ANALYSIS OF EAE MODEL FOR THE DEVELOPMENT OF THERAPIES OF MULTIPLE SCLEROSIS THROUGH BIOINFORMATIC TOOLS

Maria Zeb

Registration # 00000277868

Master of Science in Biomedical Sciences

DEPARTMENT OF BIOMEDICAL ENGINEERING SCIENCES
SCHOOL OF MECHANICAL AND MANUFACTURING ENGUNEERING
NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY,
ISLAMABAD, PAKISTAN

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Analysis of EAE model for the development of therapies of Multiple Sclerosis through Bioinformatics Tools

By

Maria Zeb

Registration Number # 00000277868

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Thesis Supervisor:

Dr. Saima Zafar

Associate Professor

Thesis Supervisor's Signature:_____

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Declaration

I certify that this research work titled "*Analysis of EAE model for the development of therapies of Multiple Sclerosis through Bioinformatics Tools"* is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

Maria Zeb

Registration # 00000277868

Masters of Science in Biomedical Sciences

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Maria Zeb
Registration # 00000277868

Masters of Science in Biomedical Sciences

Signature of Supervisor
Dr. Saima Zafar
Associate Professor
School of Mechanical and Manufacturing Engineering (SMME)
National University of Sciences and Technology (NUST)
Islamabad, Pakistan

Copyright Statement

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Abstract:

Multiple Sclerosis is an autoimmune demyelinating inflammatory disease of the central nervous system (CNS) which resulted in the severe neurological defects. Mostly MS affected the adult life in their early life and it shows huge affect on family, and on professional and on daily life. The rate of multiple sclerosis according to some research in women is four times higher than in man, though the exact reason of this is still unclear but the researchers associated this with the difference in sex that linked with the brain in the MS. The disease progression of multiple sclerosis and its developments entails fundamental steps: (1).The destruction of myelin sheath and formation of lesions, (2).Inflammation. These steps are communicated together in a collaborative way, destroying the neuron tissues and causing MS. The goal of the research is to develop the EAE model for the identification of proteins involved in Multiple Sclerosis and then develop the interventions that can improve the lives of those living with MS. Synaptosomal-associated protein 25 (SNAP-25) is a 25kD protein with 206 amino acids. The pre-synaptic terminal of neurons is composed of a t-SNARE or target SNARE molecule. Formation of neural soluble Nethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes occurs because of SNAP -25 and has a great importance. Calcium-dependent exocytosis of synaptic vesicles, proper efficient release of neurotransmitters and propagation of action potential is done by SNARE complex. The normal levels of SNAP-25 are mandatory for neurotransmission; the changes in its expression can cause many disorders including autoimmune disorder like Multiple Sclerosis. The ultimate goal of this research is to assess the behavioral changes and pathophysiology of MS.

Keywords: Inflammation, Demyelination, Multiple Sclerosis, Exocytosis, Nervous System, SNARE complex, SNAP-25

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CHAPTER 1: INTRODUCTION

1.1.MULTIPLE SCLEROSIS:

Multiple Sclerosis is autoimmune disorder and demyelinating neurodegenerative disease of the central nervous system (CNS) which resulted in the severe neurological defects. Mostly MS affected the adult life in their early life and it shows huge affect on family, and on professional and on daily life.(Hoang, · January 2010) . The absence of myelin slowed down the conduction of the action potential. Multiple Sclerosis usually shows a progressive development of neurological symptoms and behavioral defects. The real cause and the reason behind its pathogenesis still not properly clear, because lots of factors are involved. (Hoang, · January 2010). In 1868 Jean-Martin Charcot first identified this disease.(Mohamad, 2016). MS is considered as the most well- known and prevailing autoimmune disease which attacked the central nervous system. In 2008 the world health organization reported the total number of patients of Multiple sclerosis in the world which is almost 2-2.5 million and the death rate of this disease is also greater which with the number of patients 20000 around the world. The age is also a major factor in multiple sclerosis so it affected the people mostly at the age starts from 20-50years old and the record also shows that this disease is more common in female than the male. In short it attacks the young adults more than the older age people. Multiple Sclerosis is also known disseminated sclerosis and encephalomyelitis disseminate. (Mohamad, 2016).

The rate of multiple sclerosis according to some research in women is four times higher than in man, though the exact reason of this unclear but the researchers associated this with the difference in sex that linked with the brain in the MS. Apart from human analysis, researchers found that the female mice contain elevated level of the blood vessel receptor protein which is

SIPR2 in the brain region than man while doing experiments of multiple Sclerosis. S1PR2 gene causes the opening of blood brain barrier. Blood brain barrier is the structure in the brain blood vessels that helps in the blockage of harmful substances and the opening up of this causes the entry of inflammatory cells that causes the MS in the CNS. (Stephen D. Miller, 4 August 2010). Research also shows that the expression of S1PR2 gene in the brain of females is greater than in male. (Stephen D. Miller, 4 August 2010)

The disease is unpredictable, in many patients; the condition is initially characterized by progressive neurological deficits followed by progressive neurological decline. In the U.S., 250,000 to 350,000 patients have MS. (BAC., 2017). The disease is more found in women than in men and people from Northern Europe who are at high risk of MS. The diagnosis can be made by minimally invasive tests, such as magnetic resonance imaging (MRI) of the brain and cerebrospinal fluid (CSF) tests. (BAC., 2017)

There is ample evidence that relapses of MS are associated with a growing body of depressive episodes. Each relapse involved the same or very different area of the white CNS story. The remission is rarely complete and lasts longer or shorter. The disease exact reason is still not properly understood so that's why there is no treatment developed yet.(S. Schilling, 2006).

CHAPTER 2: LITERATURE REVIEW

2.1- Multiple Sclerosis Physiology

The patients with Multiple Sclerosis must show the changes physiologically which causes many bodily, mental, and scientific major defects e.g. (1) Deterioration of composition of the body along with adjustments in fats (2) The disease dangers like many heart disorders of coronary artery, non-insulin-established diabetes mellitus, metabolism of lipid metabolism, and fracture of osteoporosis are improved in the patients of Multiple Sclerosis. Loading pattern alteration is caused by the immobility of MS inside the paralyzed region and also changes the tissue shape.(Mohamad, 2016). Many factors in Multiple sclerosis like oxidative and nitrosative strain pathways are studied under the body structure and apart from this the activation of immune cell or inflammatory cell activation are also included under the body structure in the reasesch. (katz sand, june 2015).

The disease progression of multiple sclerosis and its developments entails fundamental steps:

(1) The destruction of myelin sheath and lesions formation inside the Central nervous device.

(2) Inflammation.

These fundamental steps are communicated in a collaborative way, destroying the neuron tissue and causing MS. (mohamad, 2016)

2.2- Pathophysiology of Multiple Sclerosis:

2.2.1-Destruction of myelin sheath and lesions formation:

The primary cause of multiple sclerosis is the inflammation in the CNS that causes the major damage. The feature that causes the major damage in patients of MS is the inflammation of CNS.

Many factors involve in its pathogenesis includes genetic, environmental and infectious agents that influence the causes of multiple sclerosis. All these factors cause the major injuries in the CNS which in turns form the abrasions in the white matter of neurons, brain stem and even spinal cord. White matter lesions are found very close to ventricle laterally. Signals from grey area of brain are transferred by the white matter of the brain into the whole body for collecting the information, on the other side, in the peripheral nervous system there are no lesions. (Mohamad, 2016). So the destruction of oligodendrocytes cells causes the destruction of myelin sheath and thus damages the neuron axon. The Oligodendrocytes are those cells that help in the formation of myelin sheath and also transfer the neural signals. After this destruction of myelin there is no transfer of signals, and a process of repairing in MS occurred only in initial stage named as remyelination but this process of remyelination seems useless when so many lesions are formed in the central nervous system and these damaged lesions then covered the axons of the neurons. (Jan C. Brassington1, 1998). The lesions then cause the MS and symptoms appear in the patients. The number of astrocytes in MS increases with the increase in the number of lesions, where these astrocytes are involve in biochemical processes and also present in the endothelial cells that form the blood–brain barrier to enhance the transfer of nutrients to the nervous tissue which helps in repair process of brain and spinal cord (Jan C. Brassington1, 1998).

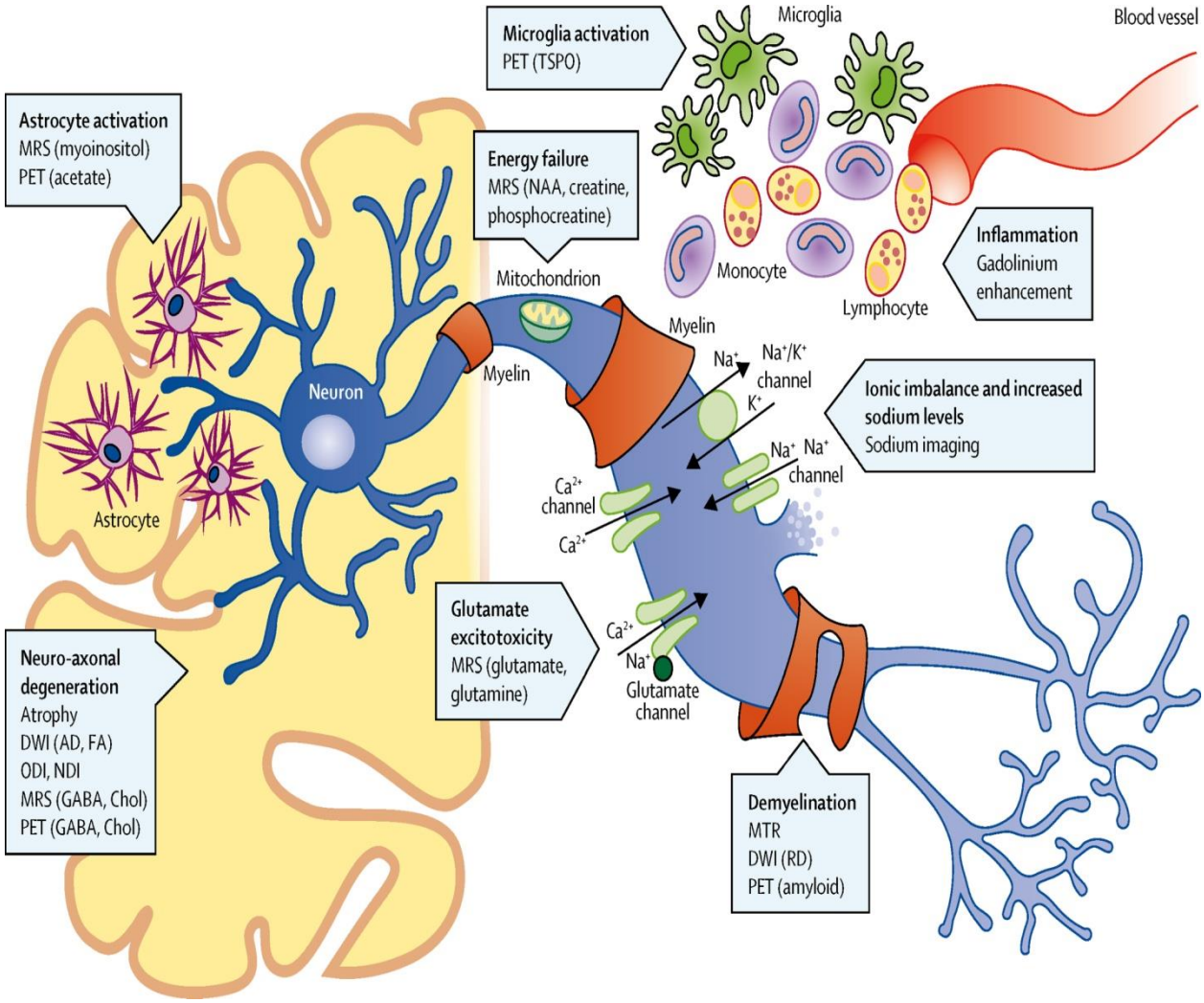


Fig 2.1: Pathogenic mechanisms and imaging targets of MS

2.2.2- Disruption of Blood Brain Barrier:

The protective barrier that prevents the entry of any foreign material into the nervous system is the blood brain barrier and its disruption causes the entry of this antigen in to the nervous system and then it initiates the symptoms of MS. The BBB is composed of endothelial cells, the cells which show the connection to the cell lining are connected by claudin and occludin, they form a

strong barrier to prevent the entry of proteins and other molecule that may cause serious autoimmune disease. The disruption of BBB due to the lymphocytes and monocytes causes the release of cytokines which activates the activation of adhesion molecules on the monocytes and lymphocytes. (Phelan, Monday, December 19, 2016)

After the breakdown of the BBB, the symptoms appear like swelling. These activated lymphocytes and macrophages attack the myelin sheath of neurons directly. (Jan C. Brassington1, 1998)

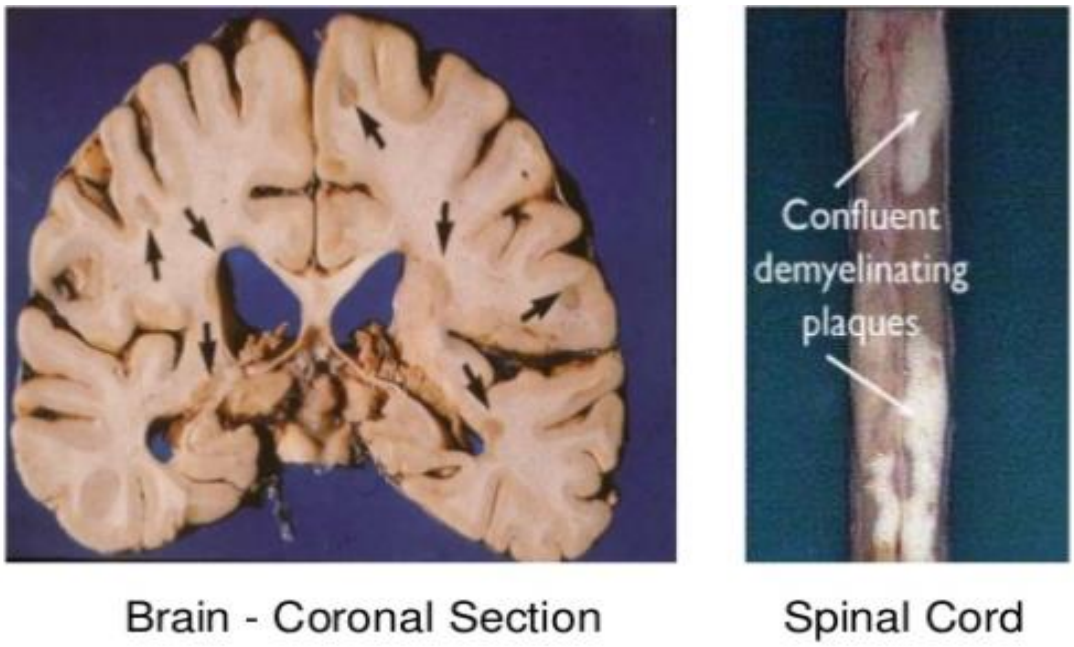


Fig 2.2: White plaques in the region of brain and spinal cord causing multiple sclerosis.

2.2.3-Inflammation

The process of inflammation is present in all the forms of Multiple Sclerosis, which starts with the T-lymphocytes or T-cells. Myelin sheath recognized the T-cells as a foreign body and T-cells starts to damage the myelin sheath causing demyelination so these T-cells which cause the destruction of myelin sheath are also called auto reactive lymphocytes. The inflammatory

processes are initiated with the demyelination of neuron sheath and they help the immune cells to release the cytokines and antibodies causes the damage of blood brain barrier which results in activation of several cells like macrophages, cytokines and also activate other destructive proteins. (Loren A. Rolak M. D., September 4, 2002) Inflammatory processes in CNS lessen the information transferred through:

(1) The release of cytokines and antibodies abruptly stops the production of neurotransmitters after the neuronal death.

