İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

PROTEIN BIOMARKERS OF MULTIPLE SCLEROSIS; STUDIES OF CEREBROSPINAL FLUIDS FROM CLINICALLY DIFFERENT SUBTYPES OF MS PATIENTS

M.Sc. Thesis by
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KLİNİK OLARAK FARKLI MULTİPL SKLEROZ ALT GRUBU HASTALARININ BEYİN OMURİLİK SIVILARINDA MS BİYOBELİRTEÇLERİ ÇALIŞMASI

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FOREWORD

I would like to express my deep appreciation to my supervisor Assist. Prof. Dr. Eda Tahir TURANLI for the inspiring support and the opportunities she provided me. It was a great pleasure for me to carry on this thesis under her supervision. I want to thank her for encouraging me to establish this thesis, which could not have been accomplished without her.

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ABBREVIATIONS

AD	: Alzheimer's Disease
ADEM	**:** Acute Disseminated Encephalomyelitis
APCs	**:** Antigen presenting cells
APOE	**:** Apolipoprotein E
BBB	**:** Blood – Brain Barrier
BMS	**:** Benign MS
CIS	**:** Clinically Isolated Syndrome
CNS	**:** Central Nervous System
CSF	**:** Cerebrospinal Fluid
CU	**:** Colorimetric Unit
EAE	**:** Experimental Autoimmune Encephalomyelitis
EP	**:** Evoked Potentials
GFAP	**:** Glial Fibrillary Acidic Protein
HLA	**:** Human Leukocyte Antigen
ICAM-1	**:** Intracellular Adhesion Molecule-1
Ig	**:** Immunoglobulin
MBP	**:** Myelin Basic Protein
MOG	**:** Myelin Oligodendrocyte Glycoprotein
MRI	**:** Magnetic Resonance Imaging
MS	**:** Multiple Sclerosis
NF-L	**:** Neurofilament Light Chain
OCB	**:** Oligoclonal Band
OLG	**:** Oligodentrocyte
ON	**:** Optic Neuritis
OND	**:** Other Neurological Disease
PLP	**:** Proteolipid Protein
PPMS	**:** Primary – Progresssve MS
PRMS	**:** Prograssive – Relapsing MS
RR	**:** Recurrence Risk
RRMS	**:** Relapsing – Remitting Multiple Sclerosis
SPMS	**:** Secondary – Progressive MS
Th1	**:** T helper 1
Th2	**:** T helper 2
TNF	**:** Tumour Necrosis Factor
VCAM-1	**:** Vascular-Cell Adhesion Molecule-1

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PROTEIN BIOMARKERS OF MULTIPLE SCLEROSIS; STUDIES OF CEREBROSPINAL FLUIDS FROM CLINICALLY DIFFERENT SUBTYPES OF MS PATIENTS

SUMMARY

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS) which widely appears in the adults. It shows heterogenous lesions in the clinical and pathological phenotype which limits the quality of life standarts. In the pathogenesis of MS, the destruction of oligodentrocytes, neurons and axons play important role. In this regard, the destruction of myelin tissue in the white and grey matter causes irreversible function defects and a progressive disorder type. It is possibly autoimmune disorder which affected by both environmental and genetic factors. Because MS is a complex neuroimmune disorder, treatment of it is against the symtoms of the disease. On the other hand, diagnosis of the disease is also depend on the presence of symptoms and thus it is needed to have some parameters that facilitate and make easier the diagnosis of MS.

Recent proteomic studies directed to find protein biomarkers in the body fluids of MS patients, to facilitate the diagnosis of the disorder. Those studies showed that there are some proteins, found in the cerebrospinal fluid and the serum of the patient, that show difference in the course and the subtype of MS. Also, the level and the kind of proteins differ, when you compare the control groups protein levels with the different subtypes of the MS.

In this study, the basic aim is, to investigate the protein biomarkers which are chosen by the previous literature findings and can help th diagnosis of MS by comparing the level of them with the control groups and the different MS subtypes. In this regard, 20 clinic isolated syndrome patient, 20 relapsing-remitting MS, 20 primary progressive MS, 20 secondary progressive MS and 20 control groups which have different neurologic disorder and should have lumber punction because of that disease (compatible with the patient group as age and sex), planned to be collected by the İstanbul University, Cerrahpasa Medicine Faculty, Neuroimmunology and the Demyelination Service. In the CSF samples and serums, Myelin Basic Protein (MBP), myelin oligodentrosite glycoprotein (MOG), Tau protein, Glial Fibrillary Acidic protein (GFAP) and neurofilament light chain (NF-L) proteins were investigated with the Western Blot technique. The findings were analyzed under "*ImageJ Analyses*" programme, to compare the levels of proteins in different groups. At the end, the evaluation of these proteins as a biomarker in the diagnosis and the course of the disease were discussed.

At the end of the study, it is observed that, four of five proteins showed statistically significant results between both RRMS (n=31) and CIS (n=25) groups to be a candidate biomarker of MS. When the protein levels were compared in RRMS group with control group (n=12), it is observed that, concentration of Tau protein %53 (p=0.0004), GFAP 67% (p=0.0034), NF – L 67% (p=0.0015) and MOG 76% (p<0.0001) proteins was increased. The same proteins in CIS groups were compared with control group and it is observed that; concentration Tau protein %46 (p=0.0029), GFAP 67% (p=0.0036), NF – L 68% (p=0.0037) and MOG 77% (p<0.0001) was increased. For the MBP, results did not show any stattistically significant data. Our preliminary results, indicates that, Tau, GFAP, NF – L and MOG proteins can be functional in the diagnosis and prognosis of MS. Further studies will continue with increasing the number of samples and serum samples of the same patients will be evaluated in order to make correlation between serum and CSF samples.

KLİNİK OLARAK FARKLI MULTİPL SKLEROZ ALT GRUBU HASTALARININ BEYİN OMURİLİK SIVILARINDA MS BİYOBELİRTEÇLERİ ÇALIŞMASI

ÖZET

Multiple skleroz (MS) genellikle genç erişkinlerde görülen, alevlenme ve düzelmelerle seyreden, merkezi sinir sistemini birçok lokalizasyonda etkileyen, genetik ve çevresel etmenlerin karmaşık etkileşmeleri sonucu oluştuğu varsayılan, olasılıkla otoimmün, inflamatuar demyelinizasyonun yanında akson kaybı ve oligodentrositlerin yıkımı ile seyreden kronik bir hastalıktır. Merkezi Sinir Sisteminin (MSS) ak maddesini çeşitli lokalizasyonda etkileyen kronik ve yaşam kalitesini sınırlayan bir doğası vardır. Hastalığın immünopatolojisinde ve genetiğinde birçok etkenler vardır. MS kompleks bir hastalık olması dolayısı ile hastalığın tedavisi semptomları gidermeye yönelik olup, hastalığın tanısının koyulması da semptomların görülmesine bağlı olarak gerçekleştirilebilmektedir. Bu nedenle hastalığın tanısını kolaylaştırıcı parametrelere ihtiyaç duyulmaktadır.

Son yıllarda yapılan proteomik çalışmalar, hastalığın tanı ve teşhisini kolaylaştırmak amacı ile vücut sıvılarında biyobelirteçlerin bulunmasına yönelik olmuştur. Bu yönde yapılan çalışmalarda, hastalığın seyrinde, beyin omurilik sıvısı (BOS) ve serumda immün yanıtı olarak gelişen, miktarlarında farklılık gösteren, çeşitli proteinler gözlenmiştir. Bu proteinler seviyeleri ve çeşitleri olarak MS altgruplarında ve kontrol gruplarına oran olarak da farklılıklar göstermektedir.

Bu çalışmada, genel olarak amaç, literatür çalışmaları sonucunda belirlenmiş, MS hastalarında seviyeleri değişen proteinlere, MS altgruplarında ve kontrol gruplarında bakarak hastalığın seyrini ve alttipini önceden belirlememize yardımcı olacak proteinlerin saptanmasıdır. Bu nedenle, İ.Ü. Cerrahpaşa Tıp Fakültesi Nöroimmunoloji ve demyelinizan polikliniğinden takipli olan, 20 klinik izole sendromlu, 20 yineleyen-düzelen (relapsing-remitting) MS'li, 20 ikincil ilerleyen (secondary progressive) MS'li ve 20 birincil ilerleyen (primary progressive) MS'li ve 20 kontrol grubu olarak yaş ve cinsiyetleri uyumlu farklı nörolojik hastalıklardan dolayı lomber ponksiyon yapılması zorunlu olunan hastaların BOS ve serumlarında myelin basic protein (MBP), myelin oligodentrosit glikoprotein (MOG), tau proteini, glial fibrillar protein (GFAP) ve nörofilament hafif zincirlerine(NF-L) bakılacaktır. Proteinlere Western Blot tekniği ile bakılarak, "*ImageJ Analysis"* programı ile seviyelerinde karşılaştırma yapılacaktır. Protein seviyelerini alt gruplarda karşılaştırılarak hastalığın seyri ve tanısı açısından biyobelirteç olarak değerlendirmesi amaçlanmıştır.

