS phenotypes, which are defined by the failure of the blood-brain barrier. BBB breakdown mechanisms are not fully known, but they tend to have effects direct to resident cells and endothelial cells of pro-inflammatory cytokines and chemokines (such as TNF, IL-1β, IL-6). Dysregulation of the BBB causes further inflammation and demyelination, followed by oligodendrocyte loss, reactive gliosis, and neuro-axonal degradation in the CNS, resulting in endothelial transmigration of active leukocytes including macrophages, T cells, and B cells. (Genaro Gabriel Ortiz 1, Nov 2014) Plaques occur both in white and grey matter and are

commonly located in the brain, the optic nerve, and the spinal cord in the CNS. Although the anatomic location is correlated with particular clinical events of MS, their total volumes are only marginally linked by the involvement of other pathophysiological mechanisms, such as the incidence of grey lesions and the regular occurrence of tissue injury, which is induced by the anatomic location of the white matter. In the early stages, severe demyelination and a variable degree of axonal loss and reactive gliosis characterize pathology. Patients with focal inflammatory plaques, with demyelinated axons, decreased number of oligodendrocytes, subsequent astrocyte proliferation of glycolysis, perivascular and parenchymal axons, and lymphocyte and macrophage infiltrations are present in general. In the progressive course, diffuse grey and white matter atrophy predominates in MS and is marked by low-grade inflammation and activation of the microglia on the bounds of the plaque in conjunction with diffuse wounds of the normal white matter. The secondary demyelination follows inflammation, microglial activation, axonal injury, and myelin damage during this course. The patterns of tissue injured in primary or secondary MS patients are usually homogeneous. They showed loss of oligodendrocyte, preferential destruction of small-scale axons, astrocytic gliosis, and demyelination. Different components of adaptive and innate immunity included demyelination and subsequent neurodegeneration with different forms of MS. Myelin sheaths are especially vulnerable to the release by activated macrophages and non-specific products such as cytotoxic cytokines, excitotoxins, or reactive oxygen and nitric oxidation. However, the most commonly observed patterns of demyelination are antibodies and associated modifications, and hypoxia tissue injuries where demyelination initiation is due to distal Oligodendrocyte process degeneration and oligodendrocyte apoptosis, while astrocyte loss of polarity contributes to the disturbance in the structural organization (Wen-Juan Huang, 2017). Classically, MS is considered an autoimmune disease that is a T cell-mediated disease that predominates with CD8 + cells in contrast with other sub-sets of T-cells, B cells, or plasma cells. Inflammatory lesions mainly consisting of cells CD8+T and CD4+T start from this disease and activate Microglia/macrophages. Evidence of function suppression restricting CD4 + T-cell responses and the tissue-damaging role of recorded CD8+T cells has been found. Indeed, the basic interaction of CD8+T cells with target cells requires MHC-I expression tightly regulated with neurons and MHC-I molecules only to respond to strong threat signaling, such as proinflammatory expression.

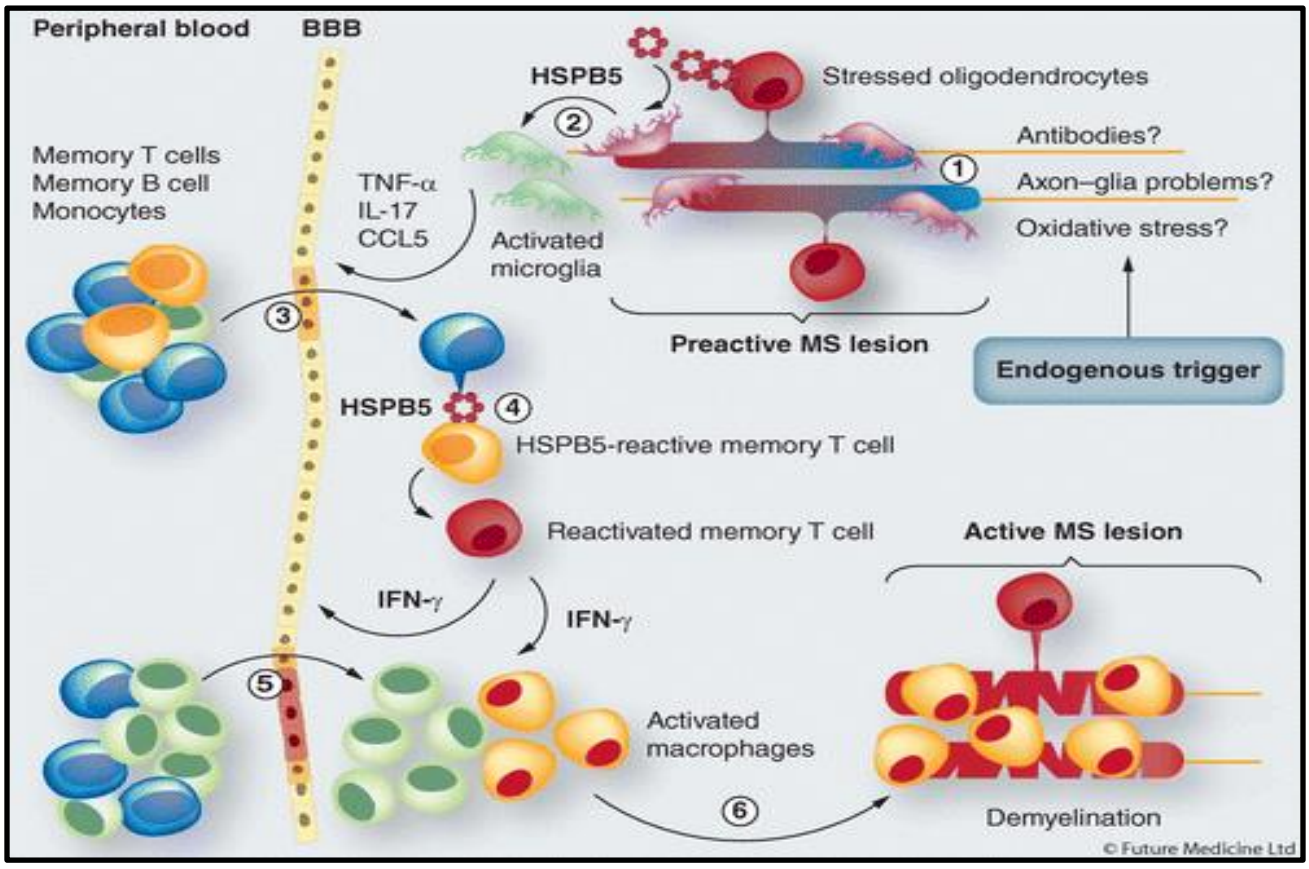

Figure 2.2: Pathophysiology of Multiple Sclerosis. The focal disturbance activates clusters of microglia in the normal CNS tissue which results in MS lesions. (1) Activation of microglia is triggered by HSPB5 and referred to as proactive MS lesions which are then stored in oligodendrocytes. (2) Activated HSPB5 results in the secretion of anti-inflammatory factors and other mediators that can disturb the BBB and generate immune surveillance and the BBB then recruits peripheral lymphocytes. (3) This result in the production of regulatory T cells for healing purpose but also secrete memory T-cells. (4) These Memory T-cells combine with the high level of HSPB5 and start secreting IFN-γ. (5) This factor disturbs the healing process and increases the recruitment of peripheral lymphocytes in perivascular infiltrate. (6) IFN-γ further initiates a pro-inflammatory response which destroys myelin sheath and oligodendrocytes and thus causing a destructive MS lesion. (Johannes M van Noort & Sandra Amor, 2013)

2.4 Pathways associated with Multiple Sclerosis

In MS myelin is degraded because the immune system mistakenly recognizes its foreign. As the immune system travels through Blood vessels which are composed of endothelial cells. These cells are joined to each other with the help of very tight junctions called tight junctions. There is also a membrane inside that is known as the Basement membrane and together all they are known as the Blood-brain barrier. BBB is very important because it stops a lot of things from entering the brain and it also stops the immune system from entering the brain. As T cells are part of the immune system and they won't be able to enter the brain.

But in MS, T cells pass the endothelial cells and breaks through the basement membrane. In this way, they can enter the brain and it is an unfamiliar environment for T cells because it should not be there. As in the brain, there are neurons and they have a myelin sheath and this sheath has a protein n its surface. So in this case, T cells go to the protein and this looks foreign to them and they start attacking it.

When T-cells recognize it foreign, they start releasing chemicals called Cytokines. These cytokines do different things in the body like they promote degradation of Blood-Brain Barrier and therefore BBB is more permeable to other T cells and they come inside. These cytokines also recruit other immune cells called B cells and they enter into the brain easily where they make an antibody for the myelin and they further degrade myelin sheath. And due to this, it results in Neuroinflammation.

There are other cells called Oligodendrocytes and when myelin is degraded they start the process of Remyelination. The function of these cells is to repair all the damage in myelin but as time goes on, remyelination becomes less effective and it results in Multiple Sclerosis.

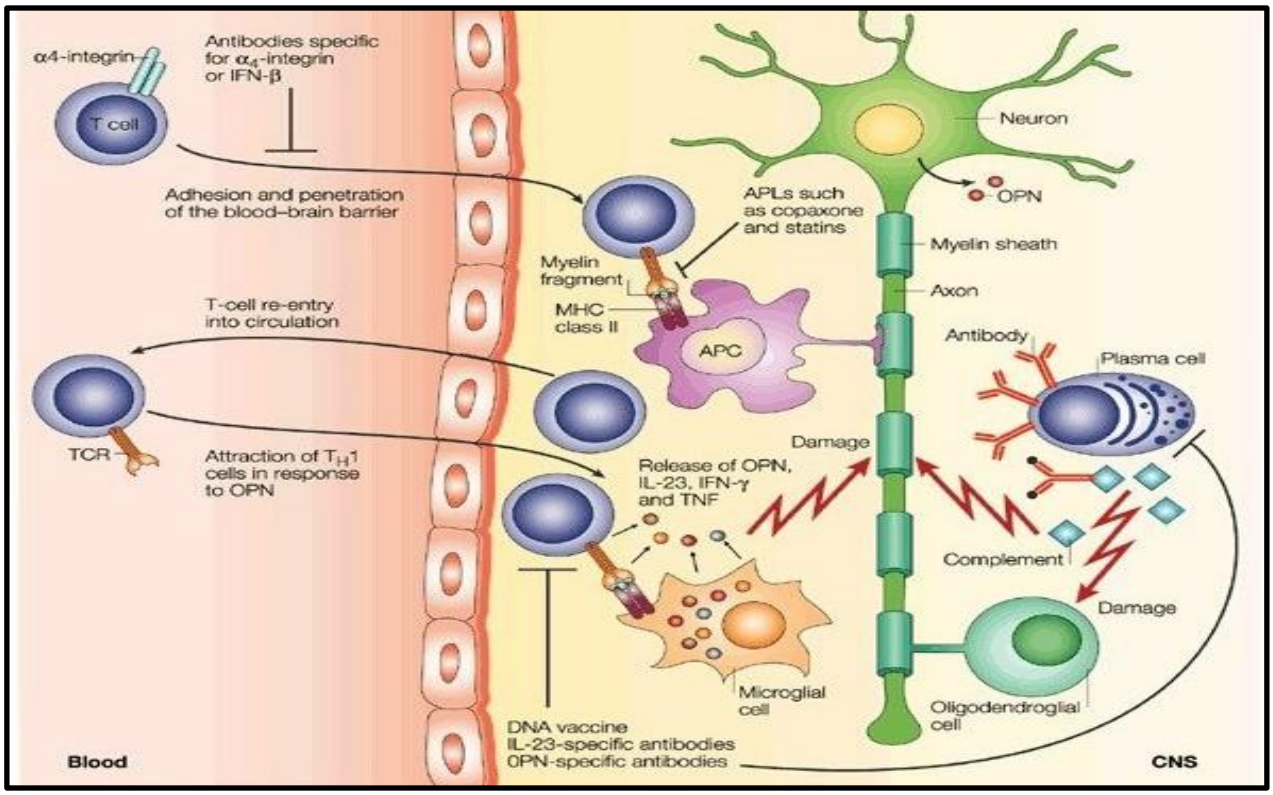

Figure 2.3: The inflammatory phase of multiple sclerosis. In the CNS, T cells, B cells, and Antigen-presenting cells including macrophages that secrete certain chemicals called Cytokines. These cells destroy the Oligodendroglial cells (which protect the myelin sheath). As a result, the injured myelin cannot conduct the neuronal signal and affect the nerve impulse. (Lawrence Steinman, 2003)

2.5 Signs and Symptoms of Multiple Sclerosis

MS people appear to get their first symptoms between 20 and 40. A large number of the signs and symptoms of multiple sclerosis (MS) can affect every area of the body. Every person is affected differently by the disease. Symptoms in certain people grow and deteriorate slowly over time. In most disease types, the initial signs include numbness or tingling in the limbs or on the face's side, muscle weakness, dizziness, unstable throat, and visual problems including distorted or double vision and partial blinking (Fig.2) The severity of these early signs declines for months or even years in most people. But remissions are usually shorter when the condition worsens in progressive forms of MS. New signs and symptoms, including abnormal reflexes, difficulty in movement coordination and control, bladder dysfunction, and neuropsychological problems such

as depression, loss of memory, and emotional instability may appear. Finally, the impairment of motor control can eventually result in full paralysis. Fatigue is one of the most frequent and alarming symptoms of MS. It is often characterized as an intense sense of fatigue, which means it has difficulty completing even the most basic tasks. The variable mood of MS patients often correlates depression with MS. Many causes increase MS symptoms such as viral infections like cough, influenza, and gastrointestinal issues. Many other factors are also found in MS. (Eva Zindler 1, 2010)