(2) The production of cytokines and antibodies elevates the myelin sheath destruction and (3) Large number of cytokines and antibodies in the body damage the axon fully. (Mohamad, 2016). Many immune refluxes are activated by these cytokines. Interleukin 21 affects many immune cells and increased the autoimmunity through several mechanism such as initiation of NK cells, helper T-17 cells improvement and follicular helper T(T_{FH}) cells increased, increasing the level of B-cell differentiation and antibody excretion and decreased of regulatory T (T_{reg}) cells so that's why it is considered as one of the most important immune factor. Interleukin-21 initiates the process of autoimunity to elevate the MS patients with alemtuzumab. (Nancy L. Sicotte, 6 Jan 2011).It also increase the autoimmunity by using different mechanisms, such as improvement and increased level of helper T-17 and follicular helper T, initiation of NK cells. (Loren A. Rolak M. D., September 4, 2002)

The process of inflammation and the processes of apoptosis had major role in MS symptoms and these processes occurred in the peripheral and CNS in the Multiple Sclerosis patients. (Loren A. Rolak M. D., September 4, 2002)

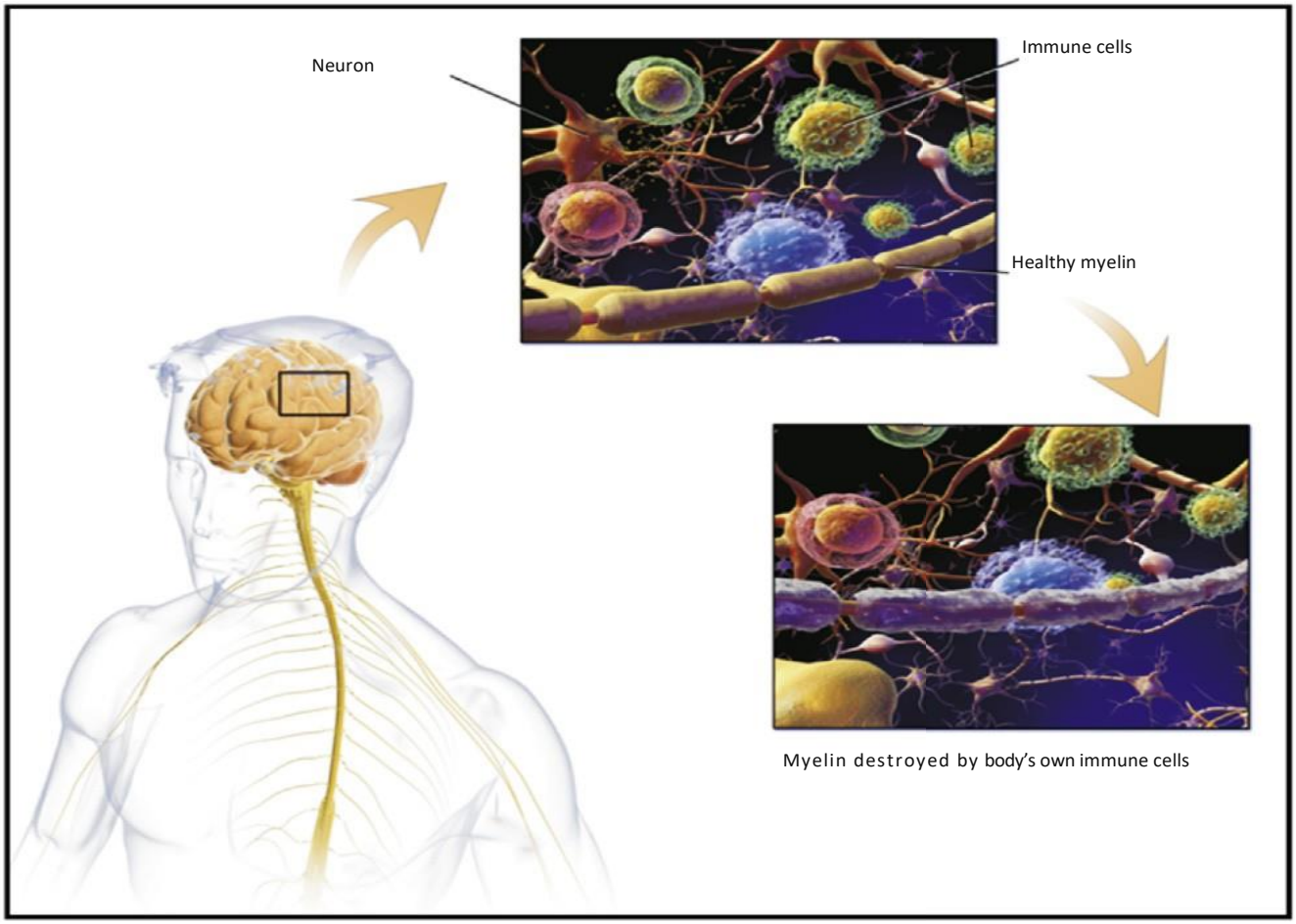


Fig2.3: Different incidence of MS. (Mohamad, 2016).

2.3- MAJOR CATEGORIES OF MULTIPLE SCLEROSIS

Neurologists grouped the patients of Multiple Sclerosis into four major groups, based on the disease course. (Goldenberg, 2012)

2.3.1- Relapsing-remitting Multiple Sclerosis:

The common form of Multiple sclerosis is RRMS and affected about 85% of the Multiple sclerosis Patients. In relapsing-remitting Multiple Sclerosis an attack is followed by the recovery

time which is known as remission and normally it starts in 20s and 30s year of age. During remission all the symptoms disappear and so there is no progression of disease during the remission period. (Katz Sand, June 2015). These patients may have an attack when symptoms flare up this condition is termed as relapses. Then a period of recovery occurs after the attack when patients got few or no symptoms called remission. It may last weeks, months or longer but the disease will not get worse during these breaks. (Katz Sand, June 2015). It can be further classified as

- **Active RRMS** in which there are relapses and the evidence of new MRI activity over the specific period of time. (Katz Sand, June 2015).
- **Not Active** as well as worsening in which there is an elevation in disability following a relapse or not worsening. (Katz Sand, June 2015)

Fig 2.4: RRMS

So demyelination in this type of Multiple sclerosis is found without any symptoms. This graph shows the disease activity which occured in RRMS, however each person's experience with this disease is different and unique (Lublin et al.2014).The new symptoms disappear following a

relapse or the new symptoms disappear which results in an increasesd level of disability. Lesions on MRI are normally displayed by arrow which occurs as a part of relapse. (Lublin et al.2014)

2.3.1.1-Mechanism of Relapsing-remitting Multiple Sclerosis

The inflammatory attack on myelin in RRMS (Insulating layer membrane that surrounds the nerve fibers in Central Nervous System) and on nerve fiber occur. During the inflammatory attack, activation of immune cells damage the localized area which results in symptom of MS. Variability of damaged areas shows that no two people have the same exact symptoms.(Lublin et al.2014).

2.3.2- Secondary progressive MS (SPMS)

Initial relapsing-remitting course is followed by this type. People with RRMS are transformed into a secondary progressive course where there is loss of neuronal functions with period of time. (Loren A. Rolak, 2003). SPMS is also further classified as

- **Active Relapse** with relapses and the evidence of MRI activity occur during a specified period of time. (Loren A. Rolak, 2003)**.**
- **Not active** with the progression and accumulation of the disability over the period of time, with or without relapses occur or without progression. (Loren A. Rolak, 2003)**.**

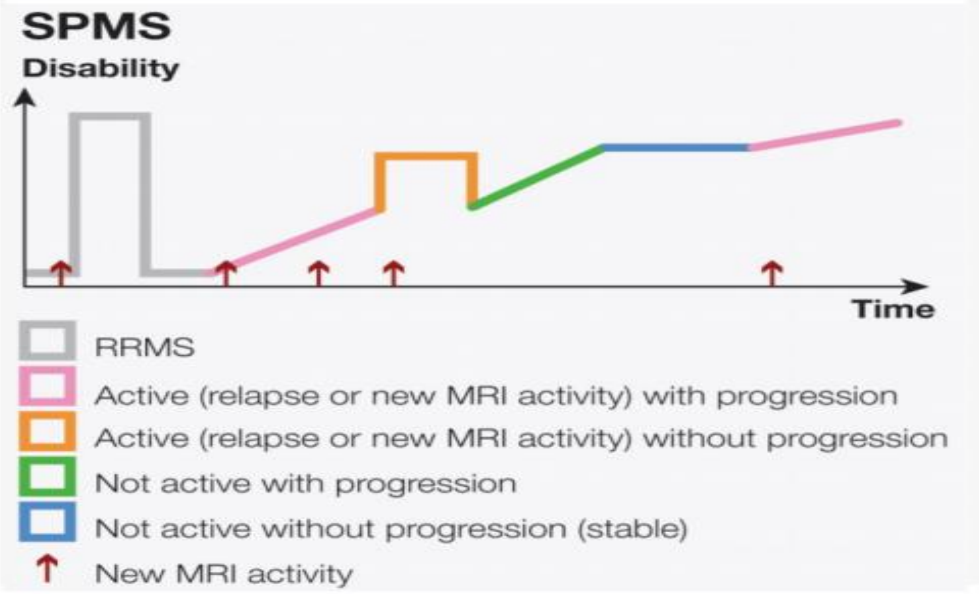


Fig 2.5: SPMS

65% of patients with relapsing-remitting MS they there RRMS condition developed into secondary progressive MS with no remission and results in progressive neurological defects.

The above graph shows the period of relapsing-remitting disease which elevates over the period of time with or without the evidence of disease activity. Occasional relapses can occur in SPMS. (Lublin et al,2014).

2.3.3- Primary Progressive Multiple sclerosis (PPMS)

In PPMS there is a worse condition of neurologic functions and apart from this there is an accumulation of disability from the onsets of symptoms without early remission or relapses. (Katz Sand, June 2015) It can further classified as

- **Active** in which the relapse or evidence of new MRI activity occur over a specified time period. (Lublin et al,2014).

- **Not active** form as well as with progression or without progression. (Lublin et al,2014).

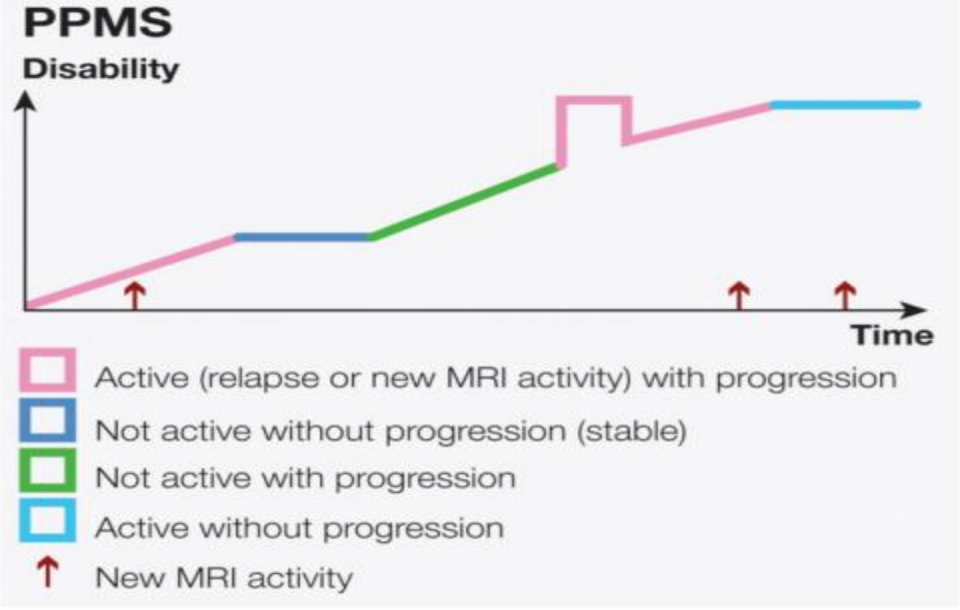


Fig 2.6: PPMS

This graphs shows the disease activity occur in PPMS. When the disease is stable, PPMS have the brief periods with or without a relapse. (Lublin et al,2014).

2.3.4- **Progressive-relapsing MS**

It is a very rare form of Multiple Sclerosis influencing 5% of patients and this form of MS is progressive from the start and there is no period of remission. (Katz Sand, June 2015)

2.4- Clinical Presentation:

An autoimmune disease in which there is demyelination of neurons in specific region like optic nerves, brainstem, cerebellum, periventricular and spinal cord region. The histopathology shows that cerebral grey matter of brain is also involved in this disease but this is not well understood

on conventional MRI. (Loren A. Rolak M. D., September 4, 2002). Thus the clinical features of this disease depend on the area of brain and spinal cord which are involved in MS. The onsets of symptoms in RRMS are gradual and the inflammatory condition may evolve over the days. (Loren A. Rolak M. D., September 4, 2002). The clinical attack may lasts for 24 hours with the non appearance of fever or any kind of infection even. While the primary progressive MS insets for 12 months by the time of diagnosis. (Loren A. Rolak M. , 2003).

First and common presentation of RRMS is unilateral optic neuritis results in gradual loss of vision, pain because of eye movement and also alters the colored vision. (Loren A. Rolak M. , 2003) Beyond 2 weeks from the onset of disease visual loss rarely progresses. The recovery of this vision problem may take 2 weeks but patients don't show full recovery. After examination, visual acuity becomes low; there is a relative afferent pupillary defect, impaired colour vision. The optic disc seems to be normal (retrobulbar neuritis) or swollen acutely on funduscopy and then it will pale and atrophic with the passage of time. (Loren A. Rolak M. D., September 4, 2002)

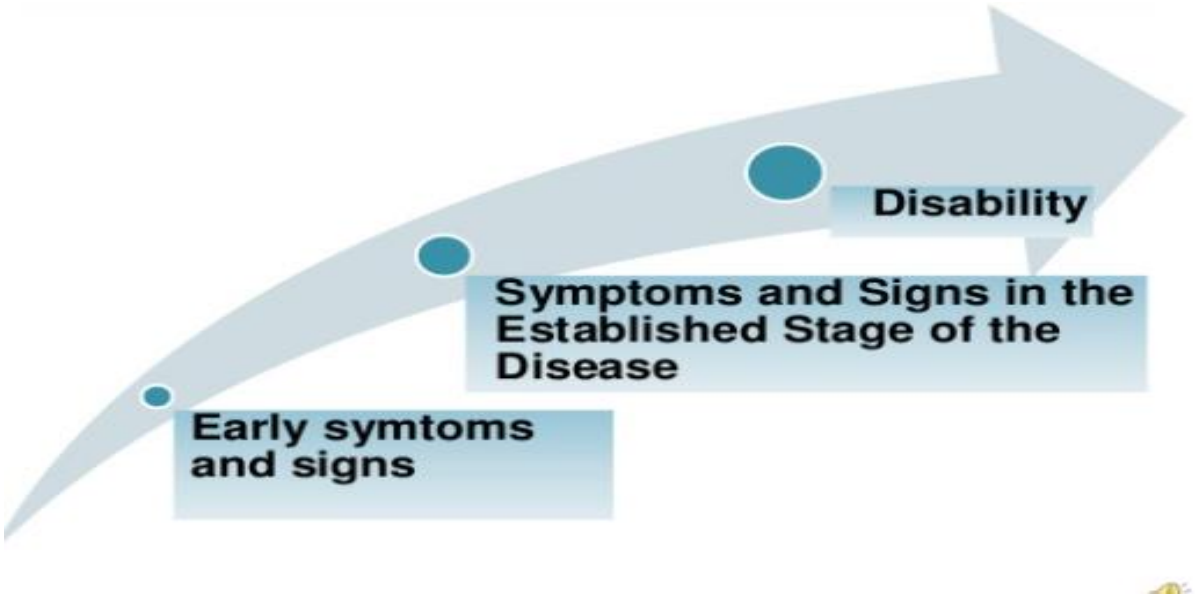


Fig 2.7: The stages of clinical manifestation of Multiple Sclerosis.