Yapılan analizler sonucunda, RRMS ve kontrol grubu arasında *Tau* proteininin konsantrasyonu kontrol grubuna oranla RRMS grubunda %53 ($p=0.0004$), *MOG* proteini 76% ($p<0.0001$), GFAP proteini 67% ($p=0.0034$) ve *NF – L* proteinin %67 ($p=0.0015$) oranında arttığı saptanmıştır. Aynı proteinler için CIS ve kontrol grubu arasında ise, CIS grubunun *Tau* proteini konsantrasyonu kontrol grubuna oranla, 46% ($p= 0.0029$), *MOG* proteininde 77% ($p<0.0001$), GFAP proteininde 57% ($p=0.0036$) ve *NF – L* proteininde ise %68 ($p= 0.0037$) oranında arttığı saptanmıştır.

MBP proteini için ise istatiksel olarak anlamlı farklar görülmemiştir. Ön bulgularımız *Tau, MOG, GFAP ve NF – L* proteinlerinin hastalığın tanı ve seyrinde işlevi olabileceği yönündedir. Çalışmalarımızın devamında örneklem sayısını artırarak ve aynı hasta gruplarının serum örneklerinde de bu proteinlerin konsantrasyonlarını karşılaştırarak, serum ve BOS arasında korelasyon yapılması hedeflenmektedir.

1. INTRODUCTION

1.1 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating disease of central nervous system (CNS) characterized by infiltration of monocytes and lymphocytes to the CNS and cause myelin damage in multiple locations and loss of neurological function. MS better described as a syndrome rather than a single disease because of its complex nature with heterogeneous clinical, pathological and immunological phenotype. Chronic nature of the disease decreases the life quality in the patients. The clinical heterogeneity of MS has been investigated and evaluated for many years, but it is certain that heterogeneity comprises to both the genetic mechanisms of the disease and the pathomechanisms involved in lesion formation. In other words, MS is a neuroinflammatory and weak neurodegenerative disease of the CNS [1].

1.1.1 Symptoms of MS

Clinical and pathological features of MS are firstly defined in 1868 by Jean-Martin Charcot. Since first observations to today, the information about the characteristics of pathological mechanisms and the genetics of the disease is still not enough in order to understand the whole frame of the disease [2]. Although, more than a century, the detailed clinical definition and causes of MS are intensively investigated, still it has an unknown pathogenesis. Because of the main target is myelin sheath and its producer oligodentroglia, it causes variety of neurological dysfunctions [3]. The symptoms of MS start with visual loss or double vision, nystagmus, sensory, and motor signs and symptoms. In the progressive forms of the disease, cognitive impairments can be seen. Some people have mild cases of multiple sclerosis with little or no disability while the others have full-symptomatic MS that confines them to a bed or wheelchair. Still others are only diagnosed with multiple sclerosis after their death and live their entire lives symptom free. This variability makes it extremely difficult to diagnose multiple sclerosis [3-4].

1.1.2 Diagnosis of MS

Diagnosis of MS is extremely difficult depending on the varieties of symptoms. There are many reasons complicating the diagnosis: More than fifty symptoms are related to MS and degree of the symptoms can develop different for each person. Most of these symptoms can be seen in other neurological diseases, also they are vague and hard to quantify. There is no single blood test or other radiological analyses that confirms the diagnosis of MS. On the other hand, in some cases, history and symptoms of the patients are enough to diagnose the disease. But some patients may need to be multiple tests and prolonged observation for diagnosis. To standardize the diagnosis procedure, there are some criteria which are a combination of clinical parameters and radiologic findings with supportive laboratory analyses.

Before standardization process, diagnosis of MS would be done by using Poser and Schumacher criteria, which are also, depend on radiological and clinical findings. But in 2001, at the International MS Society meeting, McDonald criteria are accepted for the diagnosis of the disease. McDonald's criteria made usage of advances in MRI imaging techniques and facilitate the diagnosis of MS patients who present with symptoms suggestive of the disease. In 2005, McDonald's criteria are revised by clarifying what is meant by an "attack", "positive MRI" etc. Those criteria are widely used in all over the world [5].

Radiological analyses are depended to the electrophysiological changes caused by demyelinating points creating evoked potentials (EP). This can be measured by computer tomography (CT) or magnetic resonance imaging (MRI) [5]. These techniques are very sensitive to show the white matter demyelination of brain and spinal cord [6]. Cellular and humoral immune response plays an important role in the formation of demyelination points. Therefore, plasma cell and IgG concentration is high in the demyelination centers. Thus, the increase of IgG in the cerebrospinal fluid (CSF) can be shown by oligoclonal band analyses (OCB) [7].

1.2 SUBTYPES OF MS

MS is classified into different subtypes and its variants, depending on the patterns of progressions as well as the intensity and frequency of its generated symptoms.

1.2.1 Subtypes of MS

A) Relapsing – Remitting MS (RRMS)

RRMS is characterized by the acute attacks (relapses) and following total or partly remissions. Additionally, stabile continue of the disease between the attack periods is the most common form of the disease. The initial presentation in most patients (frequency in all newly diagnosed MS is 85%) is relapsing-remitting MS (RRMS) with relapses and remissions due to self-limiting plaques of inflammatory demyelination disseminated in time and place in the CNS. Following relapses arises in an unpredictable fashion and after each attack level of disability at patient can be increased. RRMS show the tendency of turn into secondary progressive MS form in the following times [8].

B) Primary Progressive MS (PPMS)

In this form of MS, disease progression is continuous from the beginning without clear relapses. There can be plateaus (periods of stabilization) time interval. 10–15% of all MS patients are in this group and it tends to occur in older aged individuals. Usually disease progression continues until death. The female to male ratio is equal in this group, unlike other forms where females predominant by about 2–3:1 [8].

C) Secondary Progressive MS (SPMS)

This form of MS starts as a RR-MS and approximate 5 or 6 years later steady progression occurs with or without relapses. Each attack increases the level of disability. Approximately 50% of relapsing-remitting patients progress to the secondary progressive form [8]

D) Progressive – Relapsing MS (PRMS)

This uncommon form (about 5%) is progressive from the onset with superimposed relapses (with or without recovery) [8].

As it is stated above the most common form of MS is RRMS (85%), but in the progression of the disease this form turns into one of progressive forms. At the 50% of the RRMS cases, it turns into SPMS in 10 years, and at the 90% of the cases it turns into SPMS in 25 years.