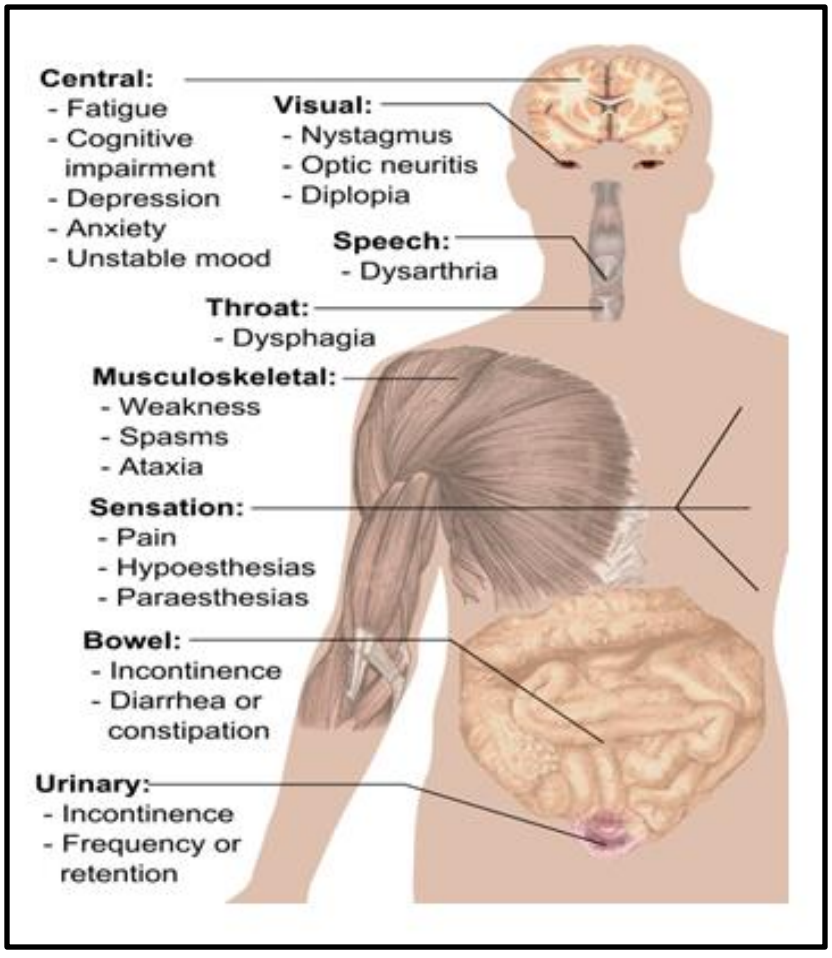

Figure 2.4: Signs and symptoms of Multiple Sclerosis

2.6 Types of Multiple Sclerosis

Four types of MS can be distinguished depending on MS duration and future MS progression. However, in 2013, the International MS Panel adopted two new categories:

(1) clinically isolated syndrome and (2) radio-isolated syndrome, but the four main types still include relapsing-remitting; primary progressive; secondary progressive, and progressive relapsing.

2.6.1 Relapsing-remitting (RRMS)

This is the most common form of MS; about 85% of people present with this pattern. RRMS presents neurological symptoms that start acute or sub-acute and can be fully or partially recovered by individuals. The observed symptoms of MS and this type are referred to as benign MS with low incompatibility, but in 40% of cases of this type, MS and the type malignant MS with a high disability develop into MS. The clinically isolated syndrome was called Benign MS, where demyelination has been reported for some signs of MS but 30 to 70% of the clinically isolated syndrome has become MS. (Pittock, 2008)

2.6.2 Primary progressive

The key progressive form involves MS impairment progression and slightly improved MS symptoms. The relapsing-remitting type (10%–20%) developed into the primary progressive type. The relapsing-remitting type converted into a primary progressive type for almost ten years. The average starting age is roughly 40, later than RRMS, and more males are affected and hence the female ratio is equal to that for men. (A J Thompson 1, june 1997)

2.6.3 Secondary progressive

Patients with relapse-remitting MS (65%) have been secondary progressive MS without remission, with a progressive neurological decrease. A Canadian study shows that within 6–10 years of onset of illness 41 percent of the people with RRMS reached the secondary progressive period, up to 58 percent from 11 to 15 years from the start. SPMS individuals can also have recurrence overlapping. (B G Weinshenker 1, Feb 1989)

2.6.4 Progressive relapsing

PRMS is characterized as a progressive disease with superimposed recurrences from the beginning. PRMS is known to be largely identical to PPMS. Progressive disease prevalence. Roughly 10%–15% of PPMS people would have recurrences superimposed. (F D Lublin 1, April 1996)

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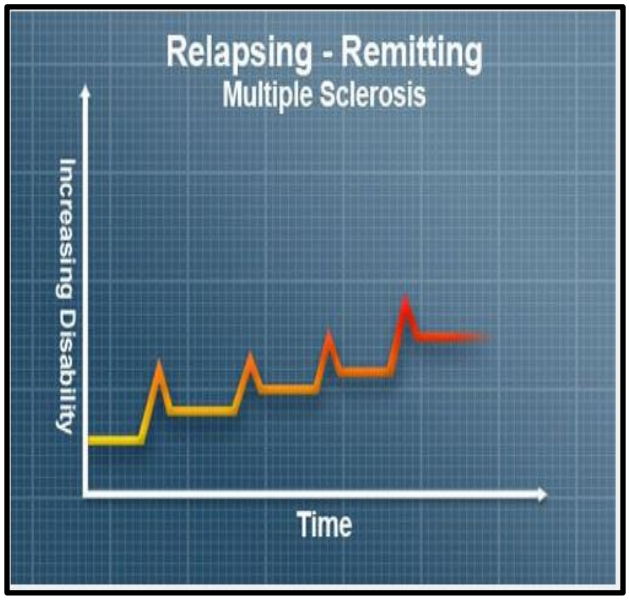

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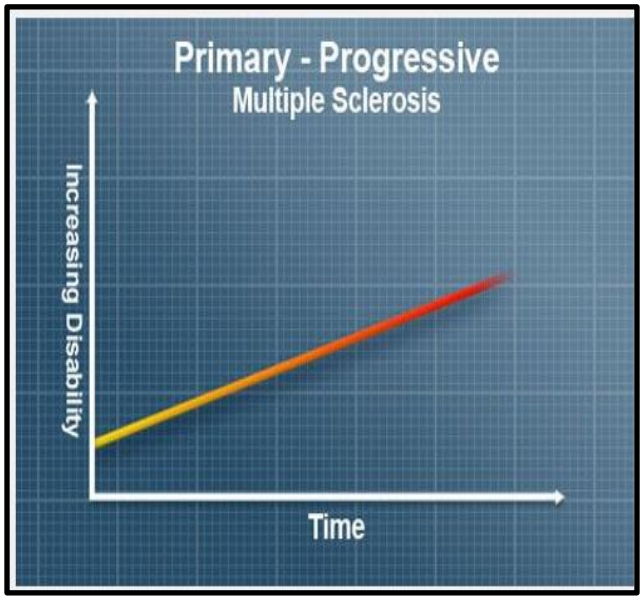

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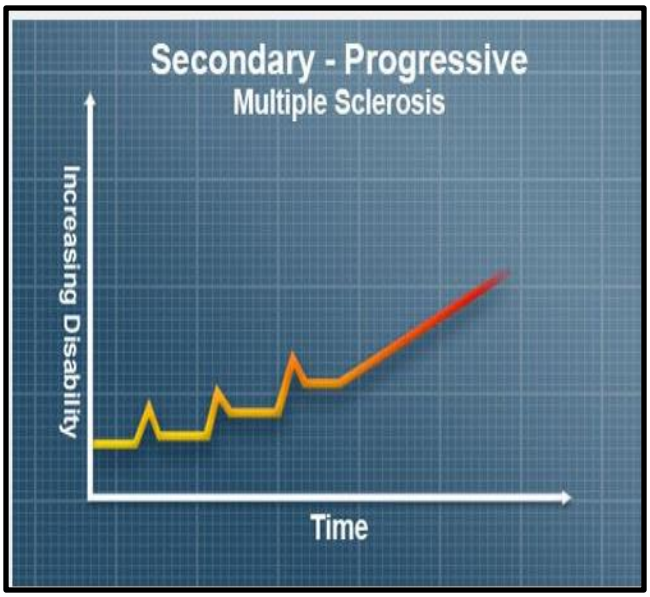

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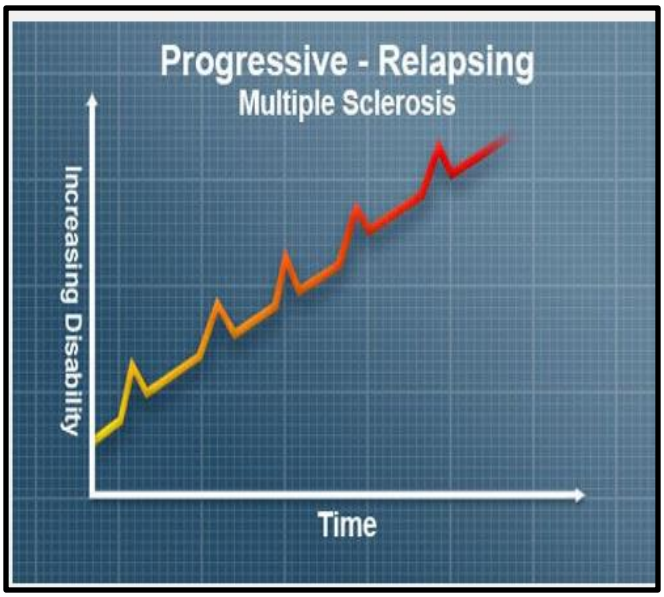


Figure 2.5: Types of Multiple Sclerosis (Charles Patrick Davis,2020)

2.7 Multiple Sclerosis Causes

No reason for the incidence of MS has so far been provided, however, a combination of environmental and hereditary factors can be included, including pollution, viral and bacterial infections, and stress. MS can be caused by several causes, and these can be summarised in microbial, viral, and other infections.

2.7.1 Microbial infection

The key processes in which MS is introduced and increases can be induced by many microbes, which increase microbial infection to cause MS, from one position to the next. MS can be induced by many microbes. The hygiene hypothesis and prevalence hypothesis can be linked to the mechanism of induction of MS. The hygiene assumes that microbial infections are protective in early life, but MS answers the early infection in the later lifetime afterward. whereas the prevalence assumption indicates that MS is dependent on factors of infection that have been reported in the regions with a high proportion of MS patients among the population. Hygiene

hypotheses. The results from MS patients support the hygiene hypothesis other than the prevalence hypothesis. (Alberto Ascherio 1, jun 2007)

2.7.2 Viral infection

Three pieces of evidence are supporting viral infections in MS: (1) oligoclonal rubber fluid in patients who suffer from MS; (2) many human demyelinated viruses; (3) animal demyelinated viral infection. Besides, no data are present on the person-to-person transmission during MS incidence, depending on MS in the temperate zone, greater distance from the equator, and increased occurrence in the vicinity of the regions with the highest MS levels. (1 C. H., oct, 2015)

2.7.3 Other Infections

Smoking and stress can also contribute to MS. It can also be caused by environmental toxins, especially solvent exposure. The MS may be associated with a polluted diet and hormonal intake. Gout disease in MS patients with lower levels of uric acid is not reported. It can also be caused by dysregulation in the neurovisceral integration of cardiovascular tone that may cause multiple clinical symptoms associated with MS. The neurodegenerative and inflammatory progress of MS is disrupted and can lead to autonomous cardiovascular dysfunction. (1 Z. S., jan 2016)

2.8 Diagnostic Approaches

No specific markers for MS diagnosis are available. The diagnosis relies largely on medical and neurological records. It is therefore critical that the attacks are properly described. In the absence of fever or another disease, these attacks are characterized as new neurology deficits of more than 24 hours that may be correlated to an anatomic site. The neurological deficiency typically develops inferiorly over two to 4 weeks and generally resolves completely or in sections throughout 6 to 8 weeks, either spontaneously or after corticosteroid therapy. In the MRI, a single anatomical region may be involved, as a monofocal attack, or more than one anatomical CNS region can be involved, which is commonly seen in multifocal attacks. In a patient who has an attack, MRI with an intravenous (iv) contrasting agent that contains gadolinium is the most significant paraclinical test to validate the diagnosis. The existence of lesions (inflammatory and

demyelinating) for differential diagnosis can also be present and lesions can be distributed throughout the CNS. Lumbar puncture (CSF) exams and basic CSF biochemistry (glucose, protein, albumin, IgG, and lactate levels) microbiological tests (cell counting, and if necessary, other microbial and ELISA tests), cytopathological assessing (screening of malignant cells), and intrathecal immunoglobulin G (IgG) synthesis are also an important test in the diagnostics of MS, Besides that, electrophysiological tests can be carried out when required (visual evocative potential [VEP] and somatosensory evocative potential [SEP]). (Bogdan F. Gh. Popescu, Aug 2013)The diagnostic analysis of a patient with suspected MS is summarised in Table 2.

Primary Tests	
1.Blood tests	Different types of tests were used to diagnose the disease.
2.MRI	(cranial, cervical, and thoracal)
3.CSF analyses	
4.In patients with optic neuritis	VEP and optic coherence tomography
Secondary Tests	
1.Evoked potentials (VEP ve SEP)	
2.Optic coherence tomography	
3.Urodynamic testing	
4.Cognitive testing	

Table 2.2: Investigations for the diagnosis of Multiple Sclerosis.

2.9 Treatment methods for Multiple Sclerosis

The therapeutic possibilities for MS depend on the clinical situation. In patients with relapse-relief disease and patients with the secondary progressive process, I discuss separately the treatment of relapse and disease-modification.