2.4.1- Initial presentation of Multiple Sclerosis:

There is gradual loss of sensory and motor functions of limbs when inflamtory lesions are caused by the myelitis in the spinal cord. Changes seem to occur from hours to days. The intensity of this myelitis is different because the normal sensory syndrome becomes serious disabling syndrome with serious attack like terra paresis. (Loren A. Rolak M. D., September 4, 2002). Lhermitte's is caused because of lesion in the cervical region with an electric shock-like sensation down the neck which is a diagnosis clue. A strong band like sensation around the trunk region occurs due to thoracic cord lesions. (Loren A. Rolak M. , 2003) But the major reason of this misinterpretation is the cardiac event and examinations of signs and symptoms may include reduced fine touch of sensory areas, sense of vibration and joint position sense with a sensory level are also reduced. Upper motor neuron are sensory signs causes a lesion with enhanced tone or spasticity and pyramidal weakness. Hemi-cord syndrome or partial Brown-Séquard might cause by myelitis. (Loren A. Rolak M. D., September 4, 2002).

2.5- Clinical Course of Multiple Sclerosis:

The symptoms and presentations of multiple sclerosis are characterized by the variability and diversity of disease. Until now there is no virtual neurologic complaint has not been found or traced towards MS. (Loren A. Rolak M. D., September 4, 2002).Most of symptoms of this disease appear abruptly within hour and sometimes within a few days and after few days these symptoms reach to their peak. The pathophysiology of this disease is still poorly understood so till now many drugs are used for the treatment of this disease for avoiding the relapses which is useless in the secondary progressive phase of the disease. (Loren A. Rolak M. D., September 4, 2002)

Clinical way of diagnosis involves the history and examination findings define the diagnosis procedure. The main evidences of the diagnosis are the dissemination in time (DIT) and dissemination in space (DIS). Still diagnostic laboratory tests for MS are not available properly. The diagnosis is based on the clinical methodology followed by investigations. (Loren A. Rolak M. D., September 4, 2002)

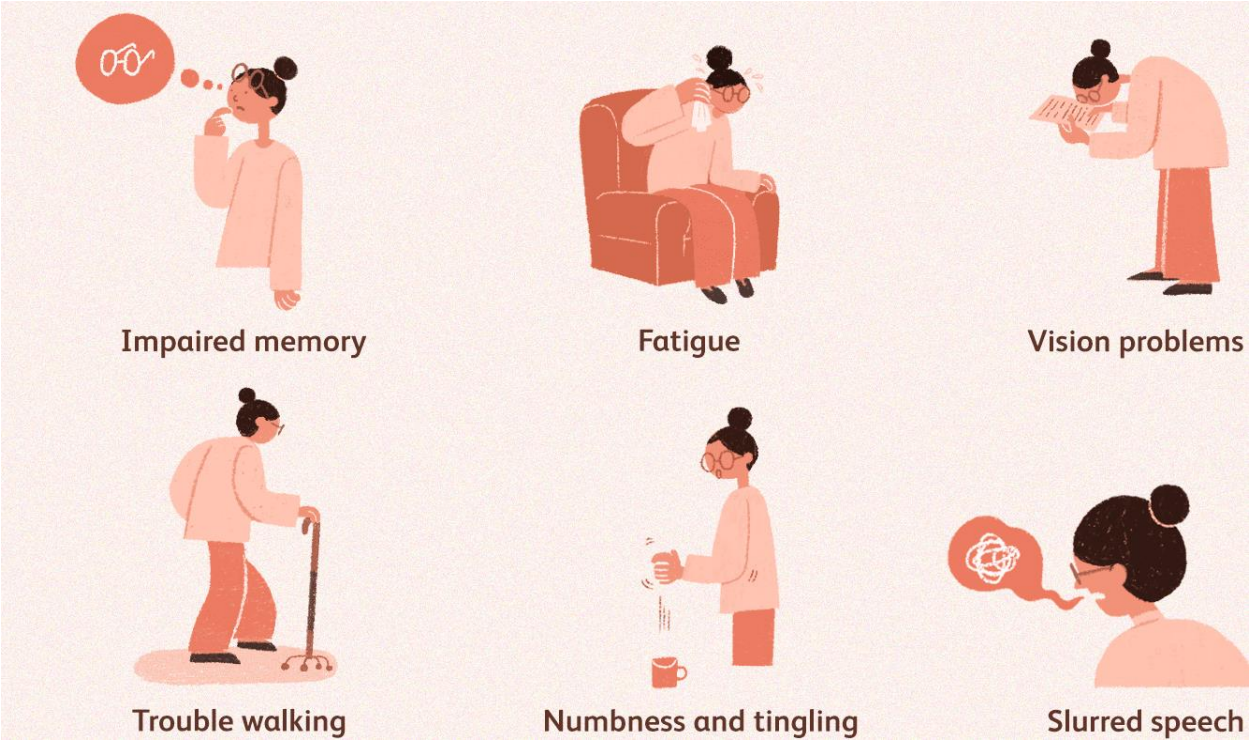


Fig 2.8: Diagrammatic representation of symptoms of Multiple Sclerosis

2.6- Pathogenesis of Multiple Sclerosis:

The exact causes of the multiple sclerosis are still unclear and undetermined. But the possible factors involve in the pathogenesis are different kind of infections, autoimmunity, some major environmental factors and the genetic susceptibility.

2.6.1- General Risk factors of Multiple Sclerosis:

MS is considered as complicated disease, and variety of genetic variants are involved in this disease. Both genetic and non-genetic are responsible for the causes of multiple sclerosis. The major risks factors that affect the lifestyle are the major environmental reasons like exposure to smoke from tobacco, obesity at the young age, viral infections, low level of sun exposure and

lack of vitamin D(Tomas Olsson, December 2016). Factors that are linked with reduced risk included oral tobacco use, increase consumption of coffee and even the serological evidence of cytomegalovirus (CMV) infection. (Mohamad, 2016) . Heritability of multiple sclerosis explains that siblings of individual having the multiple sclerosis are more prone to this disease. So the risks of familial recurrence of MS are 15%. The population studied determines that the risk is increased by 7 folds and this risk is 30% higher in monozygotic twins. (Tomas Olsson, December 2016).The genes that predispose to the symptoms of MS are still not defined completely. The inheritance appears to be polygenic with the influence of some genes (a). Genes for human leucocytes antigen (HLA) typing interleukin receptors are involved.

(b). CLEC16A (C-type lectin domain family) CD226 genes are also involved.

The age factor and also migration rate according to the explanation of migration studies are also involve in the risks factors of Multiple Sclerosis: individuals who migrated from a country with low-risk of this disease to a country with high-risk before adolescence show similarity to that of those who were born and reside in the high-risk country for MS risks. (Tomas Olsson, December 2016).

Majority countries had experienced a different level of elevation of MS among women; the research explains the major factors in genetic composition, also showed that smoking and obesity like factors is the major contributor of this disease that may change the reproductive behaviour. (Tomas Olsson, December 2016)

The immune response, specificities like the spectrum of T cells has prime importance for MS, and is greatly activated by non-heritable environmental factors. The major genetic risk variants

that are common causes of MS might be attributed to lifestyle or environmental factors. (Loren A. Rolak M. , 2003).

HLA-associated genes are also involved in MS and different classes of HLA genes are present, and they are the major modifier of this disease. In MS, the class II variant HLA-DRB1 *15:01 has a striking association with an increased risk of MS whereas variant HLA-A*02 of class 1 was associated with protection from the disease. (Tomas Olsson, December 2016).

2.6.2- Non Genetic Factors for Multiple Sclerosis:

MS showed that a sibling of an individual with MS had an almost 17fold increased risk of the disease. This risk is increased by 7 folds in the siblings showing the importance of genetic predisposition and environmental factors. (Tomas Olsson, December 2016). The gradient of latitude is an important factor that influences the environmental factors. This risk also depends upon on the age factor, at which age individuals are migrated: the risk of MS for those who migrated from a country having low-risks of this disease to a country with high-risk before adolescence was similar to that of those who were born and live in a country with a high risk of this disease. (Navikas V).

2.6.3- Environmental factors of Multiple Sclerosis:

Different environmental factors are involved in the multiple sclerosis apart from the genetic factors. Major among these area as follow:

2.6.3.1- Sun exposure and Vitamin D:

The difference in the latitude play very important role in multiple sclerosis. This disease is more common in the people living in the far away region from the equator. Different associated factors are linked with the latitude but the most common is the exposure of sunlight including both its intensity and duration. The rate of mortality according to some research is inversely related to the residential sunlight exposure, high level of exposure to sun light and greater level of skin damage. The patients with the multiple sclerosis less develop the skin cancer. The ultraviolet rays in the sunlight have the immunosuppressive effects and helps in vitamin D3 synthesis and this vitamin is then hydroxylated in the liver to to 25-hydroxy-vitamin D (25-OH-vitD). This form is the precursor of Vitamin D. Increase level of Vitamin D and the UV radiations protects from MS. Vitamins is also obtained from food and supplements. The vitamin D plays an important role and it has important affect in an autoimmune encephalomyelitis. The level of vitamin D changes according to the seasonal changes in exposure to UV radiations from sunlight.. (Katukuri Nishanth, 5 Aug 2020).

2.6.3.2- Smoking:

Smoking also influenced the serious phases of multiple sclerosis and those who smoke are at high risk of multiple sclerosis. So smokers are at high risk of this disease. The intensity of tobacco smoking develops this disease. So the smokers are diagnosed with this disease and the chances of multiple sclerosis decreases among the people or the person who stop using tobacco or cigarette. The patient diagnosed with this disease because of smoking showed more complicated and severe symptoms like vision is blurred in some cases other symptoms involve the paresthesias and motor weakness. (Katukuri Nishanth, 5 Aug 2020).

2.6.3.3- Role of Viruses causing infections in Multiple Sclerosis:

Many viruses are also love in Multiple Sclerosis, the exact reason of this disease is still unknown. There is huge diversity in the plaques formation and this diversity shows that more than one infectious agent like viruses involve in the pathogenesis of MS. So then virus attacked t human CNS first it's form the genome-virome interaction which make this disease more complicated. (Katukuri Nishanth, 5 Aug 2020) Viruses like Epstein Bar Virus, Herpes virus are the most common one. These viruses showed their specific responses in the brain, which causes the inflammation and results in demyelination. (Katukuri Nishanth, 5 Aug 2020).

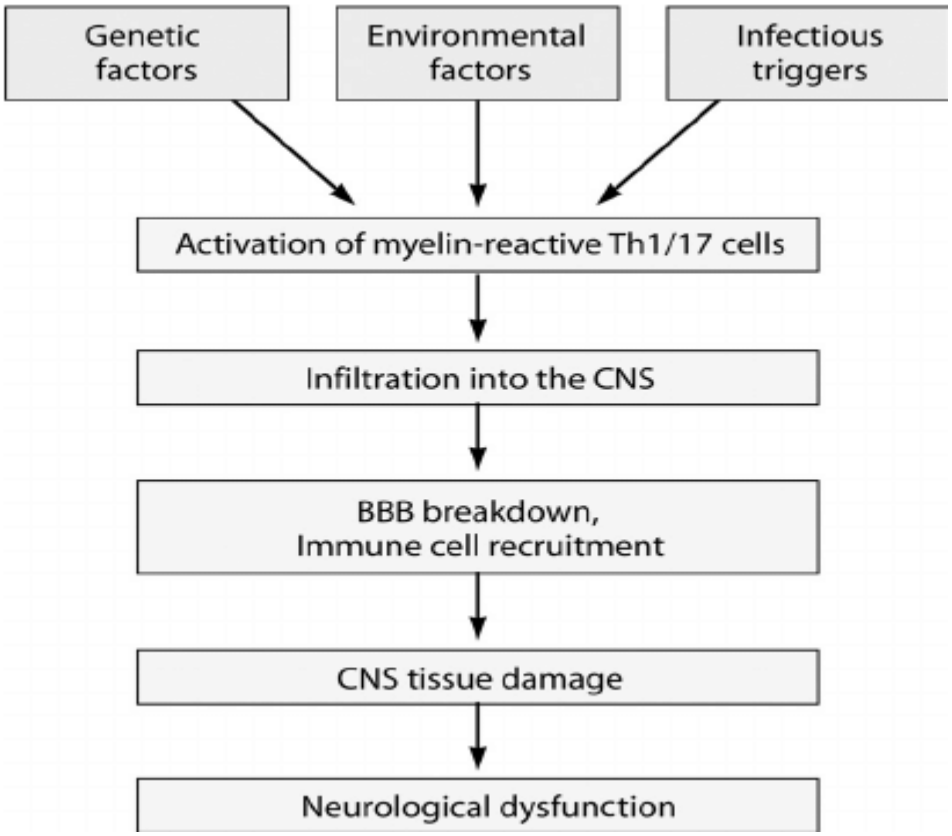


Fig 2.9: Risks factors causing neurological dysfunction. (Dimitry N Krementsov, July 2013)

2.7-Diagnosis of Multiple Sclerosis:

The progression of Multiple Sclerosis is characterized using three major clinical methods, i.e., neuropsychological assessments, neuroimaging, and biomarkers**.**

2.7.1- Neurological Assessments for diagnosis of Multiple Sclerosis:

On the basis of language skills, motor coordination including the memory that involve the learning and executive, intelligence and visual explanation all these factors are comprised by neuropsychological assessments. These tests though do not provide the exact cause of severity but gave the rough estimated line of disease severity. So for diagnosis different testing strategies have been discovered and used to estimate the different neuropsychological deficits e.g. General Practitioner Assessment of Cognition, Mental Attributes Profiling System, Mental Status Examination (MSE), Montreal Cognitive Assessment. (Jan C. Brassington1, 1998). In the early stage of Multiple Sclerosis, it is difficult to diagnose a disease because there is no definite sign and symptoms of this disease within the CNS and symptoms fluctuate over the time. (Jan C. Brassington1, 1998).

Until the development of MRI to detect demyelinating plaques in the brain, and the increased sensitivity of CSF to detect the oligoclonal abnormalities, other conditions such as neurosyphilis are occasionally misdiagnose as MS (Mahler, 1992). Similarly, the behavioral changes from cerebral MS had known to lead to an incorrect diagnosis of some other CNS or psychiatric disorder. (Jan C. Brassington1, 1998). MRI is considered as most sensitive technique available to detect the brain lesions produce by MS. (Goodkin et al 1994).

2.7.2- Neuroimaging for diagnosis of Multiple Sclerosis:

Neuropsychological examination is affirmed by utilizing neuroimaging approaches. Different methods of neuroimaging have been used to provide the specific insights of structural or functional aspects of the brain. (Jan C. Brassington1, 1998). In the past few decades the major diagnostic criteria have been formulated with some modifications including the major changes in magnetic resonance imaging (MRI) to a greater extent for some better findings towards diagnosis. (Nancy L. Sicotte, 6 Jan 2011). So the neuroimaging diagnostics involve the assessment of this disease through MRI, which reveals the area of MS lesson in the CNS and it involve the intravenous injections of a contrast material to highlight the region of lesions that shows the disease in active phase. (Katz Sand, June 2015).

Magnetic resonance imaging (MRI) is the test for diagnosing MS in combination with the blood tests. Normally radio waves are present in MRIs to find out the water content in tissues of the body realitively. Normal and abnormal tissues are detected and then it spot irregularities in the brain and spinal cord so it shows the sensitive and detailed images of CNS. They're much less invasive than X-rays or CT scans, which both use radiation. (Katz Sand, June 2015)

MRI played major role in the diagnosis and analysis of multiple sclerosis which helps in the identification of demyelination areas in the CNS. These studies of brain and spinal cord through MRI are basically critical and used to identify and then monitor the treatment response. So the MRI results predict the response of individual patient in the disease progression. In short these results may serve as important biomarkers in the clinical trial of MS therapies and had served important insights into disease pathophysiology. (Nancy L. Sicotte, 6 Jan 2011)

2.7.3-Biomarkers for diagnosis of Multiple Sclerosis:

The need of biomarkers for the diagnosis of MS depends upon the stage of disease so during the first clinical presentation of demyelination, biomarkers are used to investigate the MS disease clinically. (Harris VK, 8 December 2016)

The table 2.1**:** Summary of biomarkers used for the diagnosis of MS.