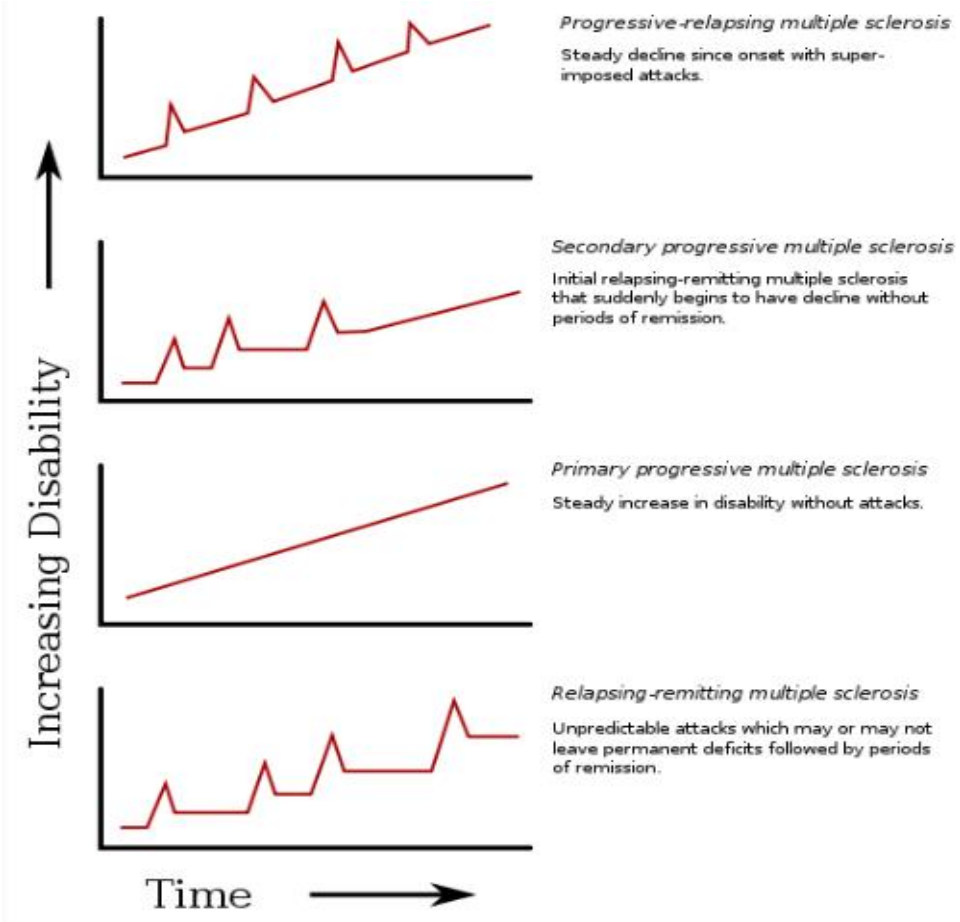

Figure 1.1: MS Subtypes

1.2.2 Clinically Isolated Syndrome (CIS) and Clinical MS Variants

Besides these 4 main forms of MS, some other variants of the disease are defined depending on the clinical onset and the neurological disturbance of the lesions. In a significant number of patients who later develop typical MS, the clinical onset starts with an acute or subacute episode of neurological disturbance due to monoregional involvement of the CNS. This form of presentation is known as clinically isolated syndromes (CIS). In some cases of CIS, MRI may reveal polyregional involvement of the CNS, in others; the disease will be limited to the corresponding anatomic site, remaining monoregional [9]. Although normal brain MRI and CSF IgG index indicates the low risk of developing MS in following 5 years but on the other hand, positive findings increases this risk 80-90% of the cases [10]. The clinical course of CIS also varies;

A) Neuromyelitis Optica (Devic's Syndrome)

Neuromyelitis Optica is a CIS variant which develops with the acute transverse myelitis and synchronic optic neuritis (ON) (inflammation/lesions in the spinal cord and optic nerves). In many patients, ON continues with the MS like relapses and remittings. Spinal cord lesions can be permanent. Its prognosis is as serious as MS and can be finalized with the vision lost. A patient with isolated optic neuritis may recover completely, may have recurrences, develop a progressive optic neuropathy, may remain symptom-free, or eventually convert to RRMS and SPMS [9-11].

B) Benign MS (BMS)

In the 20% of the patients, Benign progression occurs which means that patient remains fully functional in all neurological systems 10 years after disease onset. In the patients with 5 years asymptomatic, prognosis of BMS chance is considerably high [12].

C) Acute Disseminated Encephalomyelitis (ADEM)

ADEM is a nonvasculitic, inflammatory, demyelinated, immune response originated form of MS. It is usually monophasic and frequently seen in 6-8 years old children and adolescents. ADEM is separated from MS by this feature of the disease. It usually occurs following a viral infection but may appear after one week or month vaccination, bacterial or parasitic infection. The prevalence of ADEM in both genders is equal and its total prevalence is 0.8/100.000 per year. In the cellular mechanism of ADEM, it develops as a humoral response to myelin antigens following a vaccination or infection [13].

D) Marburg Form

This rare and malign form of MS develops with the progression of the cognitive impairments, advanced level of vision loss, dysarthria, dysphagia and respiratory problems. It is difficult to differentiate this form from ADEM [14].

1.3 EPIDEMIOLOGY OF MS

Epidemiology of MS varies between the populations and geographical distributions depending on environmental and genetic factors. One observation is that the population prevalence of MS increases with distance from the equator. It is estimated that there are approximate 34-35/100000 in MS patient in Turkey and it is more common in Caucasians (northern Europe) and north part of the world [15]. It is postulated that this distribution can be explained in part by both environmental factors (e.g. diet and vitamin D abnormalities) and population-specific genetics [16]. MS occurs two to three times more in women than men. Although the disease has a broad range of age at onset (85% of cases occur between the ages of 14 and 55), initial symptoms typically present in early adulthood (between ages 20 and 40). Another observation that, incidence of MS is increasing over time [17].

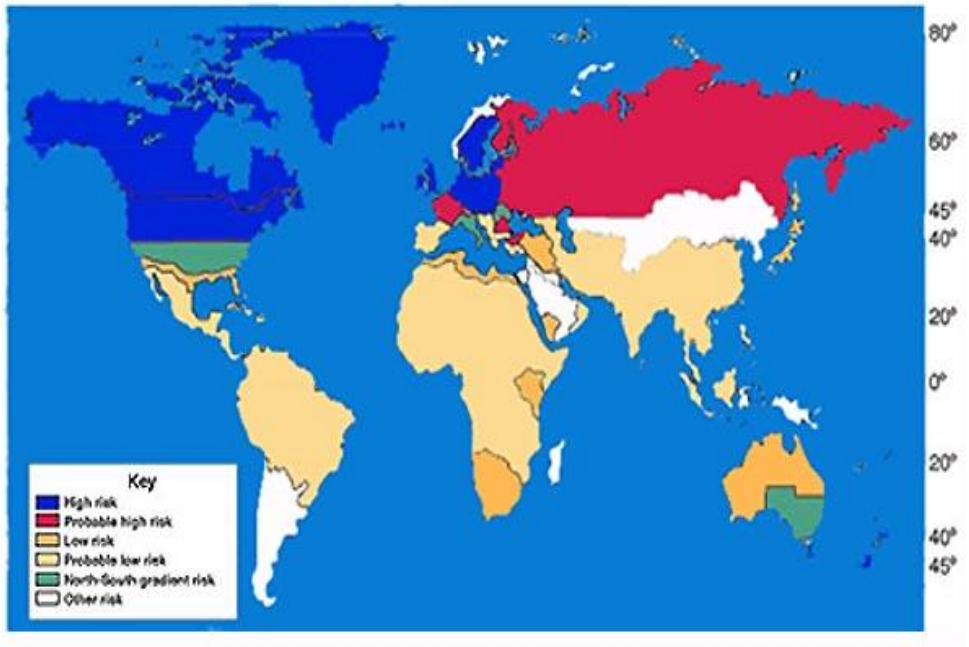

Figure 1.2: World distribution of MS prevalence

1.3.1 Classical Family Studies

Epidemiological studies, indicating familial effect to the disease concludes that first, second and third degree of familial relations 5 – 50 times increases the risk of MS development. In a family based study Sadovnick AD. et. al. showed that

monozygotic siblings (30.3%) have more risk to develop MS than dizygotes (%4.6) and siblings (5.1%) [16].

Table 1.1: MS association in twins and siblings [16]

	Monozygotes	Dizygotes	Siblings
Ebers et al, 1986	7/27 (25.9%)	1/43(2.3%)	1.9%
Sadovnick et al, 1993	8/26 (30.3%)	2/43 (4.6%)	5.1%
Mumford et al, 1994	11/44 (25%)	2/61(3.3%)	
Kinnunen et al, 1988	2/7 (28.6%)	0/6	
Heltberg & Holm,1982	4/19 (21.1%)	1/28 (3.6%)	

There are several study methods to understand the contribution of environment and genetic factors to the disease. In another family based population study, adopted children were analyzed to understand the environmental effect to the disease, Ebers GC. et. al.'s results indicated that prevalence of MS in adopted children (%0.05) were not significantly higher than general population. This result, defined that genetic sharing is more important than shared family environment [18]. In another study, twin MS patients a sibling with MS patients were analyzed and results indicated that, twin MS patients have more recurrence risk (RR) (19%) than general population [18, 23]. As it is known half-sibling share 25% of their genetic material and full siblings share 50%. The results of the half-sibling study supported the adoption data in that genetic sharing and not family environment is critical for the familial aggregation of the disease. The data also showed that the half-sibling recurrence risk (1·32%) is significantly less than that for full-siblings (3·46%) in the same family and is in fact lower than predicted by the halving of genetic sharing in oligogenic disorders [19, 20].