2.9.1 Treatment of Relapse

The reciprocal therapy is independent of whether it happens in reciprocal patients or the second phase of the disease. While almost all relapse patients show a certain degree of spontaneous recovery, most physicians prescribe relapse therapy that has a major impact on their function. The first-choice medication has been corticosteroids for several years. Corticosteroids shorten the reciprocal duration and speed up recovery, but the overall degree of recovery or long-term success is not persuasive. The most common regimen consists of an intravenous IV (500-1000 mg per day for 3 to 5 days), high-dose methylprednisolone sodium succinate course. Some clinicians replace IV methylprednisolone with oral prednisone, which is easier to use and less costly. Data have been presented, however, that demonstrate the comparable benefits in acute recurrence of oral prednisone and IV methylprednisolone. (D Barnes 1, Mar 1997)

2.9.2 Disease-modifying treatment of relapsing-remitting multiple sclerosis

Treatment aims to minimize the frequency and severity of recipients (and thereby avoid exacerbations) and to prevent or adjourn the initiation of the progressive phase of the disease in patients with repetitive multiple sclerosis. In the past, in particular, they were used to achieve this purpose, but due to low efficiency and high toxicity, they have never been generally accepted. More recently, major randomized controlled studies were conducted successfully with the following substances: beta-1a interferon, beta-1b interferon, glatiramer acetate, and Mitoxantrone. (Table 3). The US and European Regulatory Agencies for the Treatment of Multiple Sclerosis Relief-Treatment currently have 2 types of recombinant interferon beta, interferon beta-1a, and interferon beta-1b. The interferon beta-1a is a glycosylated, mammalian-cell recombinant product with an amino acid sequence similar to the natural interferon-beta sequence. Interferon beta-1b is a recombinant nonglycosylated product from bacterial cells where cysteine is replaced by serine at 17.

2.9.3 Interferon Beta

Interferons are naturally made in human lymphocytes that influence the development of protein products and modulate the immune system. The interferon β-1a includes Avonex and Rebif, but their strength and the route of injection vary. Avonex is prepackaged with an intramuscular dosage of 30 mg once a week. Rebif is supplied with 22 mg and 44 mg intakes and is subcutaneously injected 3 times a week, starting with the lower dose therapy. Betaseron, b-1b, is administered every other day subcutaneously. Interferon-b was shown to reduce the aggression rate in patients with RRMS in different class I studies and also to reduce T2 lesion burden in MRI. Long-term follow-up research, however, shows little evidence that sustainable development of impairment is slowed. (1 D. S., Dec 2008)

2.9.4 Glatiramer acetate

It is a synthetic medication that affects antigen presentation, is given as one 20 mg subcutaneous injection a day. Glatiramer acetate has been shown in many class I studies to decrease the rate of attack in RRMS patients, minimize lesion load on T2W MRI, and likely slow continuous progression in disability. For the care of any patient suffering from RRMS, glatiramer acetate should be considered. While glatiramer acetate may also be useful in patients with a disease, this hypothesis cannot be supported in any convincing evidence.

2.9.5 Mitoxantrone

It is a chemotherapeutic anthrocenedion (similar to adriamycin) that has been approved for treating relapse and secondary incremental MS in RRMS. The use of mitoxantrone in RRMS is supported in three clinical trials, including a major phase III review. However, its use in certain patients at remission five years or longer after treatment is correlated with cardiovascular toxicity and leukemia. In patients with aggressive RRMS (above two relapses per year or worse lesion load during the treatment of the primary disease agent) and potentially patients with low IFN-β responses, mitoxantrone is currently best indicated. But mitoxantrone can at most be used as a remission inducer in RRMS, provided dosing limitations. Other DMDs are required for maintenance therapy. Perhaps the best combination is a short mitoxantrone route with a DMD with a strong safety profile, such as b-interferon or glatiramer acetate. The use of mitoxantrone

in primary PPMS and subsequent phases of SPMR is backed by no robust evidence. (R Ellis 1, Apr 2009)

Agent	Dose	Major side effects
IFNB 1b	250 ug SC every other day	Flue like illness, liver function, and thyroid function abnormalities
IFNB 1a (IM)	30 ug IM weekly	Same as above
IFNB1a (SC)	44 ug thrice weekly	Same as above
Glatiramer acetate	20 mg SC daily	Injection site reactions, urticarial, lymphadenopathy
Mitoxantrone	12 mg/m2 IV every 3 months (max 140 mg/m2)	Cardiomyopathy, leucopenia, treatment-related leukemia. Infections infertility

Table 2.3: Disease-modifying drugs in multiple sclerosis

2.10 Symptomatic Treatments

Pharmaceutical and physical therapies that target the symptoms resulting from CNS damage refer to symptomatic therapies. (Table 4). These therapies are not MS-specific in general. They include bladder dysfunction anticholinergics (which can lead to cognitive decline, which involves an individualistic approach), and medications for neuropathic pain (tricyclic antidepressants or gabapentin and derivatives, typically). Cognitive disability therapy in MS is

complex and focuses on eliminating potential contributors. Several symptomatic treatments were already approved for MS. These include spasticity Sativex and walking problems fampridine. Sleep is an important component of symptomatic treatment. The prevalence of sleeping issues increases with MS. More common among those experiencing sleep deprivations are rises and anxiety, depression, and fatigue. (Markus Kipp1, Oct 2016)

Symptoms	**Treatments**		
	Pharmacological		**Non-pharmacological**
	Drug	Dosage	
Spasticity	Baclofen	10-150mg/d	Physiotherapy
	Tizanidine	2-24mg/d	Treat,concurrent, infections
	Diazepam	5-30mgm/d	
Fatigue	Amantadine	200-400 mg/d	Exclude, depression
	Modafinil	200-400 mg/d	Exclude, Hypothyroidism
Intension	Propranolol	40-120mg/d	Physiotherapy
	Gabapentin	300-2400mg/d	

Table 2.4: Symptomatic treatment of MS

2.11 Development of Multiple Sclerosis therapeutic targets using the EAE Model

Current therapies also are only partially effective and are primarily targeting the inflammatory process of the disease, while the neurodegenerative aspect possibly constitutes the biggest challenge for potential therapies. After self-sensitization in humans, attempts have been made to replicate this disease in animal models based on inflammatory disease, partially MS-like disease. Immunization of emulsions of brain tissue dissolved by saline was conducted in the early stages of this study. This led to a similar illness as previously reported in humans, but in a fraction of the animals sensitized over a long time and following several immunizations. To boost reproductiveness, powerful adjuvants, mostly complete Freund's adjuvant, have been added into the sensitization Protocol, enabling slow release from the inoculum of the sensitizing antigen and using inactivated mycobacteria for massive immune stimuli. One consequence of this adjuvant is the massive stimulation of phagocytic absorption and presentation of antigens and the subsequent CD4+ T-lymphocytes activation and expansion. Thus almost all the models used until now have a bias against a certain immune reaction mediated by class II of the MHC. The disease induced by this sensitization process has been named experimental autoimmune encephalomyelitis which is currently one of the most commonly used immunological and brain inflammation research models in vivo. EAE is the most prevalent animal model useful to explore neuroinflammatory mechanisms and is also a 'proof-of-principle model for the effectiveness of new treatment methods. In many different animals EAE can be induced (e.g. mice, rats, mini swine, guinea pigs, chickens, or primates). However, the mouse has become the most commonly used organism, partially because of the expanded repertoire of sophisticated transgenic or knockout mice. EAE's pathophysiology is focused on the immune system's response to antigens in the brain. This impact contributes to inflammation of antigen carrying structure degradation, resulting in similar neurological and pathological characteristics to MS. Three distinct methods can be seen: active induced EAE passively transmitted EAE, and recently spontaneous model EAE mouse models, enabling autoimmune processes without exogenous manipulation to be studied. AEAE in the mouse produces simple and robust results is the easiest inducible model. This model is known by many researchers in the field as the "gold standard" for neuroimmunological animal models. (Gurumoorthy Krishnamoorthy 1, Aug 2009)

2.12 Correlation between EAE and Multiple Sclerosis studies

EAE is very complex, some effective therapies in EAE also need to be tested in MS. Unsuccessful research may be prevented in EAE and to a certain degree in MS leading to bias in publishing. Although this can be assessed if there are a large number of studies with the same or very similar material, a defect/deviation against the effect size can be calculated. This is not feasible, as is often done in EAE for a few limited screening studies. In terms of the experimental conditions, EAE studies can vary widely. This involves the animal's species, strain, and sex, the era, a particular method of induction and frequency, timing, and dose under study. In genetically similar groups of animals, the majority of rodent EAE studies are conducted. This removes at least a major source of transition. However, the vulnerability of genetically identical animals to EAE can vary depending on environmental factors that cannot be monitored easily. The degree to which the intestine is colonized and the flora type will for example, largely decide EAE susceptibility. (Hiroaki Yokote, Dec 2008)

2.13 Proteomics studies in EAE model of Multiple Sclerosis

Several studies have been reported which have examined protein markers in biological fluids of patients with multiple sclerosis or animal tissues affected by EAE. These studies employed a range of proteomic methods such as MS, 2-DE, and protein arrays. One of the goals of the proteomics test is to detect protein expression changes that can influence the onset and growth of diseases in patients with multiple sclerosis. Traditionally, 2-DE and more recently MS techniques have been used to measure high protein output. Therefore the quantification and characterization of proteins are based on proteomic technologies. Typically these methods are used for calculating relative and not absolute amounts. The protein mixture is first separated by pl by acidic to basic properties with the help of 2-DE and then followed by diagonal resolution according to the size. The strength of protein spot can be compared with software help for protein levels to adjust. Because several proteins in cells undergo PTM, a single protein could be depicted on a 2-DE gel in multiple spots. An increase or decrease of the strength of a spot, therefore, represents modifications to only the level of this specific "version" of that particular protein rather than all the variants of the protein translated from the same gene. It is important to track changes in the protein expression through alternatives methods such as Western Blotting and functional alterations can be evaluated by using activity assays. (Li2, 2009)

2.14 Role of Rab 32 in the EAE model of Multiple Sclerosis

In addition to the disturbing mitochondrial dynamics, the activation of ER stress is a further hallmark of MS. In addition to promoting ER-mitochondria crosstalk in general, the mechanism also leads to neuronal death through increase the appearance of ER and mitochondria in the so-called mitochondrial associated membrane (MAM). Rab32 is a popular regulatory MAM protein. This GTPase is located in ER and mitochondria where ER-mitochondrial interactions and mitochondrial dynamics are regulated. Rab32 is inflamed after the brain in a mouse model. Our data suggest that ER stress contributes to Rab32 and takes place in the microscopic brain, consistent with a significant function for neuroinflammation. These results improve our understanding of the function of Rab32 in impairing cell survival and neuronal mitochondrial dynamics. (Yohannes Haile, 2017).

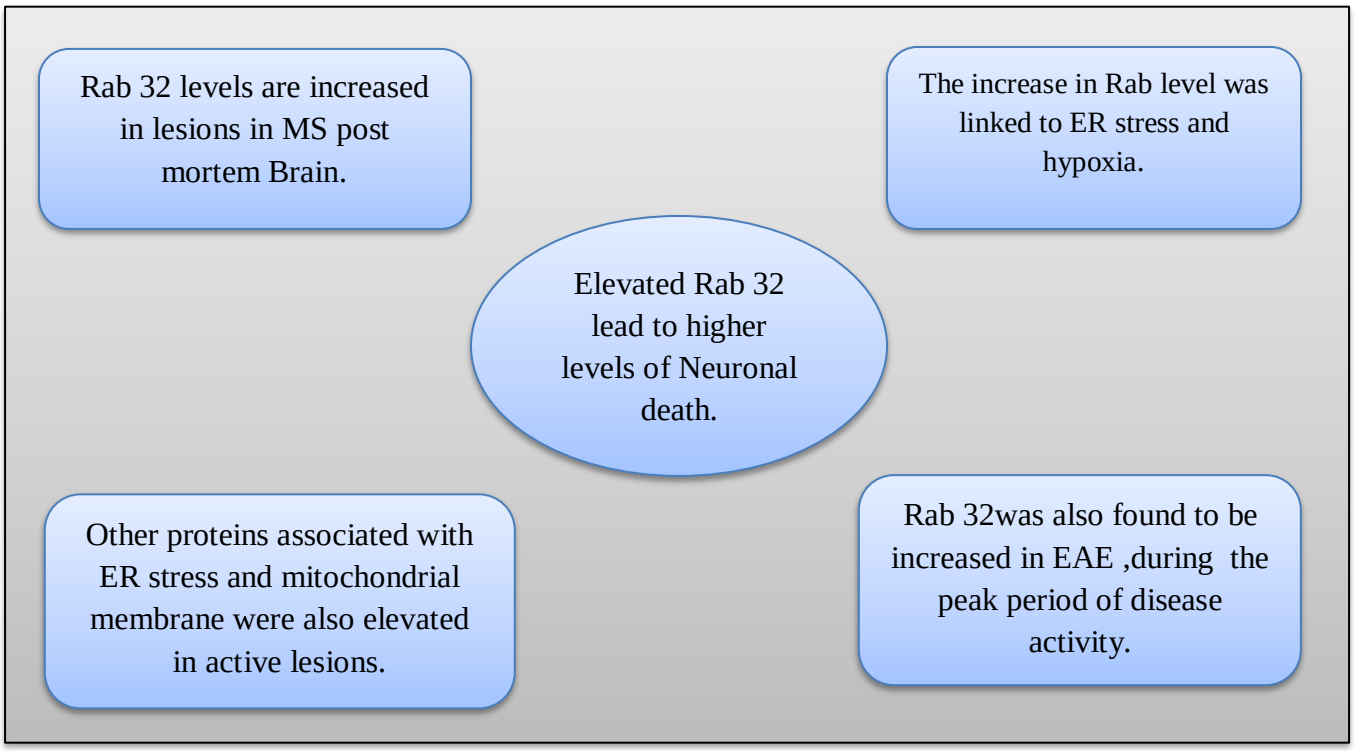


Figure 2.6: Mitochondrial Stree and ER stress associated with Rab 32 in Multiple Sclerosis

2.15 Role of HMGP1 in EAE model of Multiple Sclerosis

Because of its implication in EAE/MS pathogenesis, the high mobility box-1 group (HMGB1) protein has emerged as a possible candidate. The HMGB1-based agent shows promising results mainly from inhibition of HMGB1 in the experimental MS model, namely experimental autoimmune encephalomyelitis (EAE). HMGB1 is present in the CNS and HMGB1 endogenous cell nuclei as is in the MS patients' cerebrospinal fluid CSF, as well as the Anger, TLR2, and TLR4 receptors. Furthermore, in the active MS lesions and related animal models of experimental autoimmune Encephalomyelitis, the microglia, and macrophages expressed in cytosolic HMGB1, are increasing (EAE). HMGB1 manipulated immune function by facilitating the ripening of the activation and proliferation of T cells of DC and by leading to the functional polarization of the T cells into the pro-inflammatory phenotype of the T-helpers 1 (Th1). Glycoprotein EAE (MOG) protein (200 μg) was present in all phases of disease (before admission, onset, peak, and remission), with myelin oligodendrocytes displaying a very high degree of HMGB1 serum and a steadily decreased degree of CSF. The HMGB1 levels were only peaked during the onset stage and steadily decreased in the spinal cord homogenous. Furthermore, during the EAE progression, the nuclear expression pattern of HMGB1 was unchanged in the astrocytes and microglia. Elevated HMGB1 expression in glial fibrillary acid protein nuclei (GFAP) (astrocyte) positive cells of the regular dorsal column has also been observed (spinal cord).