Biomarkers	**Descriptions**	**Utility in Multiple Sclerosis**
NF-L	Axonal protein reflecting inflammation-mediated axonal damage.	Poor long-term prognosis is shown by CSF biomarker which further causes damage of axon.
NF-H	Acute and ongoing axonal damage is reflected by axonal protein	CSF biomarker causing MS results in accumulated axonal damage.
CH13L1, CH13L2, CHITI	These biomarkers are released by activated astrocytes and microglia in the CNS.	Biomarkers cause inflammation that is associated to disease activity.
sCDI4, sCDI63 TREM-2	Receptors from microglia and macrophages cell surface .	CSF biomarkers of microglia activation.
IgM	Oligoclonal lipid-specific intrathecal antibodies	Aggressive form of disease courses occurred because of these biomarkers.

Table 1 lists the biomarkers and their utility in MS

2.8- **Experimental Autoimmune Encephalomyelitis (EAE)**

Experimental autoimmune encephalomyelitis (EAE) is a cell-mediated autoimmune disease of T-cells in which there is an infiltration of T-cell and monocyte in the central nervous system (CNS) causing the inflammation. (ANDREW P. ROBINSON1, 2014). The myelin-producing oligodendrocytes expressed the proteins of autoimmune molecular targets are first identified which results in the primary demyelination of axonal tracks, conduction of impaired axon in the CNS and progressive paralysis of hind limb. For studying the diagnostics and potential therapeutics EAE is one the most powerful animal model. EAE has different pathophysiologic forms with different pattern of clinical presentations but it do depend on the type of animal species used and form of strain and route of immunization pathways used are the important factors. So to study the mechanisms, disease development and its histopathological characteristics variety of models have been used for this purpose to study the mechanisms towards therapeutic interventions. (ANDREW P. ROBINSON1, 2014)

Over the 60 years ago EAE in the mouse was first induced by the process of immunization with spinal cord homogenates (Olitsky and Yager, 1949). So many research had done and this led to the discovery of many encephalitogenic peptides but still mice is considered as most commonly used animal species for research and to study the different diseases because of its wide range of availability of its transgenics. Even mice knockouts are available for targeted studies. Different strains of mice can be used for studies of Multiple Sclerosis like in the SJL (H-2s) mouse model,

The induction of EAE model can be done with the CNS homogenate by the process of immunization, different other protein likes proteolipds (PLP), myelin basic protein (MBP) or encephalitogenic epitopes of PLP (PLP139–151, PLP178–191), myelin oligodendrocyte protein

(MOG92–106), or MBP (MBP84–104) in an emulsion with complete Freund's adjuvant (CFA) were also used to induce an animal model for MS. Disease showed a predictable course and after 10-15 days paralysis starts in the tail and hind limbs and then it starts progressing to the forelimbs with the weight loss. In SJL mice the disease is relapsing-remitting and paralysis course is then further studied for immunomodulatory strategies. (ANDREW P. ROBINSON1, 2014).

MOG35–55 considered as an impotent encephalitogen in C57BL/6 (H-2b) mice shows chronic progression of the disease. Many other mouse models can be used for the induction of EAE to study the mechanism of autoimmune disorder e.g., PL/J and B10.PL (H-2u) but they are normally acute and rectifying. Different types of strains and the type of immunizing antigen variations continued to identify the atypical manifestation including inflammation, mononuclear cell infiltration (MNC) and clinical presentation. Recently new clinical form of this disease is reported recently which showed a relapsing-remitting course that developed into a chronic progressive phenotype with lesions in the brain as well as the spinal cord (Levy et al., 2010). This model is the most prevailing form of multiple sclerosis displaying relapsing-remitting followed by secondary progressive disease. (ANDREW P. ROBINSON1, 2014)

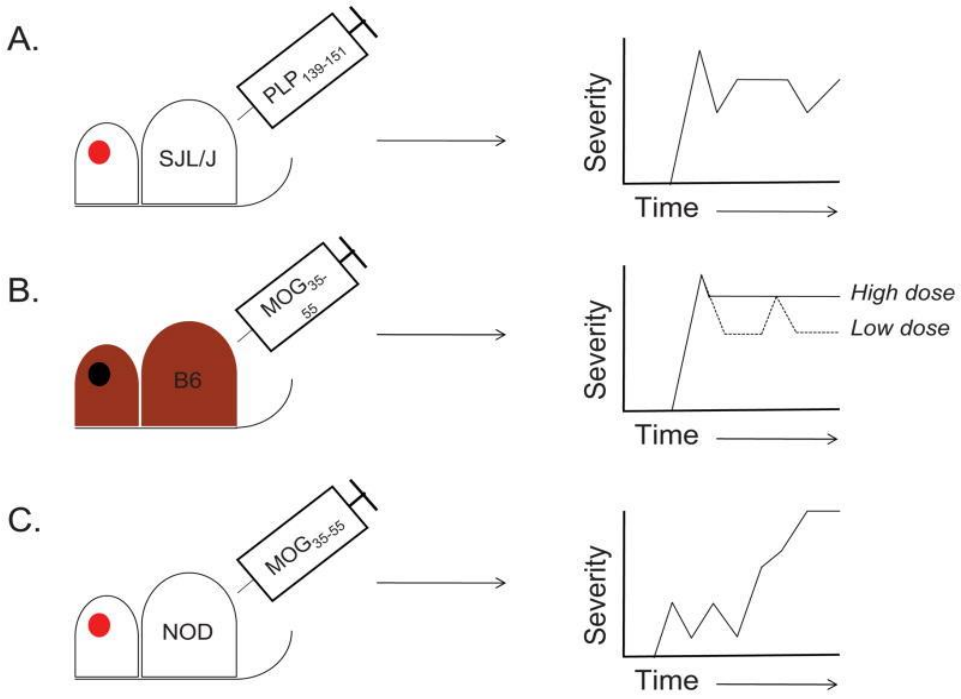


Fig 2.10: Changes in EAE model and disease course upon active immunization with encephalitogenic peptides. (Manu Rangacharia, Sep 2013).

A. Shows the SJL/J mice immunized with $PLP_{139-151}$. This results in paralytic attack at initial stage, and then causes multiple remissions and relapses. (Manu Rangacharia, Sep 2013)
B. Shows the C57BL6/J mice which were immunized with MOG_{35-55} results in chronic disease, initial attack does not resolve in case of high dose of MOG_{35-55}. MOG_{35-55} with low dose cause multiphasic relapsing-remitting disease or in some case single attack is followed by single remission.(Manu Rangacharia, Sep 2013)
C. Shows the NOD mice immunized with MOG_{35-55} results in a series of mild attacks/relapses followed by remissions, which causes the transition of this disease into its secondary chronic phase. (Manu Rangacharia, Sep 2013).

2.8.1- Experimental Autoimmune Encephalomyelitis (EAE) induction methods:

EAE is most widely used animal model for multiple sclerosis. Although the exact reason or cause of this disease is still unknown so different methods of induction were used to study the pathogenesis of this disease. The infiltration of immune cells into the central nervous system (CNS) and process of demyelination characterized the EAE model. (Phelan, Monday, December 19, 2016) The two most widely-used methods for the induction of EAE are:

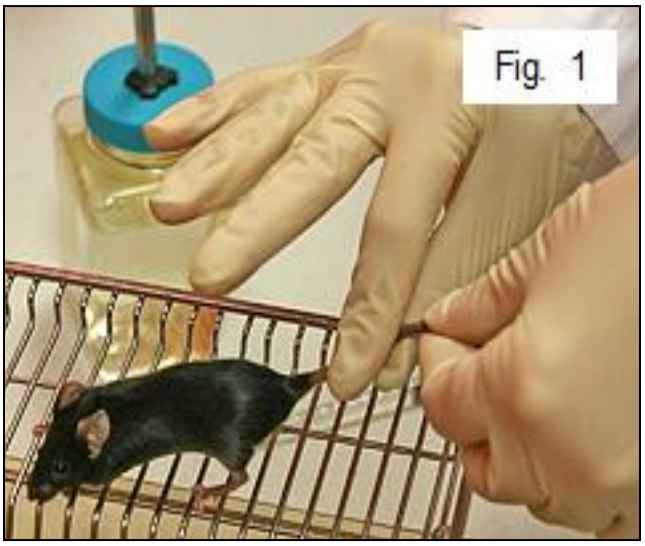
- Induction of EAE by active immunization.
- Induction of EAE by passive immunization (adoptive transfer).

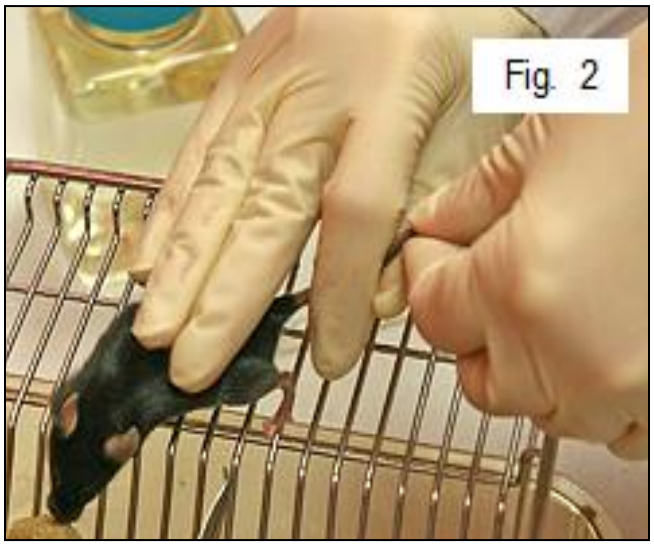
Each method possesses its own importance and potential. (Phelan, Monday, December 19, 2016)

2.8.2- Experimental Autoimmune Encephalomyelitis (EAE) by Active immunization:

The neuro antigen like myelin antigen were normally used in mouse model for active immunization of EAE model with the spinal cord homogenate and this myelin antigen along with the spinal cord homogenate activated the development of T-cells and the immune cells trafficking in the brain or spinal cord region causes the destruction of myelin in the CNS and then it results in inflammation. (ANDREW P. ROBINSON1, 2014). For EAE induction appropriate strain of mice is selected first then mouse is subjected to antigen for immunization. Peptides u8sed in the experiment can be made or synthesize in the lab or can be order from vendors for experimental purpose. Overall the induction period of EAE model through this process can be divided into two important phases. (Stephen D. Miller, 4 August 2010)

- **Induction phase :**in which the first there is notifying of myelin epitope–specific $CD4^+$ T cells followed by introduction of myelin protein or peptide in the complete Freund's adjuvant (CFA) in EAE starts the immunization. (Stephen D. Miller, 4 August 2010)
- **Effector Phase:** This phase is composed of different stages (1). First the myelin specific T-cells are activated then migrated to CNS so the T-cells are extravasated through the tight endothetial junctions composed of Blood brain barrier.(2). In the next step the myelin specific T-cells elaborate the chemokines and cytokines then the influx of peripheral mononuclear/macrophages are induced in to the CNS parenchyma. (3). Then T cells- derived cytokines activates these peripheral monocytes/macrophages and the CNS-resident microglial cells. (4). Phagocytic process of activated mononuclear cell causes the demyelination of CNS axonal tracts and activated $CD4^+$ T cells and monocytes released the inflammatory and cytotoxic eefects of cytokines. e.g., IFN- γ, LT/TNF-β, IL-17, TNF- α, and NO). The adoptive-transfer model of EAE modeled the effector phase of this MS disease in which the peripheral introduction of myelin epitope–specific $CD4^+$ T cells are activated to a simple mouse which is naive. (Stephen D. Miller, 4 August 2010)





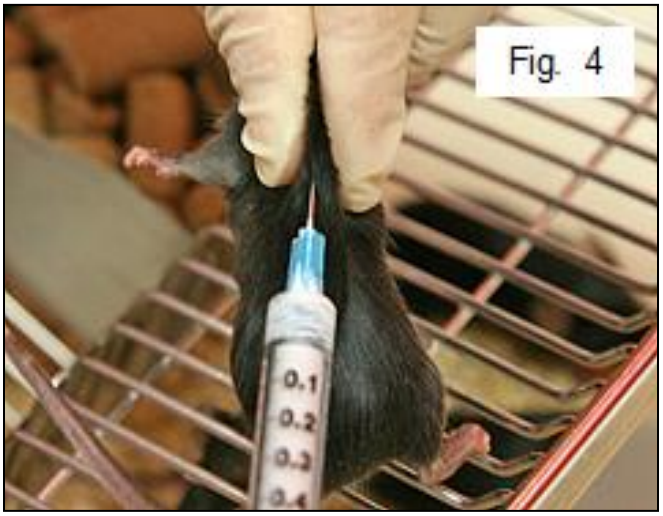


Fig 2.11: The active immunization of C57BL/6 mice.

2.9-General Benefits and Considerations of EAE models by active immunization:

EAE is most widely used model for experimental studies at lab because of following reasons (ANDREW P. ROBINSON1, 2014)

- This animal model is easily available and also inexpensive.
- We get relatively quick results.
- Compound profiling through active immunization in these kinds of mice are easy.
- Relapsing/remitting disease occured in SJL mice through Active immunization.
- Myelin oligodendrocyte glycoprotein (MOG) is an antigen used for the induction of EAE model beside this myelin basic protein (MBP and spinal cord homogenate can also be used.
- The severity of this disease is increased by the used of toxin in animal model like uses of pertussis toxin (PTX).
- The clinical score in this induction of mice of a typical reaout and ascending paralysis is shown in this disease.
- The inflammation in the CNS is visualized through histology. (ANDREW P. ROBINSON1, 2014)

The induction of C57BL/6 mice model is done with an emulsion of MOG_{35-55} in complete Freund's adjuvant (CFA), then the pertussis toxin in PBS were administered, on the first day of immunization and then again on the following days . (Phelan, Monday, December 19, 2016). On the day of 1^{st} and 2^{nd}, pertussis toxin were injected and the onset of disease for EAE after the immunization was 9 to 14 days after immunization. The progression of disease starts from day 3^{rd} to 5^{th} after this onset for each mouse. (Phelan, Monday, December 19, 2016). The peak of disease lasts for 1 to 3 days, following partial recovery. Severity of EAE is shown by only 25%

of the mice after the initial partial recovery after 20-27 days of the immunization. (Phelan, Monday, December 19, 2016)

Mice will remain chronically paralyze as mice were examined and observed for 4 weeks. There is a difference between the pathophysiology of EAE and human disease of MS but EAE is powerful and often utilized model in the development of therapies for MS. EAE contributes to the inflammatory nature of the disease and considered as best for determining the efficacy of immunomodulatory treatments. EAE had been most useful in treatment that directly affected the CNS tissue. (Phelan, Monday, December 19, 2016)

2.10- Factors affecting EAE severity:

Table 2.2: Factors that influenced EAE severity

Factors	Effects
Stress	Disease severity is reduced
Age factor (older age)	Uniform EAE insets but severity of disease is serious
Gender	Severity of this disease is more in female mice
Substrain and breeder	Varies
Dose of PTX	High dose increased the EAE severity

Within some limits EAE severity is controlled by the dosage of pertussis toxin, compensating for these factors. Higher doses of pertussis toxin generally increased the severity of EAE, lower doses reduces the severity of this disease. (Phelan, Monday, December 19, 2016)

CHAPTER 3: MATERIAL AND METHODS

3.1-Methodology:

Experimental work was performed in three different phases:

1. **Phase 1** involves preparation of Animal Model and Sample Collection.
2. **Phase 2** involves 2D Gel Electrophoresis and MS/MS analysis.
3. **Phase 3** involves Bioinformatics Analysis.