On the other hand, most investigations about the MS epidemiology indicate that migration from a high risk area to the low risk area decreases the possibility of developing MS [21]. Numerous familial aggregation studies have shown that recurrence risk ratio for MS decreases with the degree of relationship between individuals. Also familial and twin studies showed that, risk of MS development increases if there is an MS parent or siblings in family [22]. Compared to the general

population, these elevated risks suggest a strong complex inheritance of MS susceptibility [22].

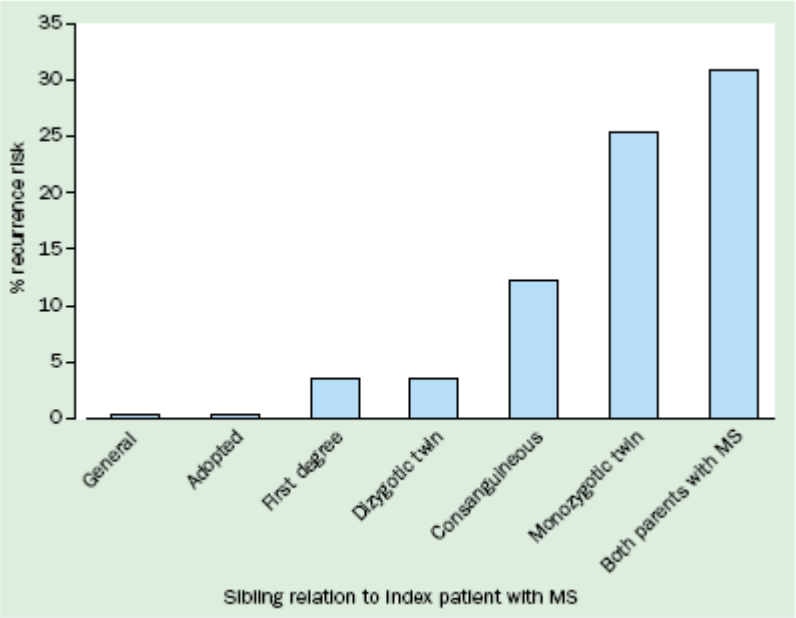

Figure 1.3: Disturbance of recurrence risk among the relatives[23]

1.3.2 Heritability in MS

The idea that genetic factors can play a role in MS is first aroused in the second half of 19th century with the notification of familial cases. The first genetic association is indicated in 1972 with the Human Leukocyte Antigen (HLA) genes. Till 30 years, still etiology, environments' and genes' contribution to the disease is not certain. MS is associated with variation in certain HLA genes on chromosome 6p21, including HLA-A, HLA-DRB1, HLA-DQB1, and HLA-DRA on chromosome 6p21.3. Initial associations were observed in the HLA class I region of the MHC and later with polymorphisms in the HLA class II region. In Northern Europeans, susceptibility has been well mapped to an extended HLA class II haplotypes DQA1*0102-DQB1*0602-DRB1*1501-DRB5*0101. In different studies, genetic susceptibility of MS is indicated as 15-20%. As a result, in comparison to total population, primary, secondary and other relatives of MS patients show high level of risk. Like all the complex diseases, interaction of genes, environment and chance factor, determines the susceptibility and progression of MS [19, 30, 31 and 32].

1.4 PATHOGENESIS OF MS

The CNS always considered as an immunologically preserved site with few lymphocytes in the case of any active or ongoing infection. However, many studies has demonstrated that a small number of T cells traffic through the CNS searching for infection or injury and that T cells activated in the periphery can penetrate into the blood-brain barrier (BBB) and enter into the CNS. Autoreactive T and B cells are normal elements of the immune system. It has been demonstrated that some of these autoreactive cells can be stimulated with myelin components in healthy individuals, but do not appear to be harmful unless tolerance of the immune system is broken and cells activated. Induction of autoimmune responses against myelin components in the CNS is thought to be occurred through mechanisms such as molecular mimicry or activation and epitope spreading. Once activated, myelin-specific T cells can cross the BBB where they proliferate and secrete pro-inflammatory cytokines which in turn stimulate microglia, macrophages and astrocytes, and recruit B cells, ultimately resulting in damage to myelin, oligodendrocytes and axons [24].

1.4.1 Pathogenic forms of MS

Regarding to main characteristics of MS lesions, etiology of MS can be classified into four pathologically distinct forms:

The first etiological cause of MS is that, T- and macrophage cells infiltrate into CNS by breaking down the BBB and cause to the myelin sheath damage. But, at the same time oligodentrocyte (OLG) cells are alive and fast, thus, total remyelination of damaged nerve cells can be observed [34].

Another etiological cause is that, activated plasma cells with the T- and macrophage cells can be seen in lesions. Loss of OLG cells and migration of progenitor cell is also observable in this etiology [34]. In both of those etiologies, main target is myelin sheath.

In The other etiological cause of the disease, there is a vasculite formation in the margin of lesion. Infiltration of T cells is less and apoptotic cell death is seen. Axon damage and migroglial activation is the main pattern of this etiology. In the last and most dangerous etiological cause of the disease, apoptotic cell death and OLG

degeneration is observable in the white matter of CNS. Besides, infiltration of T- and macrophage cells is also seen. At the last two etiology pattern, the main target is OLG cells [34].

1.4.2 Immuno – Pathogenesis of MS

Permeability of BBB to the T cells and demyelinating antibodies can be increased with the genetic (super-antigen production) and environmental (viral or bacterial infections) factors. The increase of this permeability initially starts with increasement in the concentration of adhesion molecules like ICAM-1 ("Intracellular Adhesion Molecule-1"), VCAM-1 ("Vascular-Cell Adhesion Molecule-1") and E-Selectin. Then enzymes – like matrix metallo - proteases- initiates the destruction of BBB. Activated T cells in the CNS starts the synthesize of pro – inflammatory cytokines like IFN-γ and TNF-α. Those molecules increase the expression of surface molecules of lymphocytes and antigen presenting cells (APCs). IFN-γ especially stimulates the microglia cells to activate as APCs. When a class II MHC carrying APC associates with an undifferentiated T cell and MS antigen, it can enter to one of following two pathways depending on the co – stimulatory molecule on the surface of the T cell [35].

1.4.2.1 Th1 Pathway

If the co – stimulatory molecule on the surface of the T cell is CD28 and its ligand B7-1 is present there, T cells differentiate into $CD4^+$ Th1 cells and synthesize pro-inflammatory IL-12 and IFN-γ. While IL-12 stimulates the Th1 pathway, IFN-γ inactivates the Th2 pathway. Concentration of Th1 cells are increased and starts to produce IFN-γ and TNFα.. Type 1 TNF-α cytokine initiates the death of OLG cells and damages to the myelin sheath. This damage occurs with the stimulation of TNF-α receptors leading to activation of apoptosis and necrosis. When the types 2 TNF-α receptors are activated, remyelination process starts. Macrophages which are activated by IFN-γ cytokines cause to myelin sheath damage by stimulating complement response or directly. Also IFN-γ causes to OLG damage by stimulating cytotoxic $CD8^+$ cells. This molecule initiates the *Fas* gene in the OLG cells and when the FasL on the surface of the $CD8^+$ interacts with the Fas molecules on the

OLG cells, apoptosis of OLG cells occurs [35]. In the course of time, transmission of the electrical impuls decreases because of the damage of the myelin sheath on the surface of the nerve cells, thus with the environmental factors around the cells axonal loss is increased.

1.4.2.2 Th2 Pathway

If the co – stimulatory molecule on the surface of the T cell is CTLA-4 and its ligand B7-2 is present there, T cells differentiate into $CD4^+$ Th2 cells and initiate the anti-inflammatory IL-1, IL-4 and IL-10 cytokines synthesis. While IL-4 and IL-10 stimulates the Th2 pathway, IL-1 inactivates the Th1 pathway. If extracellular infective pathogens present in the environment, they initiates the synthesis of these cytokines. Concentration of Th1 cells are increased and start to produce anti-inflammatory IL-1, IL-4 and IL-13 cytokines. These ILs, activates the B lymphocytes and by the expression of antibodies from this cells causes to re-myelination [35].

In a healthy body, normally Th1 and Th2 cell and their products are in a balanced fashion. When this balance disturbed by the environmental factors, the healthy situation turns into the one of MS patterns. Increase in the Th2 by Th1 vaccines, sensitivity of B- and T cells to body antigens and do not causing the disease by the accidently activation of Th1 cells, supports the idea of presence of autoimmune regulating mechanism in the body [38].