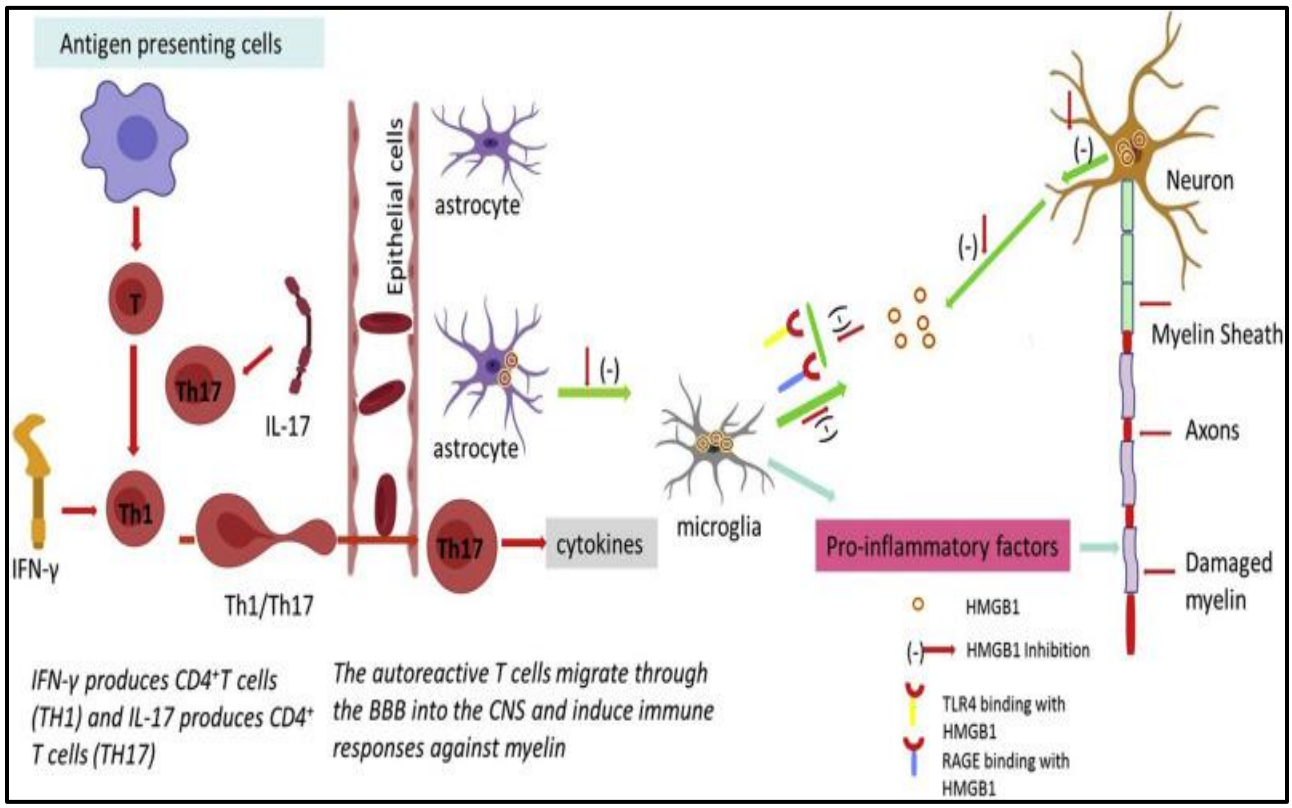


Figure 2.7: HMBP1 in Multiple Sclerosis. Activated peripheral immune cells enter the SCB by disrupting BBB, which activates microglia and astrocytes and releases neuronal disorder and demyelination cytokines. Astrocytes, microglia, and neurons release HMGB1 and lead to an EAE pathogenesis that can be blocked by the neutralization of HMGB1. (Paudel, 2019)

Chapter 3: Materials and Methods

Name	Quantity
Phosphate Buffer Saline	50ml
MOG35-55 peptide	200ug
Pertussis toxin (PTx)	400ug
Heat killed Mycobacterium tuberculosis H37RA	4mg/ml
Complete Freund's Adjuvant	1ml
Ethanol	70%
ddH2O	As required
Injection volume	0.1ml per mice
C57BL/6 mice	12
Small animal clippers	blade size 50
Test tube	15ml

Table 3.1: List of Materials used in the Experiment

3.1 Ethics Statement

All experiments were performed as per rules of the Institute of Laboratory Animal Research, National Institue of Health, USA (Eighth Edition, 2011)

3.2 Study Design

Our study design is divided into 3 phases

Phase 1: Phase 1 of this study involves the preparation of the Animal model and Sample collection.

Phase 2: This phase involves three steps which are 2D Gel Electrophoresis, Protein detection, and Identification, and MS/MS Analysis.

Phase 3: This phase involves the Characterization process which includes Protein-Protein Interaction, Protein Structural Analysis, and Protein Pathway Analysis.

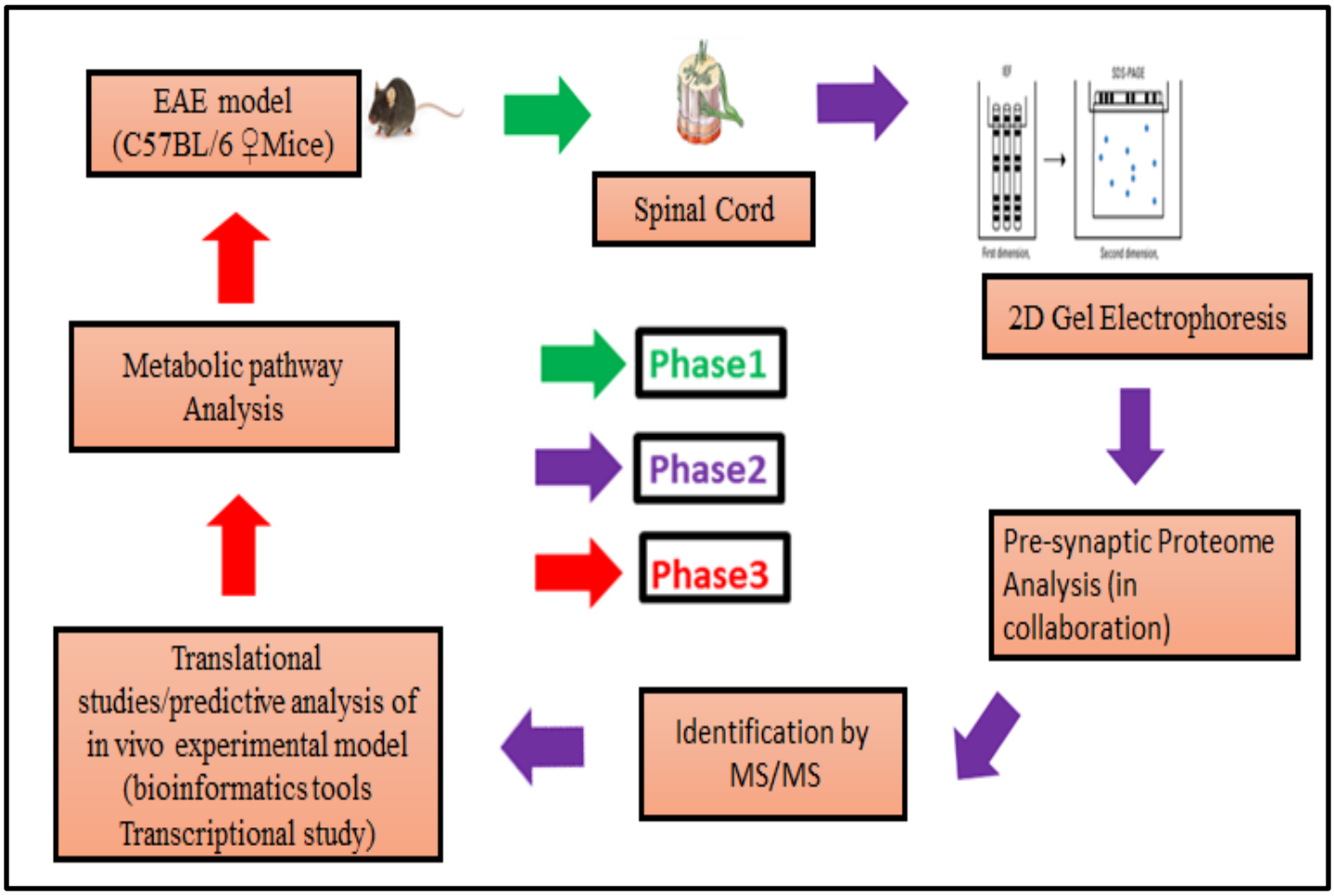


Figure 3.1: This figure shows the study design of our research which is divided into 3 phases. Phase 1, phase 2, and Phase 3.

3.3 Experimental Group

Three experimental groups were used in this method and all were matched concerning:

- Sex
- Age
- Genetic modifications
- Other conditions like housing conditions etc

3.3.1 Normal Control Group

This is the first normal control group which consisted of four mice and in this group, the Mice were free of any injection and thus remain asymptomatic. There was no injection given to them in this group.

3.3.2 No MOG35-55 Control Group

This is the second group, which also consisted of 4 four mice and in this group, the mice were treated with all the reagents of EAE except MOG35-55. The mice were given the injection of CFA only.

3.3.3 MOG35-55 Group

This is the third group which consisted of four mice and in this group, the mice received all the complete EAE injections.

3.4 Basic Protocol

1- Preparation of peptide (MOG35-55) and Adjuvant (CFA)
2- Preparation of Pertussis Toxin (PTx)
3- Injection of Antigen/adjuvant solution subcutaneously and Pertussis toxin Intraperitoneal
4- Mice were again injected with PTx after 2 days
5- Weight and clinical score are evaluated daily for symptoms
6- Between day 9 and 14, clinical symptoms will typically be detected.

3.5 Phase 1 : Preparation of an Animal Model

3.5.1 Preparation of MOG35-55 Emulsion

Each mice should be injected with 200 µl of 1:1 MOG35-55 peptide and CFA solution. As the viscous emulsion is somewhat lost during the preparation and injection so prepare the required amount of 1.5*2x. And then divide the emulsion by 2 for the measurement of the total volume. Dilute lyophilized MOG35-55 in ddH2O to 2 mg/ml at the final concentration. Typically we have 200 µg per mouse of MOG35-55 peptide. Store the peptide solution at -20 °C. Now place the 100mg of dried Mycobacterium tuberculosis in a mortar and grind it well. To obtain a 10 mg/ml CFA, add 10 ml of Freund's incomplete adjutants and store them at 4C. Before immunization, dilute the CFA stock solution with IFA to a final concentration of 2 mg/ml. Mix 1:1 with a solution of MOG35-55 peptide until 1 mg/ml is obtained at the final concentration. Draw 1 ml in 2 mg/ml of CFA and 1 ml in two 2 ml syringes of MOG35-55 solution. Calculate the number of syringes required by the number of animals immunized. For MOG35-55 the cannula 27 G and for CFA the cannula 20G is required. Stop air bubbles and use a three-way valve to connect all syringes. For a minimum of 10 minutes, send the emulsion from one syringe to the other, which is a crucial phase. And at the end, the solution should be white and stiff. The emulsion can be processed several days before the immunization. Wait for at least 30 minutes to check if the emulsions are stable. Draw the solution in one of two syringes before immunization and attach a cannula of 27 G.