3.2-Experimental Groups:

Three experimental groups were used. All groups were matched with respect to

- Gender
- Age
- Litter (when possible)
- Genetic Modifications (i.e., transgene, knock-in, knock-out)
- Other relevant conditions (e.g., gonadectomy, housing conditions)

Three groups were used and each group consist of four mice.

3.2.1- Wild type / control group:

Not a single injection is given to the mice in this group so mice remain asymptomatic. All the mice in these groups are wild type with no symptoms.

3.2.2- Control group without MOG35–55 injection :

The mice in this group were injected with all EAE reagents except MOG35–55. The mice were injected with complete Freund's adjuvant (CFA) containing Mycobacterium tuberculosis.

3.2.3- Mice model with MOG35–55 :

Mice in this group received complete EAE injections. For development of active EAE, mice **(C57BL/6)** were injected (s.c.) at flanks with $MOG^{35–55}$ peptide of 200 µg (Sigma Company) in PBS emulsified in complete Freund's adjuvant (CFA) of equal volume containing Mycobacterium tuberculosis (Sigma Company) at a final concentration of 1 mg/ml.

3.3- Materials:

3.3.1- Laboratory equipment and other materials

- Analytical balance
- Square anti-static weighing dishes,
- Disposable anti-static microspatulas
- Conical centrifuge cubes
- Corning, orange round bottom cryogenic vial of 2.0ml
- PrecisionGlide 27 × 1/2-gauge needle, gray
- PrecisionGlide 25 × 5/8-gauge needle, blue
- 1 mL syringe of Tuberculin slip.
- 3 cc Popper & Sons Perfektum glass with proper matched number syringes. Must be cleaned and properly autoclaved.
- Micro emulsifying needle, 20 gauge

- Scintillation vials
- Sterile alcohol prep pads

3.3.2- Chemicals:

Chemicals used in this study were purchased from Sigma Company and lists of chemicals used are

Table 3.1: List of Chemicals used

S.No	CHEMICALS	AMOUNT
1	Dulbecco's phosphate buffer saline (DPBS)	50ml
2	Lyophilized MOG35–55 peptide	200ug
3	Pertussis toxin (PTx)	400ug
4	Heat-killed Mycobacterium tuberculosis H37 RA	4mg/ml
5	Complete Freund's Adjuvant	1ml
6	Ethanol	70% (vol/vol)

- Dulbecco's phosphate buffer saline (DPBS) were used without calcium or magnesium.
- Lyophilized MOG35–55 peptide were used with sequence (Sequence: MEVGWYRSPFSRVVHLYRNGK; >95% purity).
- Pertussis toxin (PTx) were lyophilized in pure water and salt-free toxin were used.

3.4- Methods:

- The reagents quantities were based on the mice number used in the experiment so the number of ice and quantity of reagent were first calculated. Each mouse received 1mg/ mL total of $MOG^{35–55}$ -CFA through two 0.05 mL injections on both Days 0 and Day 7.
- $MOG^{35–55}$ and M. tuberculosis were administered at 200 µg/mouse.

Critical step: Different disease profile comes from changing the $MOG35^{–55}$, M. tuberculosis, or PTx dosages. Alteration in the cytokines, immune cell population and location of lesions depends upon the concentration of dosage only when the disease course it's onsets and clinical scoring is consistent. (Jee and Matsumoto, 2001).

3.5- Phase 1 Preparation of Animal Model:

3.5.1- M. tuberculosis and Complete Freund's adjuvant storage:

- Within the BSC, M. tuberculosis H37 RA was opened carefully ampule per manufacturer's specifications.
- The content was then transferred into a parafilm, and stored at 4°C until it is use.
- CFA ampule was to re-suspend the M. tuberculosis. CFA ampule per manufacturer's specifications was opened within the BSC. The contents are then transferred to a parafilm, and stored at 4°C for further use.

3.5.2- Preparation of MOG35–55-CFA emulsion

- Emulsifying syringes glass was attached to the emulsifying needles and then by the help of plunger it is pressed down.
- Syringe, plunger, and needle must be clean. And both of them were correctly paired.

- On the top there must be syringe and once it was assembled, each assembly note must unscrew easily, without any tightening both the syringes were tightly secured.
- Plunger was then removed from the syringe and placed it on a sterile surface within the BSC.
- 50 mL conical tube was then filled with ~1 mL more than the calculated volume of DPBS.
- Balance was cleaned by using Kim wipe sprayed with 70% Ethanol.
- MOG35–55 peptide were taken from the −20°C freezer to a room temperature prior to weighing.
- MOG35–55 peptide was then weighed out.
- Without removing any boat from the peptides, MOG35–55 peptide were weighed from the balance and then half volume of DPBS were added, buffer might be expelled out of the boat from its edges. So when the peptide was covered by the DBPS used on the experiment then we have transferred that into BSC after weighing properly. The DPBS were added then.. The end solution was clear.
- On the top of the emulsifying syringe used, the premeasured about of MOG35-55 were added and then all the solution was aspirated at the bottom of the strings plunger. Total MOG35–55 solution must be equally distributed.
- CFA were vertexes to confirm the uniform suspension.
- The predetermined amount of TB weighed out. The CFA were weigh out before transferring into the container..
- The measured volume of solution of TB-CFA was added to the emulsifying syringe.

- Process of emulsification continued for 25times on each side and syringes were placed at -20°C for 1 h.
- These syringes were placed at -4°C after 1h until emulsion become softens enough to easily pass through the needle. Then in BSC chamber re-emulsified this 25 times on each side and it become total of 50 times.
- These syringes were then stored to −20°C again for at least 1h.until this one is ready to immunize (up to 6 hrs).

3.5.3- Preparation of Pertussis toxin:

- On day 0 and then on day 2 mice were administered 0.3 mL of 500ng Pertussis toxin in DPBS.
- These things were placed in a clean BSC.
- DPBS was aliquot into the conical tube of 50ml.
- The needle is carefully pushed by using the rubber cap. The DPBS were automatically drained into the vial.
- PTx vial content was then pipette into the conical tube of 50ml.
- More than three times the DPBS with the volume of 1ml were rinsed until there will be no particles of PTx were found in the vial.
- Each syringe was filled, must avoid bubbles. Stored at 4°C.

3.5.4-MOG immunization into mice (C57BL/6) for the development of EAE

- For development of active EAE, mice **(C57BL/6)** were injected (s.c.) at flanks with $MOG^{35–55}$ peptide of 200 µg (Sigma Company) in PBS emulsified in complete

Freund's adjuvant (CFA) of equal volume containing Mycobacterium tuberculosis (Sigma Company) at a final concentration of 1 mg/ml.

- At the time of immunization and even 48 hours later each mouse received the two injections of 400ng pertussis toxin (Sigma Company). Minimum of four mice were included in each treatment group.
- For clinical score of disease animals were properly weighed, these clinical score evaluations are very important to determine the signs of disease..
- Mice for acute stage inflammation during EAE and their corresponding negative controls were sacrificed at 17 days post inoculation (dpi). Mice for chronic stage inflammation during EAE and their corresponding negative controls were sacrificed at 57 dpi.
- Few of the inoculated mice had recovered and had shown no signs of inflammation at the end, so they were grouped together in “Recovered” mice group. On the day of sacrifice, mice were perfused transcardialy with PBS to prevent contamination of spinal cord with blood followed by extraction of spinal cord tissue. Cervical region of spinal cord was extracted and snap frozen for immunofluorescence and rest part of spinal card was extracted and snap frozen for proteomic analysis.

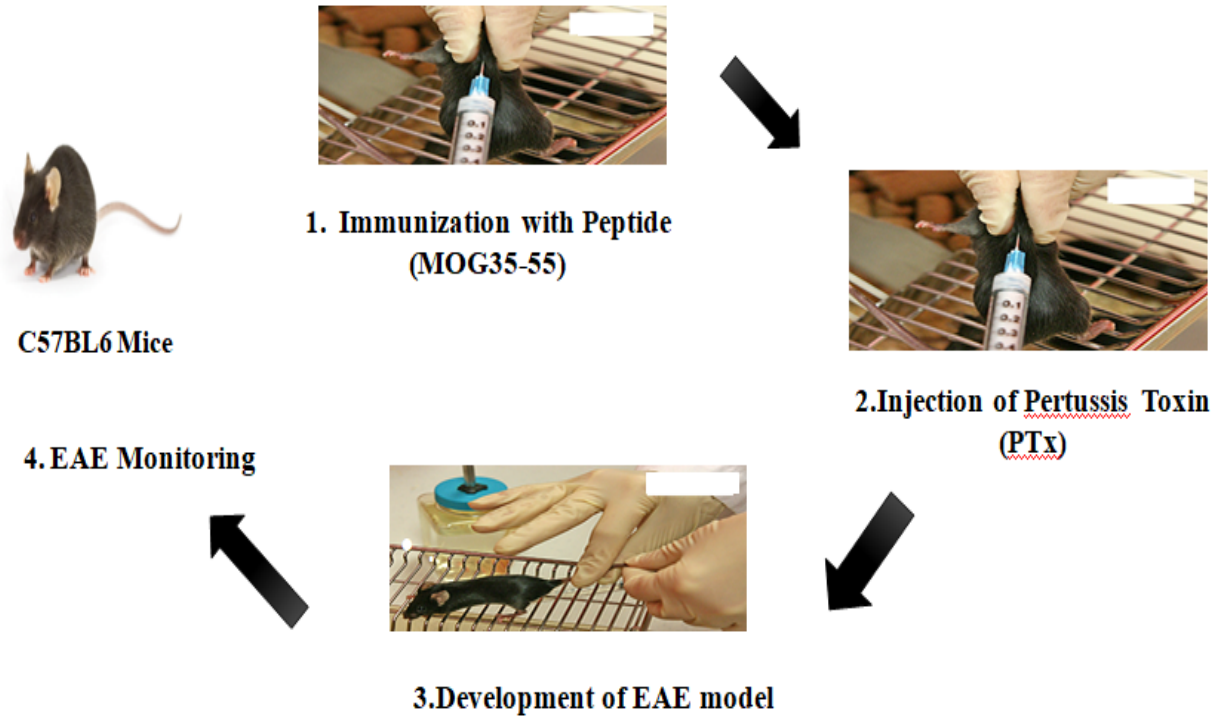


Fig 3.1: Development of Animal Model

The mouse used in this experiment was C57BL6 which is immunized with MOG35-55. MOG (35-55) is a myelin oligodendrocyte glycoprotein (MOG) 35-55 is a minor component of CNS myelin. Then two injections of pertussis toxin were injected intra-peritonically. Pertussis toxin increases the severity of disease in mouse model. Once the mouse model is developed then this model is further used for EAE monitoring.

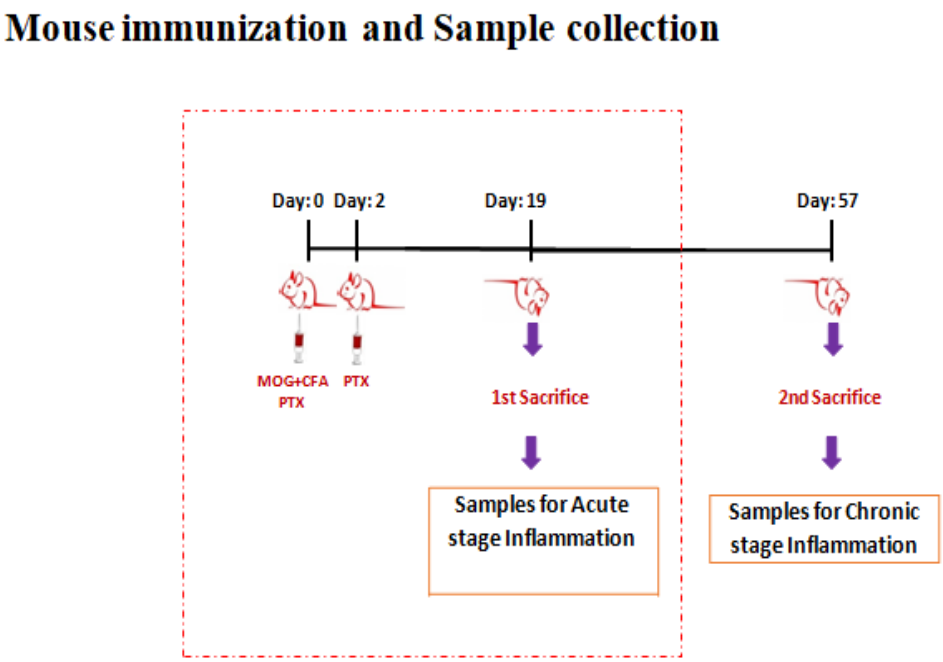


Fig 3.2: Mice for acute stage inflammation during EAE and their corresponding negative controls were sacrificed at 17 days post inoculation (dpi). Mice for chronic stage inflammation during EAE and their corresponding negative controls were sacrificed at 57 dpi.

3.5.5- **Sample preparation, SDS-PAGE and Western blotting**

- Sample preparation involves homogenization of tissue lysate (10% w/v) of frozen spinal cord tissue samples which were prepared in lysis buffer.
- The lysis buffer contain urea urea of 7M, Thiourea of 2M, 4 % CHAPS, 20 µl/ml Ampholytes, 10mg/ml Dithiothreitol (DTT), protease and phosphatase inhibitors.
- In the next step ultracentrifugation occur at 30,000 RPM for 30min.
- Bradford assay (Bio-Rad) is used to determine the protein concentration in tissue lysate. Boiling of samples occurred at 95°C for 5 min after mixing them with 4X Roti-Load(ROTH) as a sampling buffer.

- Cooling of sample was done, before the next step.
- It was then used for Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis (SDS-PAGE) and then finally western blotting.

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

3.6.1- Two Dimensional Gel Electrophoresis, Visualization and Analysis of protein spots

Separation of protein in two dimensions was achieved by 2D gel electrophoresis through two dimension process. In the first dimension isoelectric focusing (IEF) were analyzed and molecular weight with SDS-PAGE was analyzed in second dimension and the experiment was done for protein separation and different fractions of proteins were also separated from the mixture of tissue lysate through this method. Silver stain were used to visualized the protein spot after separation followed by scanning (CanoScan Scanner) for further analysis of protein spots. DECODON Delta2D software were used for the analysis of protein visually and to identify the expression of protein spots differentially between different groups which analyzed the each spot intensity individually with the help and calculated by using a cut off value of 1.5 fold change and p-value <0.05 in unpaired Student's t-test..

3.6.2- Mass-spectrometry for the Identification of protein/ peptide sequences:

- Protein spots from the silver strained 2DEgel and which show different expression were extracted and then processed for the identification of protein sequence with the Q Exactive hybrid quadrupole/orbitrap mass spectrometry. The processing involves the

silver stain destaining, disulfide bonds reduction, free cysteines alkylation, digestion of trypsin, extraction of peptide and peptide sequence identification.

- Mass spectrometers were used to identify the protein and after the identifications, of peptide count (≥2) were used to eliminate the false positive identifiers in the proteomics data, (95%) of peptide threshold.
- 0.01% of minimum FDR rate which is false discovery rate were then used for the qualification of identified proteins.