$CD4^+$ ve $CD8^+$ T- lymphocytes present in the every stages of MS. But usually number of $CD4^+$ cells is less. In the progression of MS, loss of axon or axonal injury is more important than de-myelination. There is a smooth relation between axonal loss and the number of $CD8^+$ cells. Also, $CD8^+$ T cells are more activated in the tissue damage of perivascular region. $CD4^+$ cells can protect nerve cells by secreting notrophin elements. And also in some MS cases, the dominant $CD4^+$ cells are not Th1 cells, they can be Th2 cells. With this observation and experimental studies, it can be concluded that; genetic factors play a bit more key role in deciding to which immune system will cause to tissue damage in the nervous system and how CNS will respond to this reactions and try to keep re-myelination [39].

1.4.3 Molecular Pathogenesis of MS

Studies for the genetics of MS, initially revealed many genes that are related with MS, but many of them are eliminated in the following studies due to proof of non-associated cases. Only 5% of the candidate genes are corrected with the following genetic studies. In fact, negative genes are also revealed many useful information as well as positive genes. Many strong candidate genes are eliminated by familial studies, such as; T cell receptor-α, agonist of interleukin-1 receptor; interferon α, β, γ and CCR5, variety of complement and cytokine genes.

The Apolipoprotein E (APOE) gene codes for a major lipid carrier protein in the brain. In previous studies, it is proved that the APOE protein is associated with regeneration of axons and myelin after lesions of central and peripheral nervous system. Evidence from other motor neurological diseases points out the concept that APOE allelic variants may be associated with disease severity. But also, by providing a pathway for delivery of self-lipid antigens, APOE can contribute to the pathogenesis of MS. Together with the role, this protein may have in neuronal remodelling in conjunction with the observations that axonal loss in MS is significant and that irreversible clinical disability relates to such axonal loss. APOE is an appropriate disease modifying candidate gene for MS. [25, 26, and 27]

TNF alpha is a cytokine involved in inflammation process and is a member of a group of cytokines that stimulate the acute phase reaction. The primary role of TNF is in the regulation of immune cells. TNF is also able to induce apoptotic cell death, to induce inflammation, and to inhibit tumor formation and viral replication. It is a potent immune mediator and pro-inflammatory cytokine. Disease progression in MS patients has been correlated with high TNF levels in CSF.. Only one study involving 50 patients showed any direct effect of TNF alpha on MS outcome. In that study, 14% of PPMS patients had homozygous point mutations at 308. position, but this mutation is not seen in RRMS or control group [28].

Cytokine (IL) genes are another functional genes in the molecular pathogenesis of MS. Development of many autoimmune diseases occurs in the case that an imbalance between the frequency of proinflammatory T helper 1 (Th1),anti-inflammatory T helper Type 2 (Th2) T cells, cytokines, and regulatory T cells. In MS, it is known that autoreactive T-cells target an antigen in the CNS and initiate an inflammatory process resulting to demyelination. Development of autoimmune

disease may require not only the presence of autoreactive T cells, but also that these autoreactive T cells become activated. As cytokines may drive the inflammatory process in an MS plaque, and that acute plaques are associated with acute axonal injury and therefore transient neurological dysfunction, it is feasible that cytokine polymorphisms may affect prognosis [29].

One of the milestone discoveries in MS is the proof of immunoglobulin (Ig) presence in the CSF, showing the activity of intrathecal antibody production. The roles of intrathecally produced Ig has not been fully understood yet, but results from several studies have suggested a role for Ig in the pathogenesis of the MS lesion and perhaps severity of disease [30].

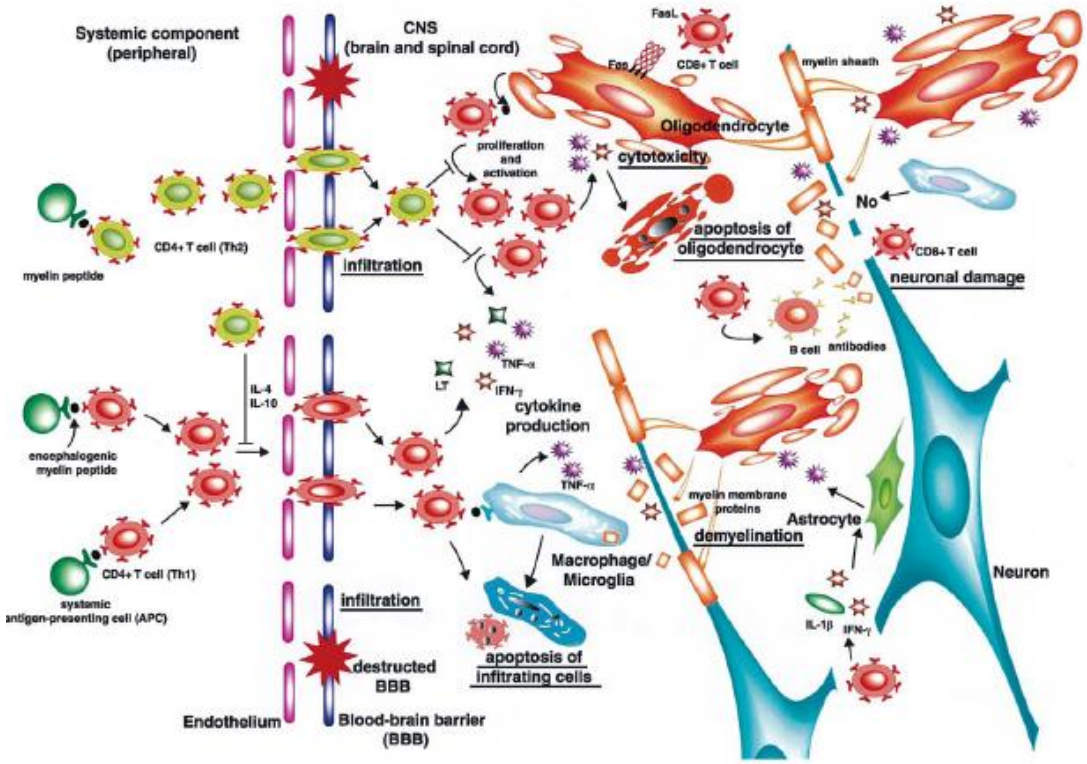

Figure 1.4: Immunopathogenesis of MS[34]

1.5 ANIMAL MODELS OF MS

Two common animal models for MS have emerged in order to simulate demyelination and remyelination in the multiple locations of the brain:

1.5.1 Experimental Autoimmune Encephalomyelitis (EAE) Model

Experimental Autoimmune Encephalomyelitis (EAE), also named experimental allergic encephalomyelitis, is an alternative animal model of MS. Animal models of human diseases are diseases of non-human species (often rodents and rarely primates) which closely resemble their human counterparts of the disease and are be studied with a view to better understanding and treating the human form. Actually EAE is not MS, nor is it a single disease in a single species, but its different forms resemble the various forms and stages of MS very closely in a large number of ways.

EAE is an acute disease of rodents and also it can be chronic-relapsing, inflammatory and demyelinating autoimmune disease. The animals are injected with the whole or parts of various myelin antigens. These proteins induce an autoimmune response in the animals - that is the animal's immune system initiates an attack on its own myelin as a result of exposure to the injection. The animals develop a disease process that the symptoms and pathogenesis resembles to MS in humans. Several proteins or parts of proteins (antigens) are used to induce EAE including: Myelin Basic Protein (MBP), Proteolipid Protein (PLP), and Myelin Oligodendrocyte Glycoprotein (MOG).

Studies on EAE have number of benefits:

- Because EAE is an animal disease, it enables researchers to study demyelination in ways that would not be morally acceptable in studies of MS in humans.
- It allows researchers to test potential treatments for MS for their efficacy and safety without putting the lives of people at risk.
- It allows researchers to experiment with different ways of inducing EAE to attempt to find potential causes of MS.
- Because the generations times of most of the EAE species are short, and because they breed very fast, large populations of such animals can be turned over in short periods of time.

Researching EAE has a number of disadvantages:

- EAE is not a human disease like multiple sclerosis and many significant assumptions are made to accept EAE as an animal model for MS.

- It is obvious that the animals involved in experiments suffer considerably and questions about the ethics of EAE are inescapable [41].