3.5.2 Development of EAE with MOG immunization

C57/BL6 female mice were purchased from NIH Islamabad having body weight ranging from 18-20 grams. All these animals were maintained under specific pathogen-free conditions and were fed in the ASAB animal house at NUST. For the development of active EAE, these female mice were injected with 200ug of MOG35-55 (Sigma) emulsified in complete Freund’s Adjuvant (CFA) supplemented with 400ug Mycobacterium tuberculosis (Sigma) to a final concentration of 1mg/ml. Emulsions were prepared using an Omni homogenizer mixer and were injected subcutaneously at 4 sites near each limb. 400ug of pertussis toxin (Sigma) emulsified in PBS were injected at the time of immunization and 48 hours later. Each treatment group had at least

four mice included. Together with a clinical outcome as previously mentioned, animals were weighed and scored daily for clinical signs of disease. Mice were sacrificed at 17 days after the inoculation for acute stage inflammation in the EAE and their associated negative controls (dpi). Mice and their corresponding negative controls were slaughtered at 57 dpi for chronic stage inflammation during EAE. Few of the inoculated mice had healed and no inflammation was seen at the end, so they were placed into the 'recovered' category. The mice were injected with PBS on the day of sacrifice so they could prevent the spinal cord from being infected with blood and removed from the spinal cord. The cervical area of the spinal cord was taken, the remainder of the spinal card was extracted and the snaps were frozen for immunofluorescence and frozen for safety analysis.

Figure 3.2: Development of Animal model with MOG Immunization

3.5.3 Preparation of Antigen Peptide

MOG 35-55 peptide was prepared by mixing lyophilized MOG in ddH20 to a final concentration of 2mg/ml and stored the peptide solution at -20C. Then CFA was prepared by placing 100mg of desiccated Mycobacterium tuberculosis into a mortar and grind into a thin powder. To obtain the stock solution of 10mg/ml, 10ml of CFA was added to the thin powder of Mycobacterium tuberculosis and was stored at 4C. On the day of immunization CFA, the stock solution was diluted with IFA to obtain a final concentration of 2mg/ml. Then the obtained solution was

resuspended well and was placed on ice. After this 1ml of CFA and 1ml of MOG35-55 was drawn by using 2ml separate syringes, one for each.

3.5.4 Preparation of Pertussis toxin

To prepare 100 microgram /ml stock solution of PTx, recast 250mg of PTx into 500ml of ddH2O and was stored at 4degree. On the day of immunization, PTx stock solution was diluted with 150 PBS. Then for injection 200 microliter of diluted PTx solution was prepared by using which contain 400ng of PTx.

Figure 3.3: Injection of Pertusssin toxin to increase the severity of the disease.

3.5.5 Sample preparation, SDS-PAGE, and Western blotting

Different buffers were used for the lysis of tissue lysates of frozen spinal cord samples. The 10%w/v of tissue lysates were taken and 7 M urea, 2M thiourea, 4% CHAPS, 2020µl/ml ampholytes, 10mglml DDT, protease, and phosphate inhibitors were added in a homogenized mixture of tissue lysates and were followed by ultracentrifugation at 30,000 RPM for a duration of 30min. Then, the Bradford test was used to assess protein concentrations in tissue lysates (Bio-Rad). After mixing samples with 4X Roti-Load (ROTH) as sample loading buffer, the samples were boiled for 5 min at 95°C. Samples were cooled down and further used for SDS-PAGE- and western blotting.

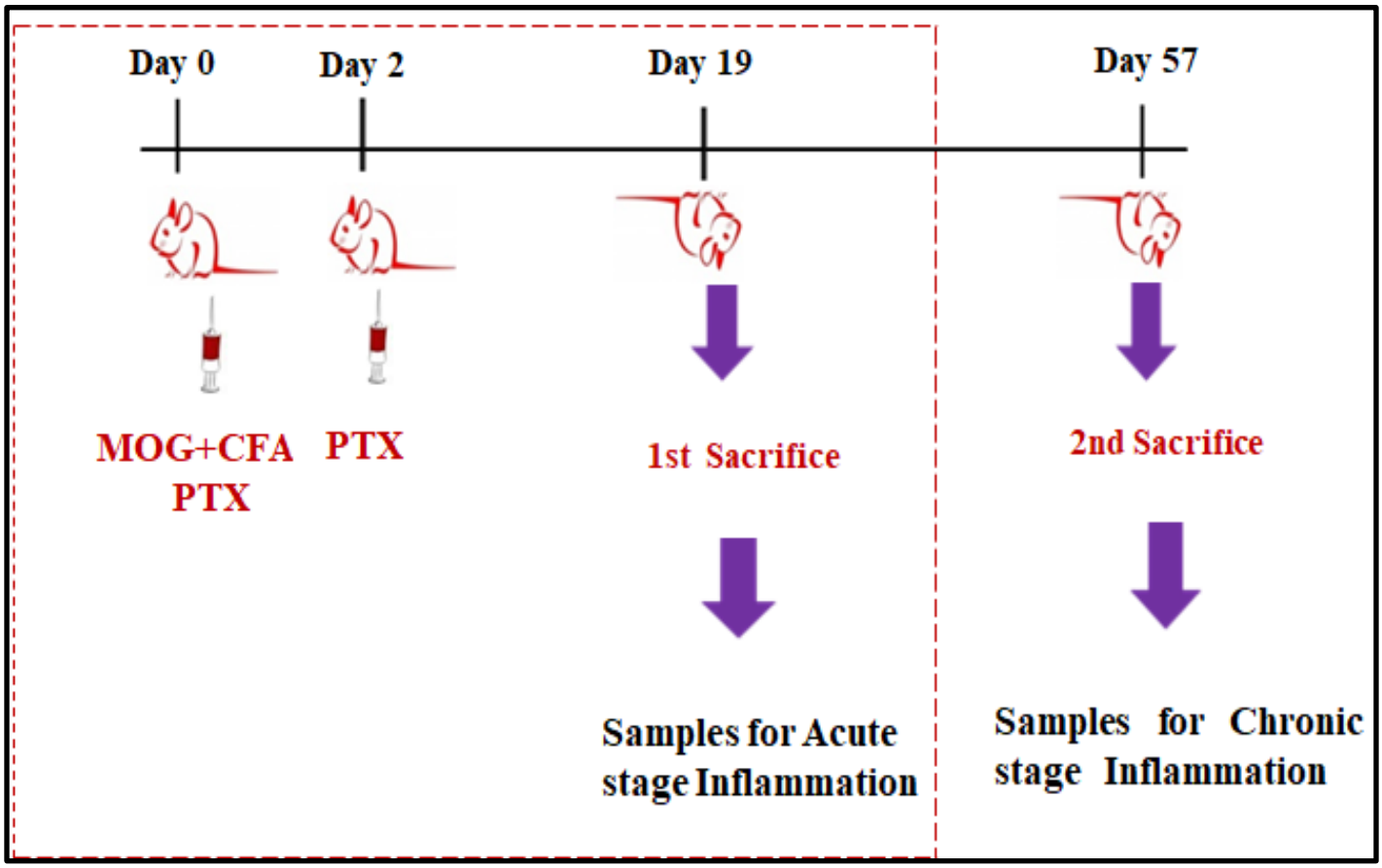


Figure 3.4: Mice Immunization and Sample collection

3.6 Phase 2 : 2D Gel Electrophoresis and MS/MS Analysis

3.6.1 2D gel Electrophoresis, visualization, and Protein spots Analysis

The primary technique for proteomics work is 2D gel electrophoresis or 2D-PAGE. It divides the complex sample mixture into two distinct protein properties. Protein in the first dimension is divided by the pI value and the relative molecular weight in the second dimension. 2D gels were used for visualization of protein spots with the help of silver spots and then screened for further analyses on protein spots (CanoScan Scanner). DECODON Delta 2D was used to check the

difference in protein expression among various groups by using a 1.5 fold change cut-off value and a p-value of less than 0.05 in the Unpaid T-test Student.

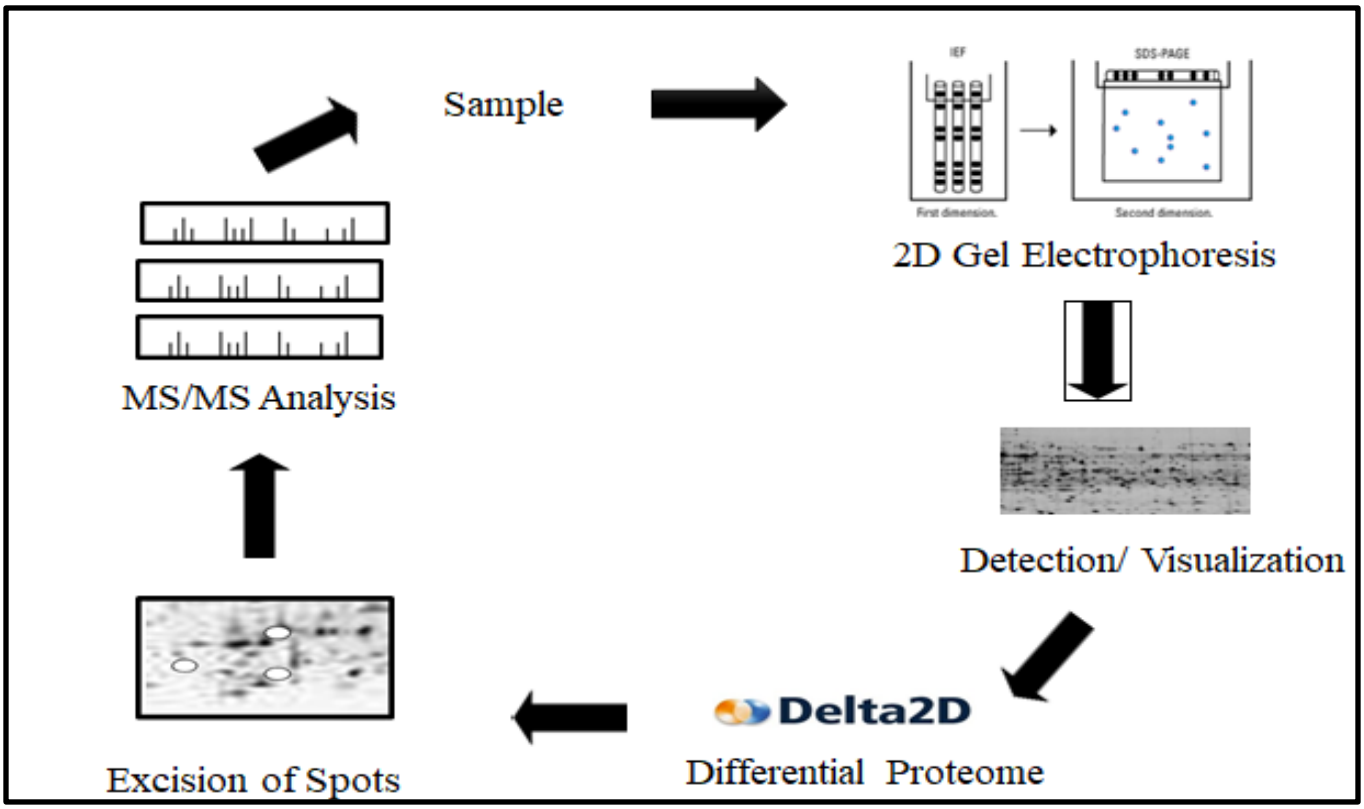


Figure 3.5: Phase 2 – Identification and Validation of Proteins.

3.6.2 Mass spectrometry for peptide sequence

Modified protein sites have been removed from a 2DE gel stained cell and re-processed (silver color, reduction of disulfide bonds, free cysteines alkylation, digestion of trypsin, extraction of the peptide, and detection of peptide sequence by using orbitrap mass spectrometry. Once proteins were identified with mass spectrometry, some careful criteria of peptide count greater or equal to 2, peptide limit up to 95%, and low fusion rate up to 0.01% were taken at the time of the suitability of the identified proteins to exclude any false identification in proteomics data for further investigations.

3.6.3 Co-immunofluorescence

Compressed spinal tissue segments (5 µm) from each EAE (acute phase), EAE (chronic phase), CFA controls, and their associated methanol-related controls (9 min.) followed by acetone (1 min.). Then PBS was used to wash the segments of tissue while the permeabilization buffer consists of (PBS + 0.2% Triton X-100) was used to expose antigenic site on tissue components for 10 minutes. After this, 5% bovine serum albumin in PBS was used to inhibit the unspecified proteins for 2 hours followed by overnight infusion with Rab7 (1: 100) as a primary antibody (pAb) purified in 5% serum and PBS overnight at 4 ° C. Secondary antibody labeled with Horseradish peroxidase (HRP) was applied for 2h and nuclei were infused with To-Pro3 for 10 minutes. Classes were shown using Confocal Laser Scanning. ImageJ software (WCIF plugin) is used to analyze the local pattern creation pattern.

3.6.4 Sucrose density gradient formation

The gradient density of Sucrose was developed to study the formation of oligomers under conditions of inflammatory stress during EAE. Spinal tissue samples were mixed with 10% w / v in 1x PBS containing 2% w / v sarkosyl. A continuous gradient of 10–50% sucrose is formed by 2mL ultracentrifugation tubes followed by placing homogenates on top of them. Ultracentrifugation is performed at 50,000 rpm in 73 minutes. at 4 ° C and five fractions were collected from top to bottom in each sample.