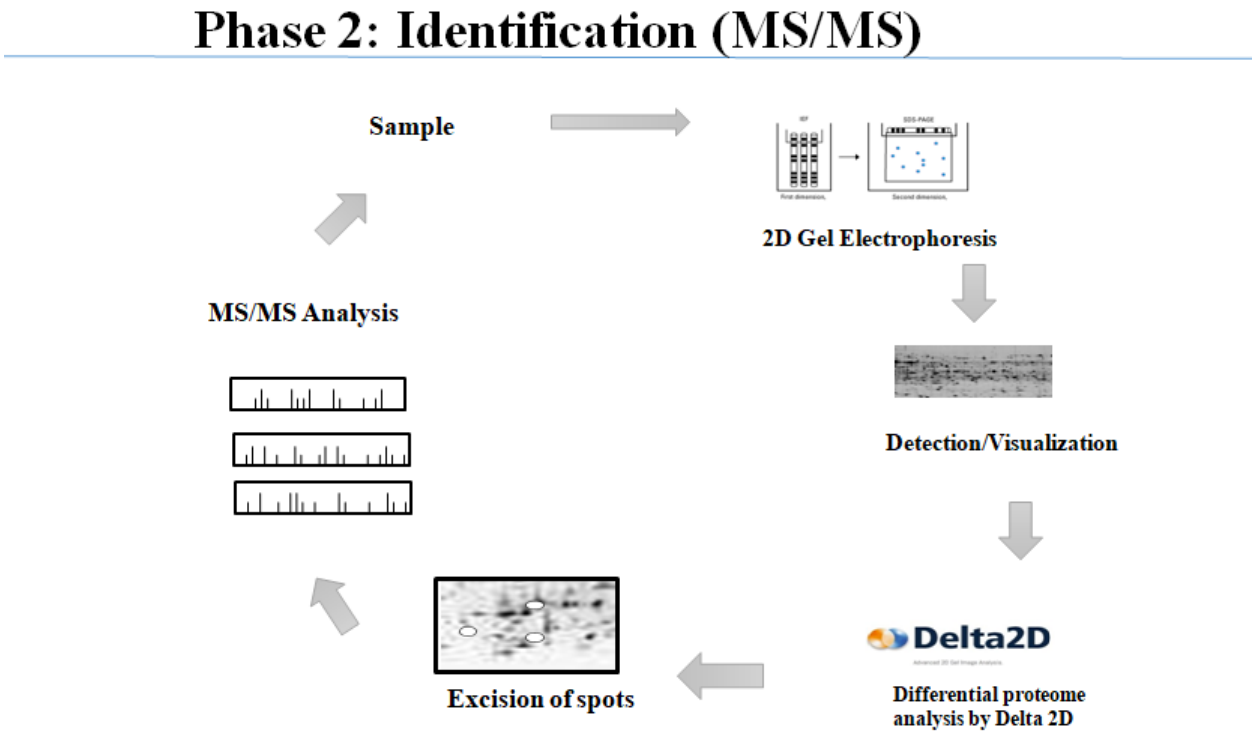


Fig 3.3: Proteins Separation by 2D Gel Electrophoresis and Identification of Protein by MS/MS Analysis.

2-D electrophoresis is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. It is the method available

which is capable of simultaneously separating thousands of proteins. This technique separate proteins in two steps, according to two independent properties: First-dimension is isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pI); Second-dimension is SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular weights (MW). In this way, complex mixtures consisted of thousands of different proteins can be resolved and the relative amount of each protein can be determined. Each spot on the resulting two-dimensional gel potentially corresponds to a single protein species in the sample. Thousands of different proteins can be separated and information such as the protein pI, the apparent molecular weight, and the amount of each protein can be obtained. At the very beginning of the 70s, two high-performance electrophoretic separations of proteins were available: i) zone electrophoresis of proteins in the presence of SDS, as described in its almost final form by Laemmli, a technique that instantly became very popular, and still is, and ii) denaturing isoelecric focusing, as described by Gronow and Griffith. As these two techniques used completely independent separation parameters, it is not surprising that it was soon tried to couple them. Two-dimensional electrophoresis was first introduced by O'Farrell in 1975.

In 2D GE proteins are separated as per isoelectric point and protein mass. Separation of the proteins by isoelectric point is called isoelectric focusing (IEF). When a gradient of pH is applied to a gel and an electric potential is applied across the gel, making one end more positive than the other. At all pH values other than their isoelectric point, proteins will be charged. If they are positively charged, they will be pulled towards the negative end of the gel and if they are negatively charged they will be pulled to the positive end of the gel. The proteins applied in the

first dimension will move along the gel and will accumulate at their isoelectric point; that is, the point at which the overall charge on the protein is 0 (a neutral charge).

In separating the proteins by mass, the gel treated with sodium dodecyl sulfate (SDS) along with other reagents (SDS-PAGE in 1-D). This denatures the proteins (that is, it unfolds them into long, straight molecules) and binds a number of SDS molecules roughly proportional to the protein's length. Because a protein's length (when unfolded) is roughly proportional to its mass, Since the SDS molecules are negatively charged, the result of this is that all of the proteins will have approximately the same mass-to-charge ratio as each other.

In addition, proteins will not migrate when they have no charge (a result of the isoelectric focusing step) therefore the coating of the protein in SDS (negatively charged) allows migration of the proteins in the second dimension. In the second dimension, an electric potential is again applied, but at a 90 degree angle from the first field. The proteins will be attracted to the more positive side of the gel (because SDS is negatively charged) proportionally to their mass-to-charge ratio. The gel therefore acts like a molecular sieve when the current is applied, separating the proteins on the basis of their molecular weight with larger proteins being retained higher in the gel and smaller proteins being able to pass through the sieve and reach lower regions of the gel.

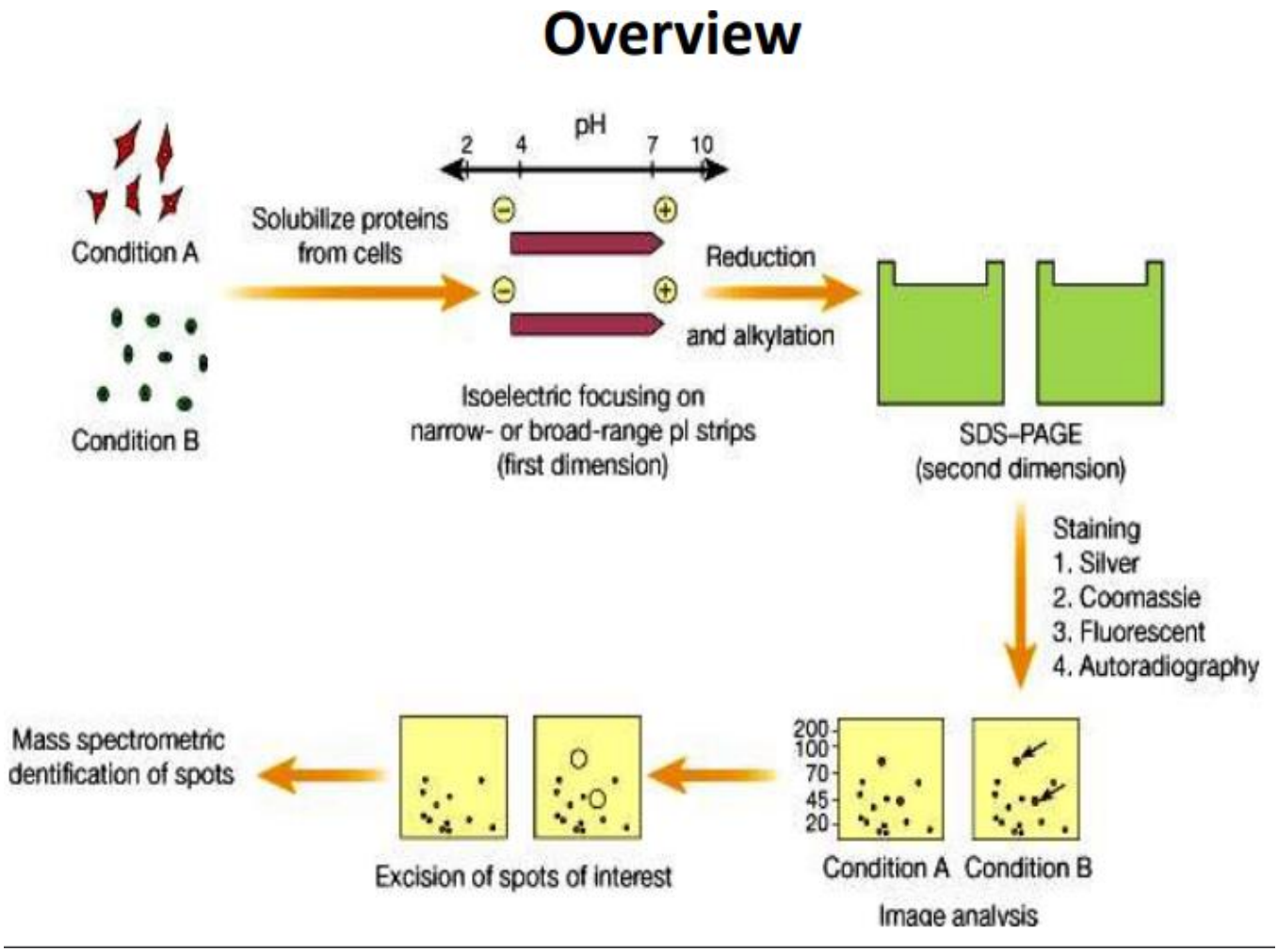


Fig 3.4: Overview of Separation of Protein through 2D Gel Electrophoresis.

3.6.3- Spinal Cord Co-immunofluorescence :

- Frozen spinal cord tissue sections (5 µm) from each of EAE (acute stage), EAE (chronic stage), CFA controls and their corresponding negative controls were fixed with methanol (9 min.) followed by acetone (1 min.). Then PBS were used to wash the sections and also

it wash tissue sections of different antigenic sites. Permeabilization buffer (PBS + 0.2 % Triton X-100) exposed this section for 10 min.

- The non specific protein were obstructed by 5% of bovine serum albumin with RAB7 for 2h and then left it for incubation overnight (1:100) as primary antibody (pAb) then diluted in 5% serum with PBS for overnight at 4°C.
- Horseradish peroxidase (HRP) labeled which is anti-goat secondary antibody was used for 2h
- To-Pro3 was used to stain the nuclei for 10 min.
- Confocal Laser Scanning was used to visualize the sections.
- Co-localization pattern of individual images is analyzed by using the software ImageJ(WCIF plugin) .

3.6.4- Sucrose density gradient formation:

Sucrose density gradient was performed in order to study the expression of oligomer formation under inflammatory stress condition during EAE. Spinal cord tissue samples were homogenized by 10% w/v in 1x PBS containing 2% w/v sarkosyl. Continuous sucrose gradient of 10-50% were made in 2mL ultracentrifugation tubes followed by layering of homogenates on the top of them. Ultracentrifugation was performed at 50,000 rpm for 73 min. at 4°C and five fractions were collected from top to bottom for each sample.

3.6.5- Statistical analysis:

Graph Pad Prism 5 was used to analyze the data. Experimental errors were displayed as SEM. Student´s t test and nonparametric one way ANOVA was used to calculate the statistical

significance followed by Turkey's multiple comparison test. Results were considered significant when *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

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 SNAP 25 and the ligand is RAB 32. The accurate prediction of the binding modes between the ligand and protein is of fundamental importance in modern structure-based drug design. The most important application of docking software is the virtual screening, in which the most interesting and promising molecules are selected from an existing database for further research.

- PDB file of SNAP- 25 and RAB 32 were generated from PROTEIN DATA BANK
- GRAMM-X was used for Docking of SNAP-25 and RAB 32.
- Docking of these two proteins gave interaction between these two and also showed different interactive site with different bond lengths.
- The interaction between SNAP 25 and RAB 32 provide pathway for their further role in pathogenesis of Multiple Sclerosis.

Phase 3: *Bioinformatics Analysis*

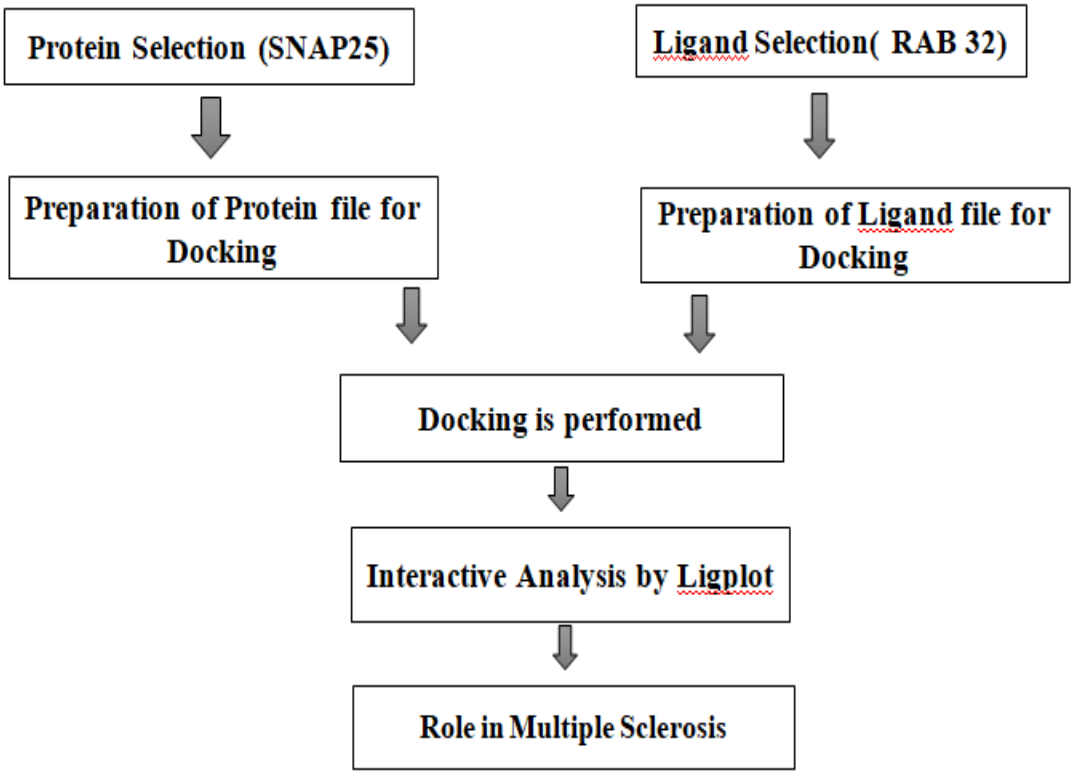


Fig 3.5: Docking of SNAP 25 and RAB 32

3.7.2- LIGPLOT Analysis:

In Bioinformatics Analysis, LIGPLOT is a computer program that generates schematic 2-D representations of protein-ligand complexes from standard Protein Data Bank file input. The LIGPLOT is used to generate images for the PDBsum resource that summarises

- Protein-Protein Interaction
- Protein-Structure Analysis

- Protein-Pathway Analysis

3.7.3- Uniprot Database:

UniProt/SwissProt database was used for functional analysis of detected proteins from the MS/MS analysis. On the basis of annotations of UniProt/SwissProt database protein candidates from high-density fraction datasets and global proteome datasets were manually separated in modules representing a singular physiological category for proteomics. So SNAP-25 sequence and fictional information was derived from this database.

3.7.4- Data Input to Ligplot:

The file format for Ligplot data entry is PDB formate. The PDB file was generated from the Protein directory of Protein Data Bank and this PDF file for specific protein like SNAP -25 was used in Ligplot for determination of ligand ligand interaction and ligand protein interaction.

3.7.5- 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

4.1- Results:

Diseases are usually results of many physiological variations at biochemical and metabolic strata. Minor differences cause major serious disorders in the broad spectrum of variability. Even changes in a single disease giving rise to its different subtypes. Likewise, differences for the progression rate amongst patients with Multiple Sclerosis, leading to the different types. Experimental autoimmune encephalomyelitis (EAE) is mostly used as an animal model for the inflammatory disease like Multiple to study the pathogenesis of the demyelination. Different types of of immunopathological and neuropathological mechanisms can be studied in EAE which further provides the pathological characteristics MS including inflammation, demyelination, axonal loss and gliosis. A complex neuropharmacology of EAE has discovered the drugs that are in use in MS, tested or validated on the basis of EAE studies. Different types of neuropharmacological. Different immunological and neuropharmacological interventions have been studied in this model so this model is considered as a great source of heterogeneity in the susceptibility to the induction.