1.5.2 Cuprizone Neurotoxic Mouse Model for MS

One of the best characterized demyelinating mouse models is that of cuprizone fed mouse model. Several hypotheses have been proposed but it is still unclear why cuprizone, a copper chelator molecule, specifically affects OLGs the cell type that synthesizes and maintains the myelin sheath of the CNS. The pathogenesis of the cuprizone model has been extensively investigated with respect to variations in the cuprizone treatment time and dose, the identification of the nerves that demyelinated, and the identification of the cellular types that are affected. Diet of 8-week-old C57BL/6J mice with 0.2% of cuprizone causes metabolic disturbance and death of mature OLGs which leads to a decrease in myelin proteins produced by OLGs such as MBP and myelin associated glycoprotein (MAG). This causes extensive demyelination of several well-myelinated nerves. The first response to demyelination is an activation of microglia/macrophages and reactive astrocytes, which occurs 1–2 weeks following cuprizone administration. As a response to demyelination and glial cell activation, at 3–4 weeks of cuprizone treatment, oligodendrocyte progenitors begin to accumulate within the lesion and become mature oligodendrocytes in 2 weeks, with remyelination occurring over the subsequent 4 weeks if, following 6 weeks of treatment, the cuprizone is removed from the diet. To date, behavioral analysis of cuprizone-treated mice has been poorly studied. [40].

1.6 BIOMARKERS IN MS

Finding biomarkers for MS is an extensive research area. The main object of proteomic studies is to find out an acceptable protein pattern that clinician can understand and differentiate the subtype or the course of the disease. As indicated above, in the pathogenesis of MS several processes are supposed to sequentially or simultaneously contribute to development of the disease. A potential biomarker that can be indicative of these processes would help to understand MS diagnostics and identification of disease course and subgroups; prediction of onset of disease;

treatment selection and improved prognosis of treatment success; and the evaluation of novel therapeutics. Though it is difficult that any one marker could function as a true indicative, biomarker combinations or patterns could provide insight into the mechanism of action of a drug and could suffice for the pre-screening of the disease before the onset [42].

CSF may be better sample in order to show the relevant inflammatory process by its localization near to most of inflammatory lesions in the CNS, although this idea remains controversial. By the liquid pattern of CSF, it is unlikely that the CSF in the lumbar cistern accurately reflects the production of the inflammatory markers in the supratentorial region, where most of the inflammation processes relevant to multiple sclerosis occurs. In addition, the intraparenchymal extracellular space may not necessarily communicate with the free CSF space. However, CSF collection does prevent biological degradation of excreted markers by the liver or by renal excretion [52].

In MS, regarding the complex interactions between immune system and nervous system, there are many of candidate proteins that can be thought to as biomarker of MS, indicating the characteristics and the subtype of the disease. In this study, five proteins have been chosen regarding their role and complex neurological function in the nervous system. Those proteins Tau, MBP, MOG, GFAP and NFL are investigated through the previous literature findings based on the concentration of them in CSFs' and serums' of the MS patients. Significant difference of those protein levels in subtypes of MS and control samples aroused the idea of candidate biomarker in CSF for MS.

1.6.1 Tau Protein in MS

The microtubule-associated tau proteins in the molecular weight 49–74 kDa play important role in stabilization and assembly of microtubules of axons. The hyperphosphorylated form of tau is the main protein in the characteristic neurofibrillary tangles of Alzheimer's disease (AD), and increased CSF tau concentration is a consistent finding as a biomarker in this disorder. Tau is primarily located in neurons, though it can also occur in glial cells as well as it can be released to the CSF. Accumulation of tau proteins in neurons as well as in astrocytes and oligodendrocytes occurs in various neurodegenerative diseases [44].

In a clinical study, it is showed that concentrations of tau proteins in CSF of RRMS patients is higher than in patients with noninflammatory neurological controls suggesting that tau could be a good marker in body fluids. In a large study including 114 MS patients (84 RRMS and 30 progressive form), 79 with inflammatory neurological disorders (ND), and 60 with noninflammatory NSs, concluding that there was no difference in CSF tau concentrations between MS patients and those with inflammatory NDs. Increased CSF tau concentrations (150%) were found only in the patients with RRMS, which contrasts with findings in the other study. No clinical scores or measures of progression were presented [45, 46].

In summary, these results suggest that tau protein has a value for further studies in serum as well as in CSF in the MS patients, because it is higher in patients with RRMS than in non-inflammatory NDs, and it might have a key function in acute axonal damage and progression. The association of tau concentrations with AD suggests that the protein could be related to cognitive dysfunction in MS as well as in AD. Also one another idea suggesting that Tau protein concentration can be vital in the progression of disease or changing the form of disease from RR- to PPMS [45, 46].

1.6.2 Myelin Basic Protein (MBP) in MS

Myelin basic protein (MBP) is a protein thought to be important in the myelination of nerves in the CNS. The variety of MBP in the CNS is very diverse; with several splice variants being expressed and a large number of post-translational modifications (PTM) on the protein.

In many studies, the reactivity of MBP in MS is evaluated with using 7-day proliferation assays of whole peripheral blood mononuclear cells. These studies showed that a slight increase in T cell responses to human MBP, when compared with MS to normal subjects or other ND patients, but the magnitude of the difference generally has been less than expected [47].

In another study, to understand the binding affinity of immunodominant MBP to class I and II type HLA molecules, different T cell types used, by using T cell cloning techniques. It is showed that, the MBP peptide bound to purified HLA DRB1*1501 molecules with its 84 to 102 amino acids fragments with high affinity. This findings also proved by animal models and thus it is understood that

autoreactivation of T cells can be induced by the recognition of MBP by specific T cells [47].

1.6.3 Myelin Oligodendrocyte Glycoprotein (MOG) in MS

MOG is another protein in the myelination process of nerves in the CNS. It is coded by *MOG* gene. It is thought that MOG serves as an adhesion molecule to provide the structural integrity of myelin sheath and also later it contributes to the development of oligodendrocyte. It is a minor type I transmembrane protein which is highly expressed in the surface of nerves and oligodendrocyte.

In many studies, findings related to anti-MOG antibodies in CSF and serums of MS patients are variable. Those studies revealed those anti-MOG antibody frequencies ranges from 0–80 % in MS patients and 0–60 % in healthy controls. A recent study indicating that IgG antibodies directed against native membrane bound, glycosylated MOG can be used as serologic markers for early inflammation in MS and it provides evidence for a possible prognostic role of anti-MOG antibodies. Furthermore, the presence of MOG-specific auto-antibodies associated or not with anti-MBP auto-antibodies in patients with a first demyelinating event is reported to be highly predictive of definite MS [48].

1.6.4 Glial Fibrillary Acidic Protein (GFAP) in MS

GFAP is an intermediate filament protein that is believed to be expressed only in astrocytes and specific for them. But later it is shown that GFAP is also expressed in other CNS cell types. Elevated CSF concentrations of GFAP have been found in MS patients when compared to other ND control, correlating with disability scales and the extent of neurologic deficits and possibly suggesting irreversible tissue degeneration. Thus it can be a good biomarker of degeneration in MS. One another observation about the GFAP is, in the same study; it is found that, the CSF of PPMS and SPMS patients have significantly lower level of this protein in respect to RRMS [48].

1.6.5 Neurofilament Light Chain (NF-L) in MS

Neurofilaments, are the main cytoskeleton proteins of axons and it consist of three components that differ in molecular size: a light chain (NF-L), an intermediate chain

(NF-M), and a heavy chain (NF-H). According to many studies, increased immunoreactivity of neurofilament has been observed, especially within active MS lesions. Neurofilaments and their differential state of phosphorylation in body fluids are potential markers for neurodegeneration in MS. Increased concentrations of NF-L in CSF have been reported in patients with RR- and PP-MS compared with healthy people and patients with inflammatory and non-inflammatory NDs. During relapse, concentrations are reported to make a peak in the third week after onset of the previous relapse, suggesting a delayed relation with disease activity. NF-L concentrations are reported to be independent of age, sex, and disease duration [48].