3.6.5 Statistical Analysis

GraphPad Prism 5 was used to analyze the data and errors were displayed as SEM. Non-standard criteria tests were used to calculate the statistical significance and the abnormalities of the ANOVA one method followed by a multidisciplinary test of Turkey. When the values of $P< 0.05$, $P< 0.01$, and $P<0.001$ were like this the findings were considered significant

3.7 Phase 3 : Bioinformatics Analysis through Ligplot and GRAMM-X Protein-Protein Docking Server (http://vakser.compbio.ku.edu/resources/gramm/grammx/)

3.7.1 : Molecular Docking through GRAMM-X

Molecular interactions including protein-protein, enzyme-substrate, protein-nucleic acid, drug-protein, and drug-nucleic acid play important roles in many essential biological processes, such as signal transduction, transport, cell regulation, gene expression control, enzyme inhibition, antibody–antigen recognition, and even the assembly of multi-domain proteins. These interactions very often lead to the formation of stable protein–protein or protein-ligand complexes that are essential to perform their biological functions. The tertiary structure of proteins is necessary to understand the binding mode and affinity between interacting molecules. However, it is often difficult and expensive to obtain complex structures by experimental methods, such as X-ray crystallography or NMR. Thus, docking computation is considered an important approach for understanding the protein-protein or protein-ligand interactions. (Alejandra Hernández-Santoyo, 21st May 2012)

Bank (PDB) and Worldwide Protein Data Bank (wwPDB) have over 88000 protein structures, many of which play vital roles in critical metabolic pathways that may be regarded as potential therapeutic targets — and specific databases containing structures of binary complexes become available, together with information about their binding affinities, such as in PDBBIND, PLD, AffinDB and BindDB molecular docking procedures improve, getting more importance than ever. (Alejandra Hernández-Santoyo, 21st May 2012).

Molecular docking through GRAMM-X is a widely used computer simulation procedure to predict the conformation of a receptor-ligand complex, where the receptor is usually a protein which is HMGB1 and the ligand is RAB 32. The docking steps includes :

1- First the PDB files of HMGB1 and RAB 32 were generated from the Protein Data Bank (PDB).
2- GRAMM-X was used for the docking of HMGB1 and RAB 32.
3- Docking of these two proteins gave interaction between these two and also showed different interactive sites with different bond length.

4- The interaction between HMGB1 and RAB 32 provide pathway for their further role in pathogenesis of Multiple Sclerosis.

3.7.2 Interpretation of Data using LIGPLOT

Fasta files of HMGB1 protein and RAB 32 were downloaded from Uniprot and then uploaded on the PDBsum server. Further data was validated which gives all the information about the 3D structure of each protein. The interaction between the proteins and the interaction of the protein with the ligand was also examined through LIGPLOT. And then the data was examined through Scaffold.

3.7.3 Determination of 3-dimentional structure of protein through Ligplot

The main tool used in determining the structure of protein is LIGPLOT that gives information about protein structure, its interaction with ligands and other molecules and also determine their role in multiple sclerosis. The structure of a ligand in any complex with the target protein that involve in Multiple Sclerosis is considered as high source of information for understanding the ligand targets complementarity. So the prediction of 3D structure of protein is very important in determining the function the protein whether the protein can interact with other ligands or not and how they interact with the other molecules. Gene structure is basically one-dimensional in which a sequence of nucleotides which is linear coded for a specific linear sequence of amino acids linked to each other in a head to tail rule manner (amino-carboxyl). This process is called translation in which the information present in the nucleotides is converted or transformed into amino acids using the genetic code thus it "expands" the single dimensional genetic code into a fully realized three-dimensional protein structure. 3-D structure of SNAP-25 gives information whether protein is misfolded are not. Accumulation of misfolded proteins causes the symptoms of disease. The original LIGPLOT program focused on specific type of interactions, most commonly between ligand and protein, including interactions with water molecules or with a specific residue. Other types of interactions might be also plotted, such as interaction with the dimerization surfaces and specific interactions with specific domains. The aim of research is to develop 3D coordinates of a protein and to study the ligand complex structure.

Chapter 4: Results

The present experimental study involves the characterization of HMGB1 protein in the progression of Multiple sclerosis using Bioinformatics tools including LIGPLOT and Scaffold. LIGPLOT is a bioinformatics program used to generate schematic illustrations of the Protein-Ligand interaction. This Program generates 2D and 3D depictions of Protein-Ligand interaction from the Library of Standard Protein Data Bank. The output obtained from Ligpot gives information about the Ions, Intermolecular forces, and the bonding between Protein and Ligand in the form of a colored and black and white file. This software can be used to show other interactions in Nucleic acid and proteins. Furthermore, it facilitates a rapid inspection of enzymes, proteins, and ligand complexes and also has a wide range of applications in Proteomics. This software custom generated data on the specific proteins that include details of secondary structure, protein-protein interaction, and binding sites.

A scaffold is a proteomic software that is used to visualize and evaluate the data generated from MS/MS proteomics experiments. It is used to check the biological relevance of samples used in Experiments. The data generated from Scaffold gives brief knowledge about Post Translational Modifications and the Specific Peptide sequences. It also gives an illustration of the data in the form of spectrum details and counts. This software not only identifies proteins intuitively but also generates a detailed list of targeted proteins. Furthermore, it compares the proteins in our samples with the already existing proteins in the Mass Spectrometry Library.

An important characteristic of this software is that it gives information about the sequence coverage of protein fragments that can be described as the number of Amino acids that are present in the specific protein sequence that matches the peptide sequence in our MS/MS data. The peptide sequence data we submitted in the software for protein identification, match with sequences of some proteins. The matched peptides represent the unique segment of the proteins and evaluate the probability of peptide sequences generated from the chosen protein. There is more than one peptide sequence that matches the same protein but is located in different parts of proteins. These sequences are indicated by red color in the MS/MS Data. The amino acid that constitutes the unique sequences gives the percentage coverage of that protein. The greater the coverage value, the greater the probability of identified protein. This software also generated a mass spectrum which is a graphical representation of relative intensities of the ions against mass

to charge ratio. The most intense ions show high abundance and are referred to as base peaks. All other peaks are measured relative to the base peak.

In this study, the data was investigated through LIGPLOT to identify the 2D structure of HMGB1 present in the disease course of Multiple Sclerosis. Furthermore, the data was assessed by Scaffold Mass Spectrometry to identify the unique peptide sequence of HMGB1 which represents the elevated level of HMGB1 present in the MS.

4.1 Phase 1: Clinical Assessment of EAE model

(a) **(b)**

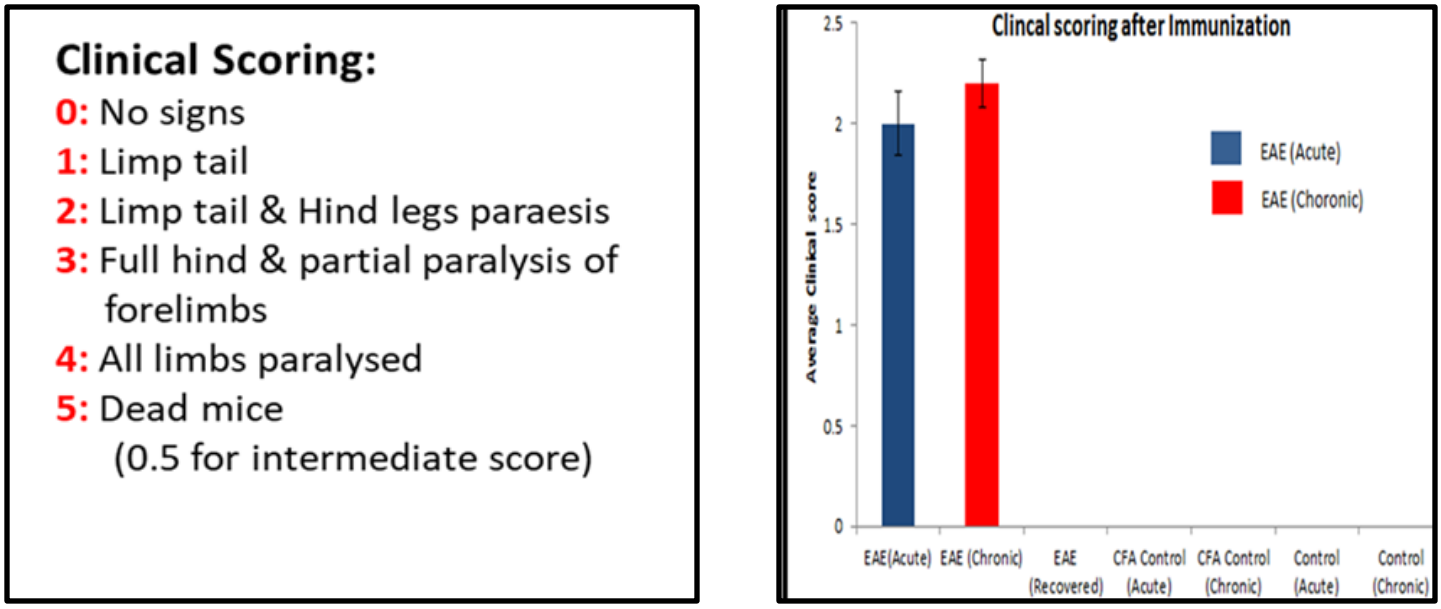


Figure 4.1: The above figure shows the clinical scoring of an EAE model. **(a)** Scoring of mice from 0 to 5 based on disease severity. **(b)** This shows the scoring after immunization with the acute stage given a maximum score of 2 while in the case of chronic EAE maximum score was 2.3.

The present study identifies the 17 differential proteins that have a potential role in Multiple Sclerosis. HMGB1 protein was further validated by Scaffold Mass Spectrometry for interactive studies. The FASTA sequence of HMGB1 was downloaded from UNIPROT and then the 3D structure was generated and Interaction with the Ligand was studied.

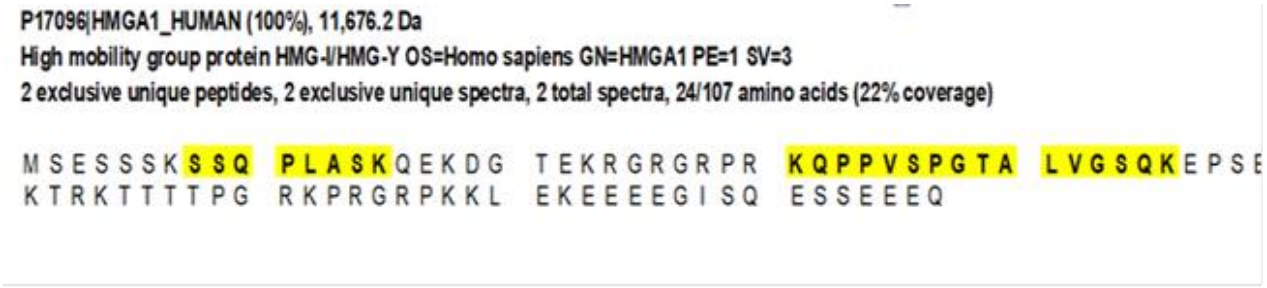
Spot	Protein name	Access. #	Mole. Weight (KDa)	pI	Peptide count	Seq. Cover. (%)	Fold Change	P Value	Regu.
Identified altered proteins during the early stage of EAE									
120	Calcineurin B homologous protein 1	P61022	22.43	4.9	8	45.60	3.92	0.002	↑
161	Synaptosomal-associated protein 25	P60879	23.31	4.6	9	51.90	2.81	0.004	↑
543	cAMP-dependent protein kinase type II-beta	P31324	46.16	4.9	12	44.70	2.13	0.042	↑
552	Secernin-1	Q9CZC8	46.32	4.6	21	50.20	1.93	0.015	↑
552	Ribonuclease inhibitor	Q91VI7	49.81	4.6	15	50.40	1.93	0.015	↑
633	Junction plakoglobin	Q02257	81.80	5.7	12	18.10	3.55	0.008	↑
708	Serpin B6	Q60854	42.59	5.5	16	45.00	3.03	0.001	↑
714	UPF0160 protein MYG1 (fragment of 47-380 a.a.)	Q9JK81	37.45	5.7	11	35.50	-2.61	0.018	↓
736	Ig gamma-1 chain C region, membrane-bound form	P01869	43.38	6.0	8	26.00	1.6	0.013	↑
752	Eukaryotic translation initiation factor 3 subunit I	Q9QZD9	36.46	5.3	17	60.00	4.43	0.002	↑
1065	6-phosphogluconolactonase	Q9CQ60	27.25	5.5	12	57.20	4.10	0.004	↑
1072	Ig gamma-1 chain C region, membrane-bound form		43.38	6.0	6	24.20	3.79	0.0008	↑
1074	High mobility group protein B1	P63158	24.89	5.6	2	12.60	-2.39	0.018	↓
1202	Growth factor receptor-bound protein 2	Q60631	25.23	5.8	9	43.30	2.09	0.018	↑
1225	EF-hand domain-containing protein D2	Q9D8Y0	26.79	5.0	9	38.30	13.54	0.009	↑
1278	Myosin light chain 3	P09542	22.42	5.0	12	65.70	2.74	0.011	↑
1330	Peroxiredoxin-4 (Fragment of 41-274 a.a.)	O08807	26.47	5.8	8	39.40	2.91	0.0006	↑

Figure 4.2: This table shows the list of 17 identified proteins in MS. The level of HMGB1 protein in this disease is downregulated.