4.2- Clinical Assessment of EAE model:

MOG-immunized C57BL/6 mice developed the signs of EAE around 15 days after immunization by displaying loss of tail tonus. The maximal clinical symptomatology, that is indicative of the acute phase, occurred at day 19 when the average clinical score reached 2. From this period on the animals slightly improved their mobility but did not completely recover from paralysis. The

clinical scores that declined to an average of 1.5 did not significantly change until the 30th day that was chosen as the end point of the experiment. Variation in body weight showed an expected course characterized by a significant weight drop during the acute phase. This loss was followed by a progressive weight recovery. Animals with EAE reached weight values similar to the normal control group at the 30th day following immunization. The average clinical score for chronic EAE model is below 2.5. Above 2.5 clinical scoring is ethically prohibited.

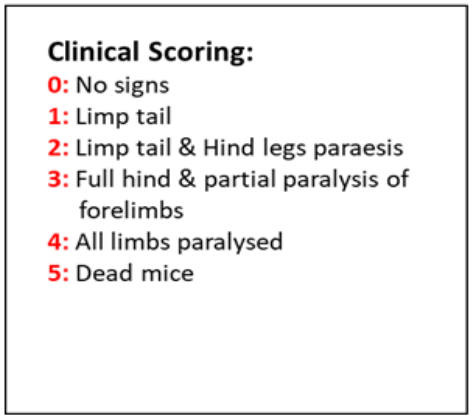


Fig a

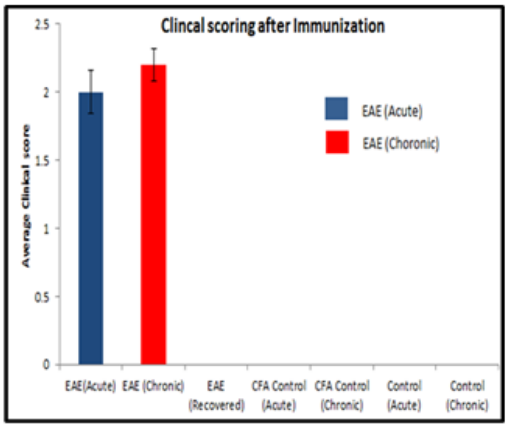


Fig b

Fig 4.1: Clinical Scoring of EAE model in which 0 sets for No signs, 1 is set for limp tail, 2 is set for limp tail and hind legs paraesis, 3 is set for full hind and partial paralysis of forelimbs, 4 is set for all limbs paralyzed and 5 is set for dead mice.

Fig 2 shows the average clinical scoring of acute and chronic stage EAE model.

4.3- Identified Differential Proteome

This study involves the characterization of different proteins identified in EAE, the characterization of SNAP-25 through bioinformatics tools including Ligplot and Scaffold shows its role in the progression of multiple sclerosis. Different proteins are involved that causes the multiple Sclerosis like SNAP 25, High mobility Protein. In the current study, we aim to define the mechanistic way behind the progression rate through this software.

Table 4.1: Differential Identified Protein

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	↑

Table shows that 17 differential proteins were identified from EAE model and SNAP 25 is further validated through bioinformatics analysis .Furthermore the data was assessed by Scaffold

Mass Spectroscopy to identify the unique peptide sequence of SNAP-25 which represents the level of SNAP-25 in MS.

4.4- Scaffold Results:

4.4.1- Identification of unique sequence through Scaffold:

The given fig shows the unique sequence of amino acid which does not appear anywhere else in the genome. The yellow color sequences are the identified unique sequences while green sequences are the modified sequence which was obtained after post translation.

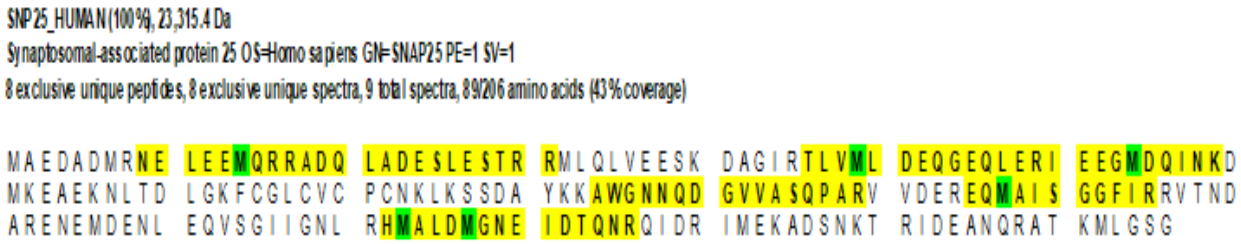


Fig 4.2 shows amino acid sequence of Synaptosomal associated protein 25 with 100% probability with the size 23,315.4 Da. SNAP-25 consist of 8 exclusive unique peptide sequence, 9 total spectra out of which 8 are exclusive unique spectra with 43% coverage of aminoacids means 89/206 amino acids expand. Highlighted yellow portion in the graph shows the identified amino acids which matched with the given MS/MS spectra. Yellow highlight indicate the peptides that were confidently identified by the fdr(false discovery rate) cut off that were specified. Green highlights indicate the post translational modification.

Sequence Coverage	Protein	Accession	Category	Bio Sample	MS/MS Sa...	Prob	%Spec	#Pep	#Uni...	#Spec	%Cov	m.w.
	Synaptosom...	SNP25_HUM...	02	A_Noor_211...		97%	0.0053%	1	1	1	5.3%	23 kDa
	Synaptosom...	SNP25_HUM...	04	A_Noor_211...		100%	0.049%	8	8	9	43%	23 kDa
	Synaptosom...	SNP25_HUM...	07	A_Noor_211...		96%	0.0051%	1	1	1	5.3%	23 kDa

Fig 4.3: Biological replicates of SNAP 25

Valid	...	Sequence	Prob	Masc...	Masc...	Masc...
☑	1.0	(R)NELEEMQR(R)	99%	24.3	25.0	24.3
☑	1.0	(R)RADQLADESLESTR(R)	98%	21.1	25.0	21.1
☑	1.0	(R)RADQLADESLESTRR(M)	100%	30.5	25.0	30.5
☑	1.0	(R)TLVMLDEQGEQLER(I)	100%	72.1	25.0	72.1
☑	1.0	(R)IEEGMDQINK(D)	100%	34.2	25.0	24.9
☑	1.0	(K)AWGNNQDGVVASQPAR(V)	100%	106.0	25.0	106.0
☑	1.0	(R)EQMAISGGFIR(R)	100%	41.2	25.0	39.5
☑	1.0	(R)EQMAISGGFIR(R)	100%	29.0	25.0	29.0
☑	1.0	(R)HMALDMGNEIDTQNR(Q)	100%	69.5	25.0	69.5

Fig 4.4: shows total 9 biological replicates with sequences were identified. 7 biological sequences showed 100% of probability.

Valid	...	Sequence	Prob	Masc...	Masc...	Masc...	NTT	Modifications	Observed	Actual Mass	Charge	Delta ...	Delta ...	Rete...	Intensity	TIC	Start	Stop	# Ot...	Other Prot...	Spectrum ID
☑	1.0	(R)NELEEMQR(R)	99%	24.3	25.0	24.3	2	Oxidation (+16)	532.74	1,063.46	2	-0.00096	-0.90			386100	9	16	0		Elution from: 14....
☑	1.0	(R)RADQLADESLESTR(R)	98%	21.1	25.0	21.1	2		530.93	1,589.77	3	0.0029	1.8			818500	17	30	0		Elution from: 20....
☑	1.0	(R)RADQLADESLESTRR(M)	100%	30.5	25.0	30.5	2		582.96	1,745.87	3	0.0022	1.2			964200	17	31	0		Elution from: 19....
☑	1.0	(R)TLVMLDEQGEQLER(I)	100%	72.1	25.0	72.1	2	Oxidation (+16)	838.91	1,675.81	2	-0.00084	-0.50			775600	46	59	0		Elution from: 27....
☑	1.0	(R)IEEGMDQINK(D)	100%	34.2	25.0	24.9	2	Oxidation (+16)	596.78	1,191.54	2	-0.00052	-0.44			286400	60	69	0		Elution from: 15....
☑	1.0	(K)AWGNNQDGVVASQPAR(V)	100%	106.0	25.0	106.0	2		835.41	1,668.80	2	0.00071	0.43			804300	104	119	0		Elution from: 21....
☑	1.0	(R)EQMAISGGFIR(R)	100%	41.2	25.0	39.5	2	Oxidation (+16)	612.81	1,223.60	2	-0.00073	-0.59			969000	125	135	0		Elution from: 25....
☑	1.0	(R)EQMAISGGFIR(R)	100%	29.0	25.0	29.0	2	Oxidation (+16)	612.81	1,223.60	2	-0.00073	-0.59			1169000	125	135	0		Elution from: 25....
☑	1.0	(R)HMALDMGNEIDTQNR(Q)	100%	69.5	25.0	69.5	2	Oxidation (+16),...	592.93	1,775.76	3	-0.00066	-0.37			582500	162	176	0		Elution from: 17....

Fig 4.5 shows the result of biological replicates of SNAP 25.According to Scaffold about 98% of probability of sequence is required for its accuracy. The Mascot score for a protein is the summed score for the individual peptides, e.g. peptide masses and peptide fragment ion masses, for all peptides matching a given protein. For positive protein identification, the mascot score has to be above the 95% confidence levels.

4.4.2- Determination of expression of protein though Mass spectrometry:

The graph below shows the mass spectrum of SNAP-25 protein with different peaks and top-bottom MS/MS spectra determine the expression of Synaptosomal associated 25 protein.

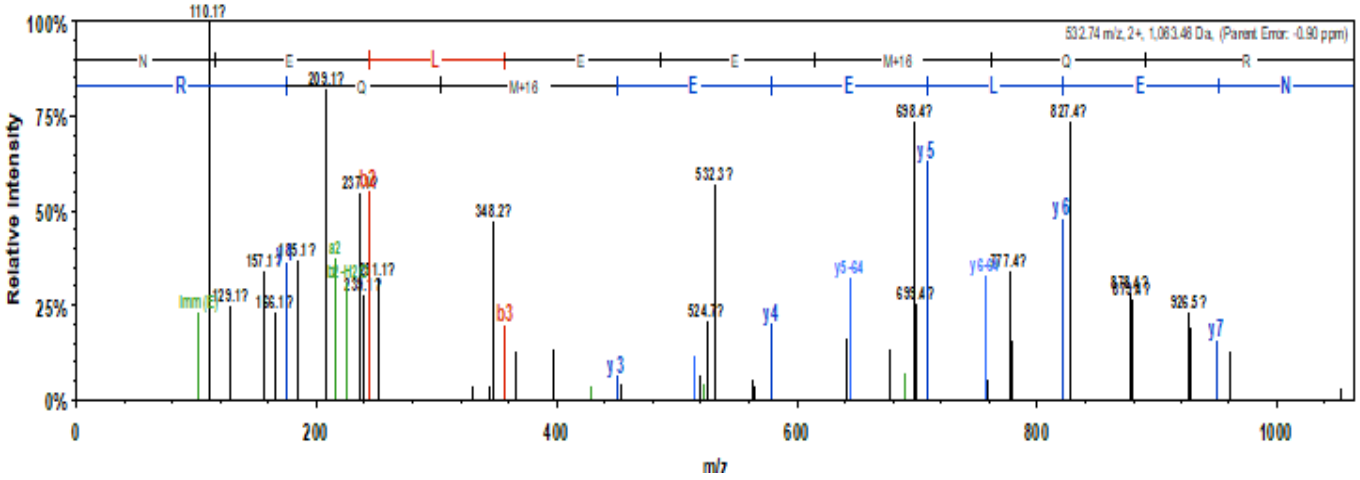


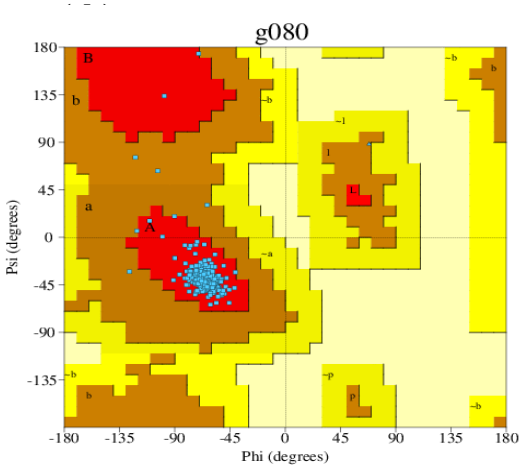
Fig 4.6: Mass spectrum of SNAP-25

B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	115.1		98.0		N	1,064.5	532.7	1,047.4	1,046.5	8
2	244.1		227.1	226.1	E	950.4	475.7	933.4	932.4	7
3	357.2		340.2	339.2	L	821.4	411.2	804.4	803.4	6
4	486.2		469.2	468.2	E	708.3		691.3	690.3	5
5	615.3		598.2	597.3	E	579.3		562.2	561.2	4
6	762.3	381.7	745.3	744.3	M+16	450.2		433.2		3
7	890.4	445.7	873.3	872.3	Q	303.2		286.2		2
8	1,064.5	532.7	1,047.4	1,046.5	R	175.1		158.1		1

Fig 4.7: B ions and y ions of Synaptosomal associated 25 protein, the B ions are the charges that retained on the N-terminus and Y ions are those products when the charges are retained on the C-terminus These spectra are graphical representation which explains the difference between calculated masses as well as the masses obtained through mass spectrometry analysis. During spectrum and peptide similarity accurate matching outcomes are gained when peaks combine well with mass accuracy of employed mass spectrometer. This graphical representation of MS/MS spectra, in which red-b ions represents fragment ions whereas blue ion indicates y ion. While green represents water, grey ones are not discovered. B and y ions assigned to this sequence,. Longer peptide to have tendency to have high sequence score in sequent.Potential ions which matched the spectra are shown in red and blue lines are mention in tabular form in fragmentation table. Like wisely, the y-ion values determined by the spectrum from y2 to y8. While green represents immonium ion or neutral loss of NH_3 or water, grey ones are not discovered.That each of peaks that's labeled as a wire b corresponds to that matched the theoretical fragmentation for this peptide, we are looking for the coverage of y & b ions. We got coverage of y8 y7 but missing y6 then y5 to y1 they have pretty low abundance We have corresponding b ions that gives us a little confidence in this identification.We are looking for good signal to noise means that signal from our peak is significantly above the noise level. How many peaks that we detected are labeled or matched to the theoretical fragmentation in this case of all of them did because they are colored.

4.5- LIGPLOT Results:

Structure of protein is important to determine the function and role of protein in specific disease. LIGPLOT determine the Secondary structure of SNAP-25 protein which consists of 5 different chains of different amino acids and 66 residues



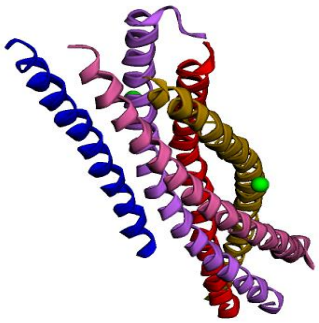


Fig 4.8 Fig 4.9

Fig. 4.8: Ramachandran plot calculations for SNAP-25 protein computed with the PROCHECK program. Fig 4.9: 3-dimensional structure of SNAP 25.

4.5.1- RAB 32 interaction with Culprit Synaptosomal-associated protein 25 :

Protein and ligands interactions are important for all processes occurred in living organisms. Ligand binding capacity regulates the biological functions.