1.7 Aim of the Study

The aim of this study is to investigate the protein biomarkers in different clinical subtypes of MS and control groups. To this end, clinical subtypes included 20 patients from each type of clinically isolated syndrome (CIS), Relapsing remitting MS (RRMS), Primary progressive MS (PPMS) and secondary progressive MS (SPMS) patients. Additional 20 people were analyzed as disease control group which included other neurological but non-inflammatory disorders. Chosen proteins which are Tau, MBP, MOG, GFAP and NF-L were analyzed in the CSFs' of patients and control groups by western blotting. Obtained results were analyzed under densitometry and *Image J* analyses programme in order to measure the differences semi - quantitatively. Thus, the results were converted to numeric values in order to compare with study groups. After CSF experiments, significantly different proteins in CSF will be analyzed in serum samples of same patients. Thus, correlation of CSF and serum levels of those proteins will be done. Findings from this thesis are expected to reveal that, there should be different protein patterns among the MS subtypes as well as control samples. Thus, these protein patterns can be candidate biomarkers in order to help the diagnosis, differentiation and prognosis of MS.

2. MATERIALS AND METHODS

2.1 Materials and Laboratory Equipments

2.1.1 Used Equipments

The laboratory equipment used in this study is listed in Appendix A.

2.1.2. Used Chemicals and Markers

The chemicals and markers used are given in Appendix B together with their suppliers. The compositions and preparation of buffers and solutions are given in Appendix C.

2.1.3 Case and Control Group

Study group is composed of 31 RRMS, 25 CIS, 4 PPMS and 1 SPMS patients who came to the Istanbul University, Cerrahpaşa Faculty of Medicine, Neuroimmunology and Demyelination Service and diagnosed according to the revised McDonald's criteria (2005). Diagnosis was based on radiologic findings like MRI and brain tomography, clinical findings and supportive laboratory analyses. The age of the patients ranged from 15 to 55 years. There were 20 females and 10 males.

Control group composed of 6 patients who came to the Neurology department because of other non-inflammatory neurological diseases and apoplexy patients came to the Neurosurgery department in Cerrahpaşa faculty of Medicine. Ages and genders of the control group were compatible with the patient groups.

All the samples are coded and numbered before the studies and blind experiments were followed. To obtain the other statistical data, patients are questioned for the age of onset, age at LP, familial disease history, and used drugs in treatment, job and education. The experimental design of this study has been reviewed and approved by the Ethics Review Committee of the Cerrahpaşa Medical Faculty of Istanbul University.

2.2 Collection and Storage of CSF, Serum, Plasma and Blood Samples

Sample collection of patients and control groups is done at the Neuroimmunology and Demyelination Service clinics. Collection of CSF is done by lumbar punction. Lumbar punction (LP) is a diagnostic and therapeutic procedure in order to collect CSF for further analyses. To remove the CSF, first the patient placed in right or left lateral position and his/her neck – chest distance is reduced as possible as, approximating a fetal position. LP is performed in the interspaces between the lumbar vertebrae, usually at the L4 – L5 level. Before the procedure local anesthetic is applied to that region. With a spinal needle, 3cc of CSF is taken in to sterile, dry, empty tubes. Than 3cc CSF is separated into 500µl * 6 sterile eppendorph tubes. Eppendorph Tubes are kept at -80^{0}C until analyses.

For further analyses, other body fluids were collected from patients and stored. For expression profile studies, blood samples were taken into PAXGENE RNA (PreAnalytix - Qiagen-BD™ Company) tubes in order to study RNA and stored first 24 hour in -20^{0}C then in -80^{0}C freezer. To study on plasma proteins, blood samples are collected in P100 Blood Collection System (BD™) for plasma protein preservation. Then tubes are centrifuged at 4000 rpm for 15 minutes, thus plasma is separated and tubes were stored at -20^{0}C. Serum samples are also taken by using mechanical serum separator tubes and after serum was separated, samples were aliquated in to sterile eppendorph tubes and stored at -20^{0}C. For further genomic analyses blood samples were taken into EDTA tubes and stored at -20^{0}C.

2.3 Oligoclonal Band Analyses

Oligoclonal bands (OCB) are the protein bands reflecting the presence of immunoglobulin proteins in the samples. OCBs can be seen in both serum and CSF samples. Oligoclonal band in CSF but not in serum indicates the CNS production of immunoglobulin. Serum can have also immunoglobulin but increased level of IgG in CSF rather than serum also indicates the presence of a neurological disorder. The presence of OCB in CSF is an important indicator of MS. Protein electrophoresis and comassie blue staining method is used to analyze the OCB presence. In this study, OCB analyses of CSF samples were done in Istanbul University, Faculty of Medicine, microbiology laboratories.

2.4 Protein Precipitation from CSF

Precipitation of proteins from CSF samples was done by following Trichloroacetic acid (TCA) protocol. TCA is widely used in biochemistry for the precipitation of macromolecules such as proteins, DNA and RNA. Week chlorine bonds in TCA bind to positively charged amino acid in proteins and helps to precipitation.

Frozen CSF samples are dissolved in ice, 1 volume of TCA (100%) stock was added to 4 volumes of protein sample in 1,5 ml sterile eppendorph tube and incubated in ice for 15 minutes. Then it was centrifuged at 14.000 rpm for 10 minutes and supernatant is removed to leave protein pellet intact. Pellet was washed with 200µl ice cold acetone and centrifuged again at 14.000 rpm for 5 minutes; this washing step is repeated again with 200µl acetone. Than pellet was dried by placing tube in 65^0C for 5 minutes to drive off acetone. Finally, pellet was suspended in 120 or 150µl sterile dH_2O and mixed by vortexing for 5 minutes.

2.5 Bradford Assay

Bradford Assay was performed before all the SDS-PAGE and Western Blot Analysis. Dr. Marion Bradford firstly described the use of Coomassie Brilliant Blue Dye in a colorimetric reagent for the detection and quantification of total protein in 1976. In the acidic environment of the reagent, protein binds to the coomassie dye, which results in a spectral shift from the reddish/brown form of the dye (absorbance max. at 465 nm) to the blue form of the dye (absorbance max. at 610 nm). The difference between the two forms of the dye is greatest at 595 nm, thus it is the optimal wavelength to measure the blue color from the coomassie dye-protein complex. Besides, the blue color can be measured at any wavelength between 575 nm and 615 nm. Bradford protein assay has been associated with the presence of certain basic amino acids (primarily arginine, lysine and histidine) in the protein. Van der Waals forces and hydrophobic interactions also participate in the binding of the dye by protein. The number of coomassie dye ligands bound to each protein molecule is approximately proportional to the number of positive charges found on the protein. Free amino acids, peptides and low molecular weight proteins do not produce color with coomassie dye reagents. The assay is performed at room temperature. The measurement was accomplished at 595 nm by using a 96 well plate. For measurement in a working range 100-1500 µg/ml, 10 µl protein samples were prepared from protein stocks in a 1:10 ratio, using

distilled water as diluent. Bovine serum albumin (BSA) dilutions in a range of 0.125 mg/ml to 2 mg/ml diluted with distilled water were used as protein standarts. 5 µl of BSA standarts and protein samples were loaded in a 96 well plate. 200 µl of Bradford Reagent was added onto each well and homogenized via pipetting. After incubation at room temperature for 2 min, the absorbance values were measured at 595 nm by Microplate reader 3550-UV (Biorad).

2.6 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) allows separation of proteins according to their sizes. %12 Separating gel was prepared with 4 ml of %30 acrylamide, 2.5 ml of 1,5 M Tris pH 8.8, 100 µl of %10 SDS, 100 µl of %10 APS, 8 µl of TEMED, and 3.3 ml of dH2O. Separating gel was transferred into SDS gel cassette via pipetting. A thin layer of isoprophanol was added onto the gel in the casette. The gel was let at room temperature for 30 min for polymerization. Next, the isopropanol in the cassette was discarded with a tissue paper and upper part of the gel is completely cleaned from the isopropanol by dH2O. %5 stacking gel was prepared via mixing; 670µl of %30 acrylamide, 500 µl of 0.5 M Tris pH 6.8, 40 µl of %10 SDS, 40 µl of %10 APS, 4 µl of TEMED, and 2.7 ml of dH2O. The stacking gel was added onto the polymerized separating gel in the cassette and left 45 min at room temperature for polimerization. 5 µl of ~10 mg/ml protein samples was mixed with 4x loading buffer and denatured at 95°C for 5 min. ~20µl of each sample was loaded onto SDS-PAGE gel. As protein ladder, FERMENTAS 14.1.3 was used. Measurement of loaded total protein is formulated depending on concentration of sample, used sample buffer and volume of buffer with the given formula below in figure 2.1. 7µl of protein ladder was loaded onto the SDS-PAGE gel. Electrophoresis was accomplished in Tris-glycine running buffer at 90 V, for first 10 min. and then voltage is increased to 130 V for 90 minutes. The content of the running gel is given in the below table 2.