4.2 Identification of unique peptide sequences through Scaffold

For protein identification, one sample from the EAE group and one sample from the control group were analyzed by using MS. The generated data files were converted to the SF3 file format and exported to the Scaffold database search tool (version).

a



b

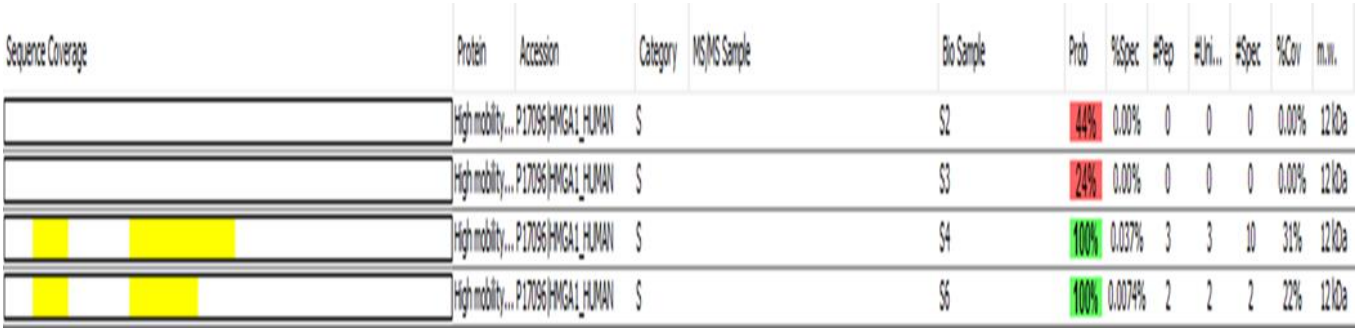


Figure 4.3: This figure shows the exported raw MS/MS data of HMGB1 protein obtained from the Scaffold database. This shows the identified peptides of the HMGB1 protein. **(a)** shows that HMGB1 protein was obtained from homo sapiens with a molecular weight of 11,676D. There were 2 exclusive unique peptides including SSQPLASK and KQPPVSPGTA, from which two exclusive unique spectra were identified with an amino acid of 24 out of 107 and with a 22% coverage. Amino acids that matched MS/MS spectra are in yellow. **(b)** It shows the probability and sequence coverage of HMGB1 protein.

4.3 Mass Spectrometry

MS was used to calculate the mass to charge ratio of molecules present in the sample of Multiple sclerosis patients. This was done to investigate the quantitative profiling and interaction of HMGB1 protein.

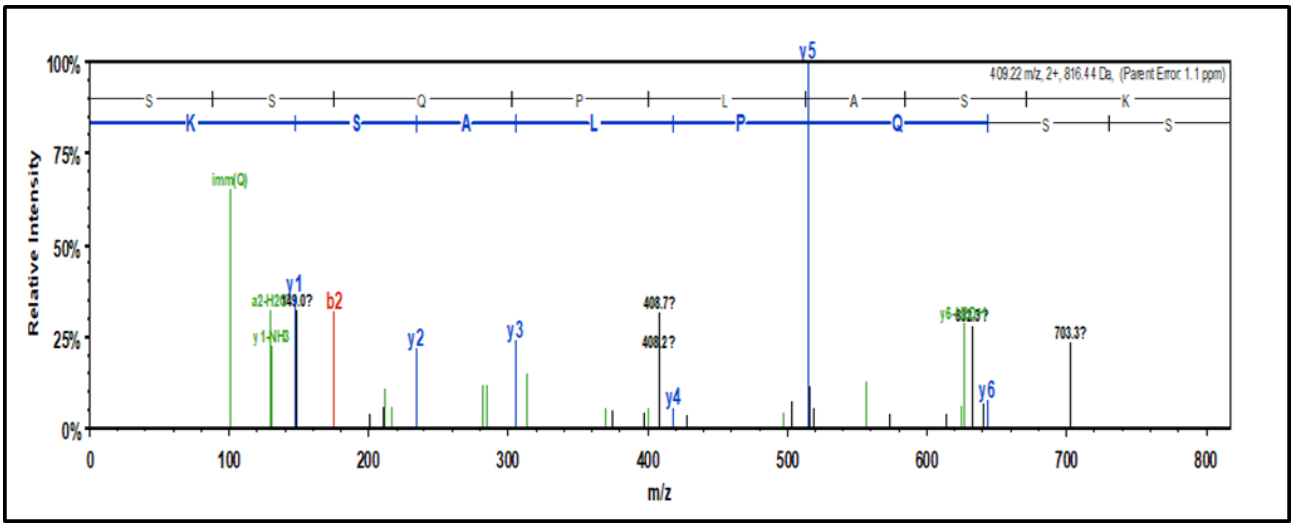


Figure 4.4: A graphical representation of sample spectrum produced by Mass Spectrometry. This is a simplified mass spectrum of HMGB1 protein which shows the mass to charge ratio on the x-axis and relative intensity on the y-axis. The peptide data were searched against the UniprotKB homo sapiens reference proteome using the MS/MS ion search engine. In the above graph, b- ions are numbered from left to right on the N-terminus, and y-ions are numbered from right to left on the C- terminus

.

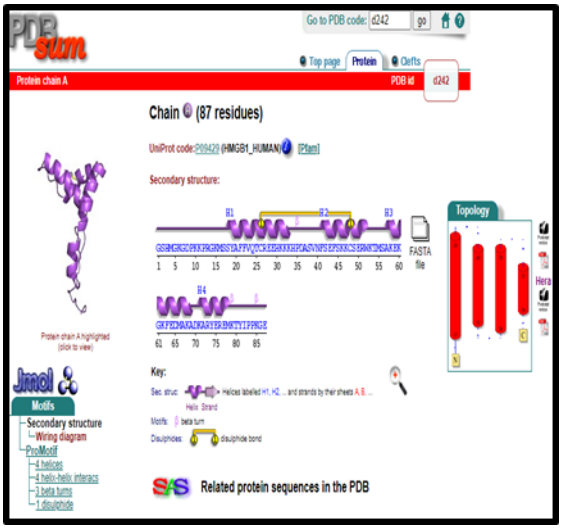
B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	88.0			70.0	S	817.4	409.2	800.4	799.4	8
2	175.1			157.1	S	730.4	365.7	713.4	712.4	7
3	303.1		286.1	285.1	Q	643.4	322.2	626.4	625.4	6
4	400.2		383.2	382.2	P	515.3		498.3	497.3	5
5	513.3		496.2	495.3	L	418.3		401.2	400.3	4
6	584.3	292.7	567.3	566.3	A	305.2		288.2	287.2	3
7	671.3	336.2	654.3	653.3	S	234.1		217.1	216.1	2
8	817.4	409.2	800.4	799.4	K	147.1		130.1		1

Figure 4.5: This is the fragmentation table that displays the same information as the above spectrum in a spreadsheet format. Here the potential ions which match the spectrum are colored. Red boxes show the b ions while Blue ions show the Y ions and Green boxes refer to the neutral loss or similar fragmentation patterns.

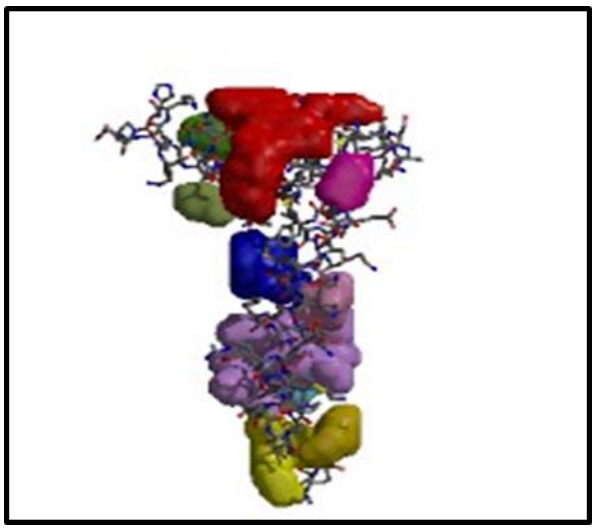
4.4 Identification of the 3-dimensional structure of HMGB1 protein

The human HMGB1 protein structure was used for the characterization of mice HMGB1 protein model using LIGPLOT software. Structural assessment of predicted HMGB1 protein model was performed by PDBsum server.

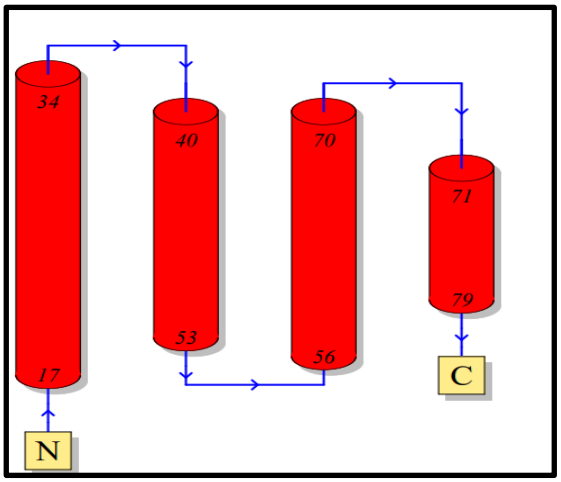
a



b



c



d

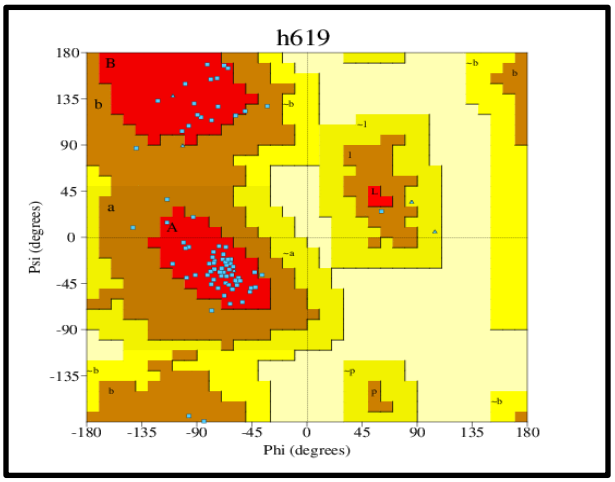
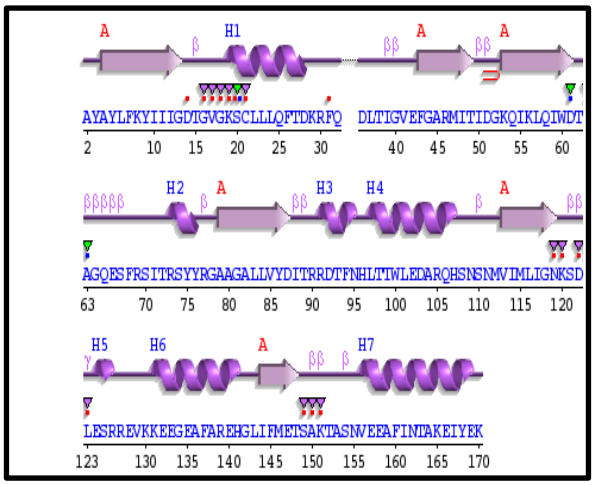


Figure 4.6 : This figure shows the HMGB1 protein model generated by the PDBsum entry Pfam **(a)** Above figure shows the secondary structure of protein including the alpha helix strand in purple color and gamma turns. **(b)** Solid surfaces are shown as clefts based on volume and the red one is the largest. **(c)** Schematic diagrams demonstrate the topology of protein in terms of how the beta-strands are arranged in beta-sheets and the relative disposition of the alpha helices (red cylinders). **(d)** PROCHECK's Ramachandran plot showing the major protein chain distribution.

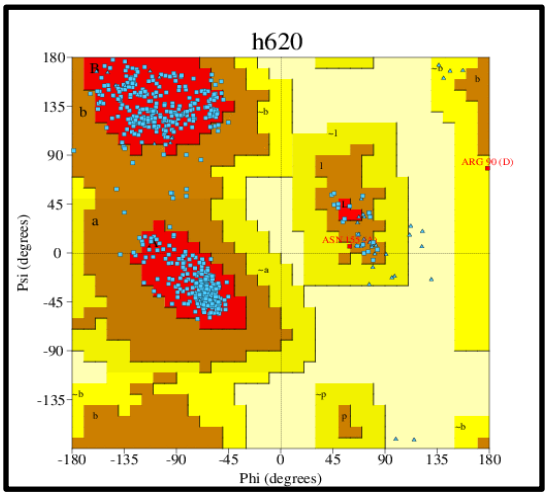
4.5 Identification of the 3-dimensional structure of RAB 32

For further analysis of the 3D structure of RAB 32 PDB structure was downloaded by blasting its sequence with Blastp via PDB data source.

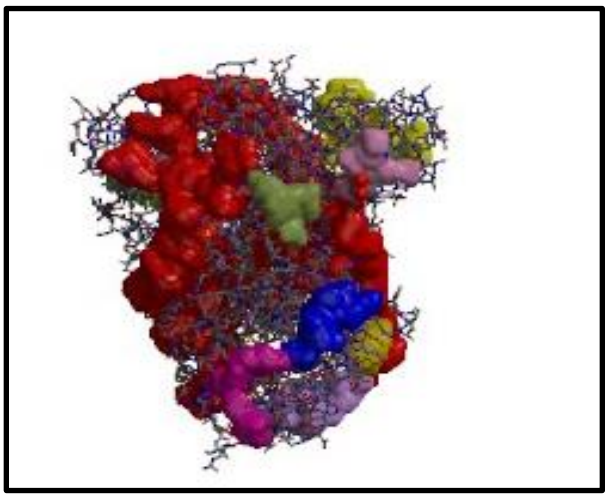
a



b



c



d

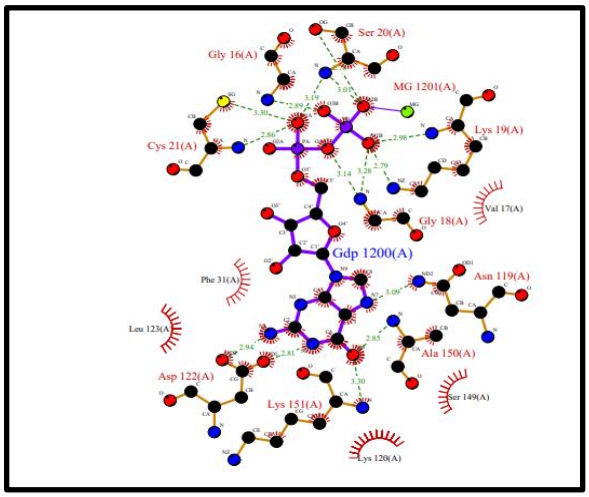


Figure 4.7: RAB 32 protein structure generated by PDBsums server and interaction plots. **(a)** Diagram of protein secondary structure including alpha helix strands and other motifs. **(b)** Shows the distribution of protein's main chain by Ramachandran plot from PROCHECK. **(c)** clefts are shown here according to volume as a solid in different colors, with the largest shown in red. **(d)** LIGPLOT diagram illustrating the interaction of the protein with the ligand GDP showing the green lines as hydrogen bonds and nonbounded contacts by the brown rays.