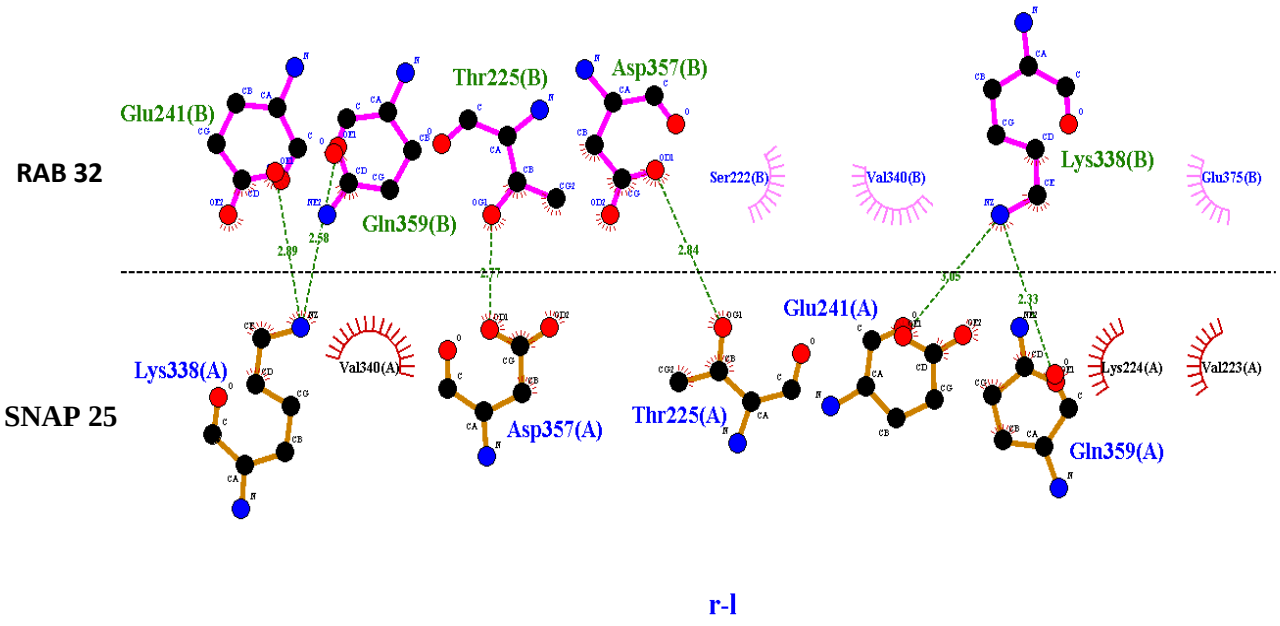


Fig 4.10: Interaction of SNAP 25 and RAB 32.

It shows first hydrogen bond was formed between Lys338(A) [Lysine 338 amino acid residue of chain A of SNAP 25 protein] with Glu241(B) [glutamic acid 241 amino acid residue of chain B of RAB 32 ligand] with the length of 2.89and Gln359(B)Glutamine 359 amino acid residue of china B of RAB 32 with bond length of 2.54. While second hydrogen bond of length 2.93 was formed between Asp(A) [Aspartic Acid 357 amino acid residue of chain A of SNAP 25 protein] and Thr 225(B) [Theronine 225 amino acid residue of chain B of RAB 32 ligand]. Total five interactive site of SNAP 25 shows interaction with different bond length with RAB 32.Three non-ligand amino acid residues involved in hydrophobic interactions were represented by red circular lines. The covalent/elastic bonds formed were represented as thin purple lines. DIMPLOT was applied on LIGPLOT results to study chain A and B interactions across protein-protein interfaces. The horizontal dashed black line represents the interface.

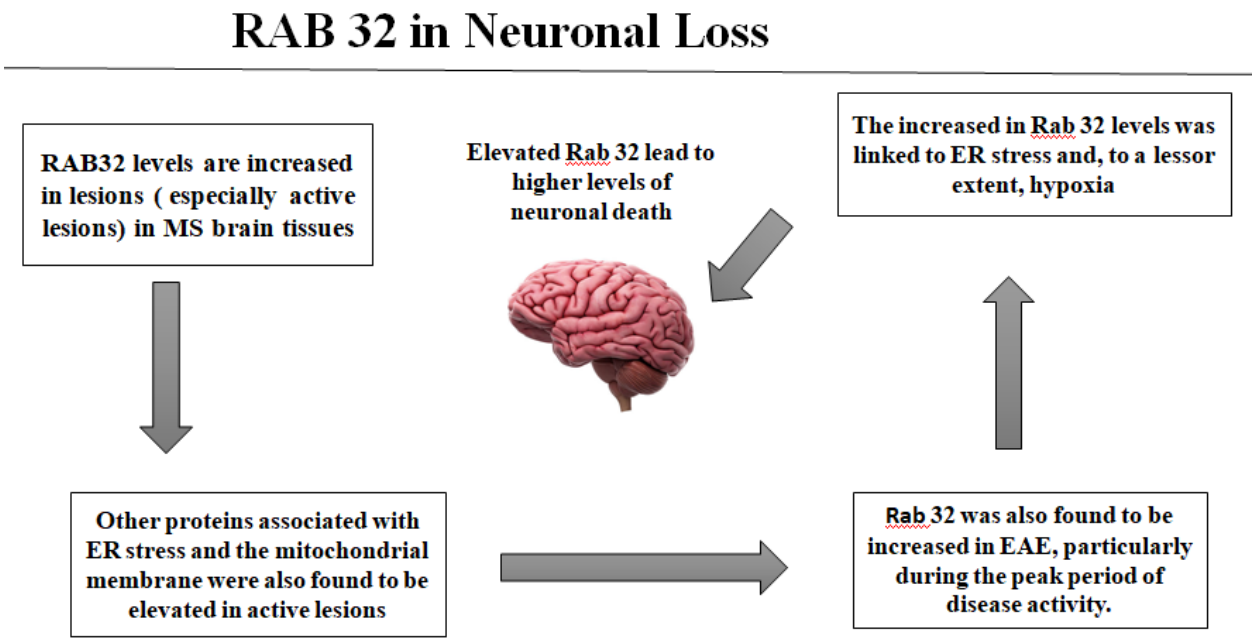


Fig 4.11: Diagrammatic illustration of RAB 32 role in Multiple Sclerosis.

CHAPTER 5 - DISCUSSION

Multiple sclerosis is an autoimmune and progressive demyelinating neurodegenerative disease of the central nervous system (CNS) that causes the neurological defects.MS presents in adult life and it showed a major impact on the life activites. It is considered as inflammatory disease of the central nervous system (CNS) in which there is destruction of myelin sheath results in demylenation. It was found through studies that two million people worldwide and some 100 000 people in the United Kingdom were affected with this disease. (Stefan Bittner, 15 April 2014)

EAE is the best model for studying MS mechanisms to test or develop drugs from pathogenesis point of view. The model was produce by active immunization of protein that causes the demylenation and inflammation. The study focused on many aspects of Multiple Sclerosis in EAE model and protein study through different tools. The purpose of study is to develop animal model of MS that can easily identify the pathological mechanisms and also targeted mechanisms for therapeutic intervention. (Stefan Bittner, 15 April 2014)

The EAE pathophysiology explains the reaction of immune cells against the specific antigens. These reactions cause the inflammation and destruction of structures carrying the antigens resulted in neurological and pathological features. Different approaches can be used for the induction of EAE model are: Actively-induced EAE (aEAE; active immunization), passively transferred EAE (pEAE; transfer of encephalitogenic cells from an immunized animal), and more recently spontaneous EAE mouse models (sEAE) which allowed the study of autoimmune mechanisms without exogenous manipulation. The induction of aEAE is easiest in mice induction that we got fast and quick results. Many researchers named it as "gold standard" of neuroimmunological animal models. (Dimitry N Krementsov, July 2013)

Subcutaneously the animal was immunized with an emulsion of $MOG^{35–55}$ through active immunization along with complete Freund's adjuvant (CFA). On the day of immunization intraperitoneal injections of pertussis toxin were given to the mice, this pertussis toxin basically facilitate the induction of EAE model. After immunization the T-lymphocytes that are myelin specific were activated in the peripheral region and then they starts migrated towards CNS across the blood brain barrirer. When the T-cells entered into the CNS, these cells were then reactivated by the infiltrating antigen-presenting cells that causes the inflammatory cascades, some other cells like monocytes and macrophages are also involve in the demyelination and death of axon. The mice C57BL/6 strain was used for this immunization process and the antigen Myelin Oligodendrocytes glycoprotein (MOG) were used. After immunization the mice showed the acute and chronic progressive disease stage. The most commonly used mice strain is C57BL/6 because of presence of variety of transgenic mice and also multitude knockouts. After using the above mentioned protocols for immunization of C57BL/6 mice with MOG_{35-55} peptide[10] the monophasic disorders appear. First symptoms in EAE occur after the recovery over the next 10-20 days. For immunization MOG_{35-55} peptide was not enough to induce this disease so the adjuvants such as CFA were used to enhance the immunogenic potential of of MOG_{35-55} peptide.

The CFA components activated the mononuclear phagocytes thus phagocytosis of these molecules were induced and also results in the secretion of cytokines. This resulted in the prolongation of the presence of antigens and also caused the efficient transfer to lymphatic system.

After immunization of mice the tissue lysate were extracted from the CSF of spinal cord and proteins were extracted from the tissue lysate for further bioinformatic analysis that defines its pathogenic role in the demyelination.

Synaptosomal-associated protein 25 (SNAP-25) is a 25kD protein with 206 amino acids. Its gene location is on chromosome 20. The pre-synaptic terminal of neurons is composed of a t-SNARE or target SNARE molecule. Formation of neural soluble Nethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes occurs because of SNAP -25 and has a great importance. Calcium-dependent exocytosis of synaptic vesicles, proper efficient release of neurotransmitters and propagation of action potential is done by SNARE complex. (Zahid, Date: 14 October 2016). It is also important for learning, movement, memories formation and normal brain functioning. The normal levels of SNAP-25 are mandatory for neurotransmission; the changes in its expression can cause many disorders including autoimmune disorder like Multiple Sclerosis. (Zahid, Date: 14 October 2016).

Different interactions between proteins and their cognate ligands are important for understanding of biological systems. Ligplot determines the interaction of different ligands with Synaptosomal-associated protein 25 kDa (SNAP-25). These ligands interaction are important to determine the functional and mechanistic way towards the cause of multiple sclerosis. The Structure of protein is important to describe the function and role of protein in specific disease. Ligplot determine the Secondary structure of SNAP-25 protein which consists of 5 different chains of different amino acids and 66 residues. The aim of study is find out the changes.

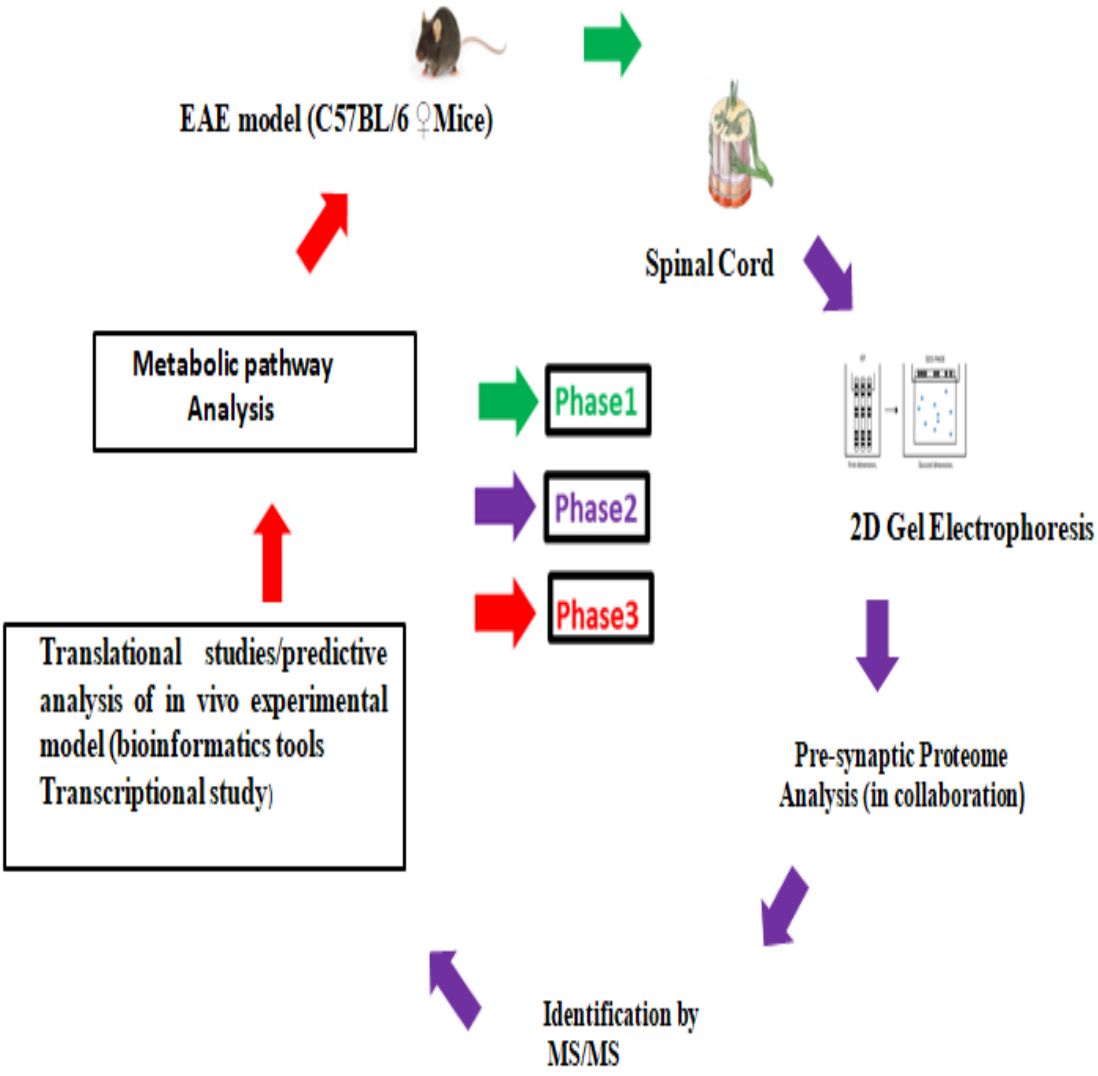
This research is based on graphical interpretation of identified proteins through LIGPLOT that automatically generate numerous 2D diagrams of ligand and protein interactions from 3D coordinates. The hydrogen bonds interactions and different contacts of protein with different ligands are portrayed through Ligplot. The main-chain and side-chains determination through ligplot facilitated the series of analyzing the function of protein in pathogenic pathway. The ultimate goal of the research is to develop the EAE model for the identification of proteins like

specific SNAP 25 proteins involved in Multiple Sclerosis and then develop the interventions that can improve the lives of those living with MS. Synaptosomal-associated protein 25 (SNAP-25) is a 25kD protein with 206 amino acids. **SNAP-25** not only plays a **role** in synaptogenesis and the exocytotic release of neurotransmitters. The over-expression of SNAP25 may destroy the synapse and thus stops the release of neurotransmitters.

CHAPTER 6: CONCLUSION

MS is a complex multifactorial disease. Though this disease is an autoimmune disorder but many other factors are involved in the pathophysiology that causes the demyelination of neurons or neuronal death. The exact causes of this disease are still unknown. EAE model of multiple sclerosis has a vital role in the development and then validation of treatments for MS and it also helps in understanding the pathogenesis of MS. Though the pathogenesis of EAE model understanding is still unclear but results of this research helped in determination of multiple causes which results in demyelination of neuron. Studies also identifies 23 different protein in EAE model out of which SNAP 25 is further analyzed through bioinformatics tools which will give pathways towards therapeutic success. Exact mechanism behind the cause of MS is still unclear, but some studies suggest that there are some proteins which are involved in Multiple Sclerosis. Our proteomics approach endeavor a comprehensive list of known and novel interacting proteins involved in MS. This study also highlights the role of SNAP 25 protein in the progression of MS for further diagnostic strategies. EAE model of Multiple sclerosis will further investigated to detect symptoms at more early stage in the CSF of MS patients Silencing of interacting protein (SNAP 25 and RAB 32) can induced considerable changes in patients having Multiple Sclerosis. These observations could also help to explain the unknown physiological role of SNAP 25 in Multiple Sclerosis and deserve close attention in scope of neurodegenerative diseases

Experimental Design of the Project



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