RAB 32 interaction with High Mobility Group Box 1 Protein

As the already culprit protein was RAB 32 in the case of Multiple Sclerosis. When we interact RAB 32 with our targeted protein that is High Mobility group Bx 1 it is shown that there are 7 interactive sites where RAB 32 makes the bond with the HMGB1 with the bond length of 3.06, 3.14, 3.13 and respectively.

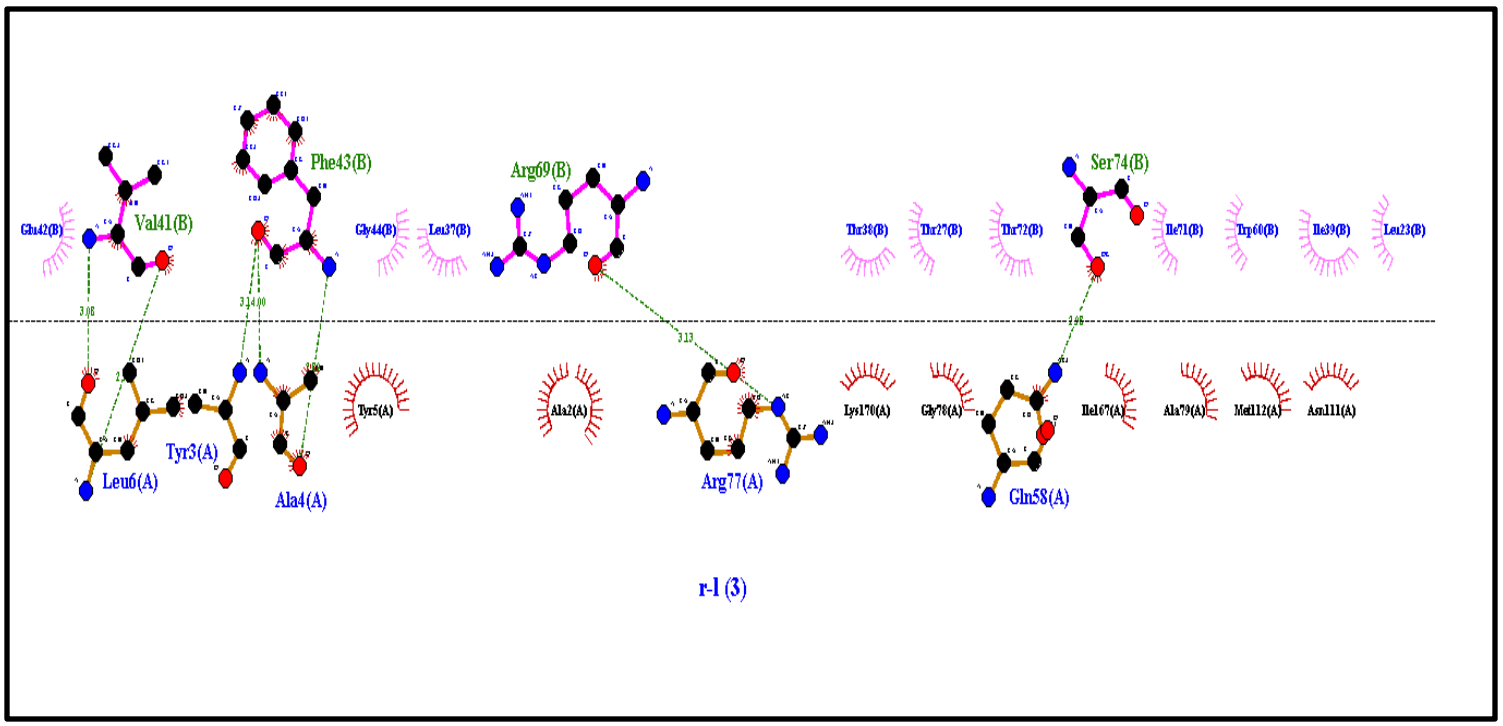


Figure 4.8: The interaction of RAB 32 with the High Mobility Group box 1 where RAB 32 has 7 interactive sites with HMGB1.

Chapter 5: Discussion

Multiple Sclerosis is a neurodegenerative, demyelinating, and chronic disease of the CNS in which [myelin] sheath is damaged. Although the exact cause behind the disease is still unknown, the pathophysiology of autoreactive T lymphocytes is known to be essential. The actual MS cause is multifactorial and it includes both predisposition and environmental factors such as infectious agents exposure, smoking, and Vitamin deficiency. These agents can cause a cascade of events in the immune system which leads to neuronal cell death and neuronal dysfunction. (MohamedKoriem, 2016)

MS must be identified early because it gives us a chance to seek treatment and to prepare ahead. MS is accurately diagnosed by assessing the medical history of the patient and by using different imaging treatment methods such as Magnetic resonance imaging (MRI), Lumbar puncture examination for analysis of CSF, blood sampling, and evoked potential. It is also important to obtain a background of the onset of symptoms, all neurological conditions, and as well as other diseases such as thyroid and diabetes. Moreover, it is also necessary to check the background of already taken medicines, drugs, and food items. Furthermore, evoked potential including visual, auditory, brainstem, and somatosensory potentials provide information on CNS demyelination and optic nerve. CSF examination of myelin fundamental protein and immunoglobulin (IgG) and blood samples analysis can be useful for diagnosing vitamin deficiencies. (Nazem Ghasemi, 2016).

Currently, only three groups of drugs are approved for a disease-modifying therapy for MS and those are Interferon-beta (IFNB), glatiramer acetate (GA), and Natalizumab. In the United States and other European countries, Mitoxantrone has also obtained an active MS license. In chronic MS type, the rate of relapse is reduced to about one-third by IFNB and GA. IFNB also reduces

the progression rate of disease by about 12 months in the short term. These figures are average and hide the findings that some patients do better than others. Those with MS can therefore hope that the often-quoted figures will achieve a better result. However, the downside is that certain individuals do not react to these therapies. So we cannot predict that who is going to respond to therapy. (Konrad Rejdak, 2010)

The MS treatment is only partially efficient so far and efforts to develop new therapeutic methods continue to extend our understanding of the disease's pathophysiology. In recent decades, a variety of various EAE models have been identified with active immunization protocols. Although models of mice are the most commonly used organism. Immunizing C57BL/6 mice with MOG35-55 is one of the most wide-spread EAE models and can also be used as a reliable model for research.

The present study demonstrates the development of a mice model C57BL6 with MOG35-55 peptide and CFA as described above in materials and methods. The protocol mentioned is regarded as a basic experimental neuroimmunological model and can also be modified for other applications as well. The above experimental procedure can also easily be applied to different EAE protocols by changing the type and amount of protein and as well as by varying different strains of mice. Moreover, the protocol mentioned can be used for experiments with the adoptive transfer.

HMGB1 protein has a dual role in the progression of Multiple Sclerosis. As a transcriptional regulator and nucleosome stabilizer, HMGB1 may also contribute to nuclear homeostasis by being released passively from apoptotic/necrotic cells or actively released from monocytes and subsequently binding on receptors including Anger, TLR-2, and TLR-4. The proinflammatory

development of cytokine, T-cell proliferation, and cell migration has been shown to mediate action that can be pathogenically important for autoimmune disorders including MS. (1 Z. S., jan 2016)

The present study mainly focuses on the identification of specific proteins involved in the EAE model of Multiple Sclerosis emphasizing the initial phase of the disease. This study found a collection of 17 proteins in the progression of MS of which 2 proteins were validated by Bioinformatics tools including Ligplot and Scaffold Mass Spectrometry. This study shows that in MS patients the HMGB1 serum levels are higher which indicates a role in MS pathogenesis in this inflammatory-like cytokine. In this study, we found that protein levels for HMGB1 have been substantially increased in RRMS patients as compared to controls. Moreover, the further investigation of studies reveals the potential therapeutic effect of HMGB1 protein as a neutralizing agent against an experimental model of model MS by decreasing the demyelination, inflammation and blocking the activation of microglia. Moreover, the data was analyzed through LIGPLOT software, and the interaction between the and protein-ligand was checked. It was seen that there are a total of 7 interactive sites between RAB 32 and HMGB1 protein which indicate the role of HMGB1 in Multiple Sclerosis. After that data was further analyzed through MS/MS spectrometry. Future investigations on the pathogenesis and progression of this autoimmune disease will provide a deep understanding of the disease mechanism.

Chapter 6: Conclusion

Multiple Sclerosis is an inflammatory condition in which the myelin sheath in the brain and spinal cord is damaged. Treatments are only partly successful as they are available to control the progression of the disease. Autoimmune encephalomyelitis is therefore an effective model for researching basic mechanisms of brain inflammation and immune-mediated damage to CNS tissue and for determining whether any therapeutic strategies can block the pathways involved in MS. EAE has helped to develop, validate and test MS medicines and, even more significantly, to understand the pathogenesis of MS. Furthermore, in conclusion, this study found a collection of 23 proteins involved in the EAE model of MS, of which two proteins were further validated by bioinformatics tools including LiIGPLOT and Scaffold. HMGB1 protein plays a major role in the progression of Multiple Sclerosis. In this study, we found that protein levels for HMGB1 have been substantially increased in RRMS patients as compared to controls. Moreover, the further investigation of studies reveals the potential therapeutic effect of HMGB1 protein as a neutralizing agent against an experimental model of MS by decreasing the demyelination, inflammation and blocking the activation of microglia. Moreover, the data was analyzed through ligplot software, and the interaction between the and protein-ligand was checked. After that data was further analyzed through MS/MS spectrometry. Future research can assess the role and accurate contribution of the differentially expressed proteins involved in the disease course of the EAE model of Multiple Sclerosis.

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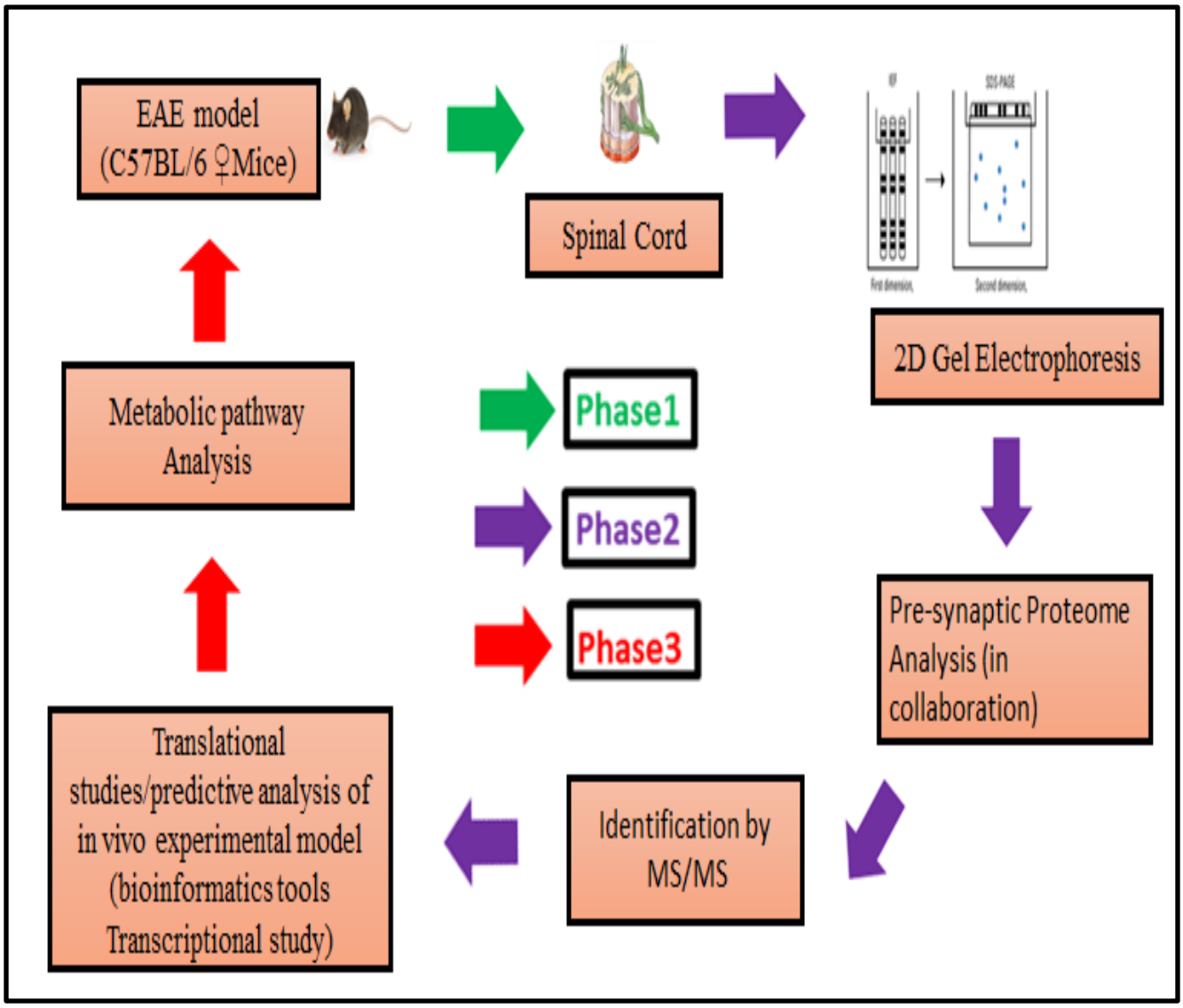

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