$[L_P]$: Loaded protein concentration

S_V : Sample volume

M_V : Sample – buffer mix volume

$[P_{Total}]$: Total protein concentration

V: Loaded volume

$$[L_P] = \frac{S_V}{M_V} \; x \; [P_{Total}] \; x \; V$$

Figure 2.1: Loaded total protein level formula

Table 2.1: Content of Tris– Glycine Runnnig Buffer

Ingredient	**Concentration**	**Amount used in solution**
Tris-HCl	0,125M	3g
Glycine	0,192M	14,4g
%10 SDS	%0,1	10ml
dH2O		1 L

2.7 Western Blotting

The protein samples separated on SDS-PAGE according to their molecular weight and they were transferred to a nitrocellulose membrane and analyzed for presence of target proteins. Transfer was performed in a semi – wet, manual, capillary blotting system by means of diffusion of transfer buffer through sponges, napkins and Whatmann papers (Transfer buffer: 3 g of Tris 25mM, 14.4 g Glycine 192mM, 200 ml of %20 Methanol, 0.05 g of %0.05 SDS, and 1l of dH2O). Transfer is performed overnight at 4^{o}C.

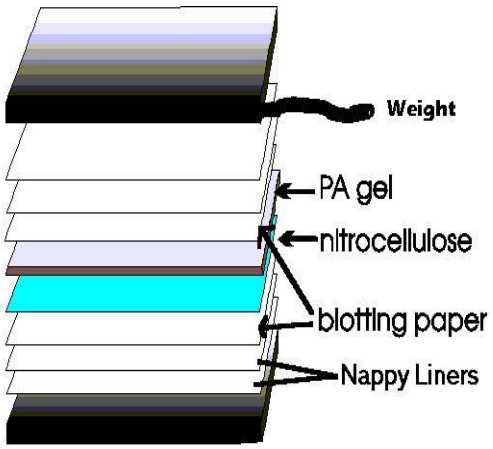

Figure 2.2: Illustriation of capillary western blot module

After blotting is completed, Ponceau S method is used in order to analyze whether proteins are transferred from gel to the membrane. To do this Ponceau S staining solution (0.1% (w/v) Ponceau S in 5% acetic acid) is used. Solution is slowly added on to the membrane and it is shaked for at most 10 seconds and than it is cleaned with dH2O. If Ponceau S stain is not cleaned with only dH_2O then 0.005% NaOH is added to the cleaning solution and shakes for 10 seconds.

After Ponceau S stain completely removed from the nitrocellulose membrane it was washed twice with TTBS buffer at room temperature each for 10 min. [TTBS buffer: 4.38g of NaCL, 6.05 g of Tris, and 500 ml of dH2O (pH of the buffer was set to pH 7.4)]. Next, the membrane was incubated at room temperature for at least one hour with blocking buffer which included 3% BSA. Then, the membrane was washed with TTBS at room temperature each for 10 min (TTBS buffer: 90µl of % 0.05 Tween20 and 180 ml TBS). Then, the membrane was first incubated with first antibody blocking solution at room temperature for one hour. The first antibody blocking solution included 5µl of first antibody (200µg/ml) with 10 ml blocking buffer. After treating the membrane with first antibody, the membrane was washed again three times with TTBS buffer at room temperature each for 10 min. Secondary antibody blocking buffer was composed of 2 µl of AP conjugated secondary antibody (1:5000), %3 BSA blocking buffer. The membrane was incubated in secondary antibody blocking buffer for 1.5 hour at room temperature. Next, the membrane was washed three times with TTBS and once with the NBT/BCIP substrate buffer at room temperature each treatment was for 10 min. Lastly, the membrane was incubated in NCBT/BCIP solution until the protein bands were detectable (200µl NCBT/BCIP stock solution + 10ml NBT/BCIP buffer; NCBT/BCIP substrate buffer: 3 g of 0.1 M Tris-HCl pH 9.5, 1.461 g of 0.1 M NaCl2, 2.541 g of 0.05 M $MgCl2.6H_2O$, and 250 ml of dH_2O). After the substrate incubation was over, membrane was washed with dH_2O and let it to dry.

2.8 Scanning of Membranes with Densitometry

Quantitatively analyses of protein bands were achieved by using densitometry scanning. Protein bands on the membranes were scanned by using BioRad GSH 800 densitometry and *MagicScan32 V4.5* software. To make the scanning procedure Standart, all the scanings are performed in the same settings:

Degree of whiteness: 255

Degree of background color: 200

Degree of blackness: 1.50

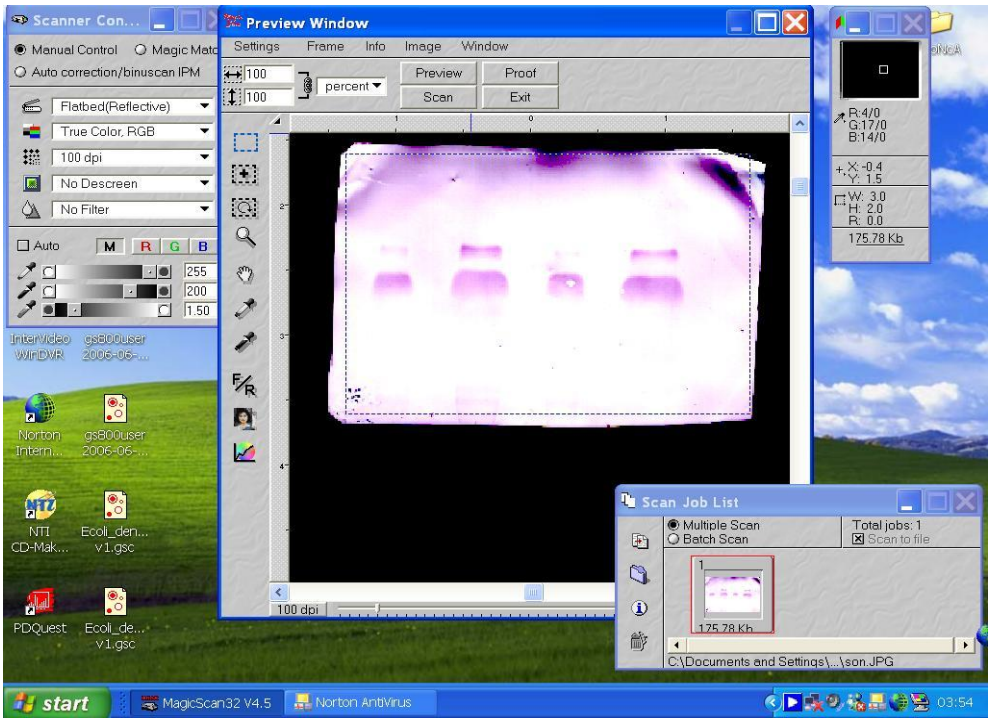

Figure 2.3: Scanning of the membranes

2.9 Analysis of Samples with *ImageJ* Anaylsis Programme

Scanned protein bands on the membranes are analyzed by using *ImageJ* analysis programme to obtain quantitative measurement. Programme measures the density and thickness of the protein bands depending on the gathered light. To make the analyses procedure standart, all the analyses are performed in the same settings. For each protein band, same number of pixels (5000) counted and mean values of the measurements are obtained from the programme.

Figure 2.4: Analyses of samples with *ImageJ*

2.10 Statistical Analyses

Statistical analyses were done by using *Student's t test*. *Student t test* is a statistical hypothesis test in which the test statistic has probability distribution. It is applied when the population is assumed to be normally distributed but the sample sizes are small enough that it relies uncertain estimate of standart deviation (SD) rather than on a precisely known value. Analysis programme were used from http://www.physics.csbsju.edu/stats/t-test.html online web page.

3. RESULTS

3.1 Sample Characteristics

52 samples were analyzed including 31 RRMS, 25 CIS, 4 PPMS, 2 SPMS and 12 control patient. Female to male ratio in study group is almost 2:1.

Gender	**Number**
Female	19
Male	43

Table 3.1: Female to male ratio

3.2 Protein Precipitations and Bradford Assays

TCA protein precipitation protocol increased the concentration of total protein minimum of 10 times. Concentrations of total proteins in samples were measured by Bradford assay. Seven standarts protein concentration ranging 0.125 mg/ml to 2 mg/ml were used.

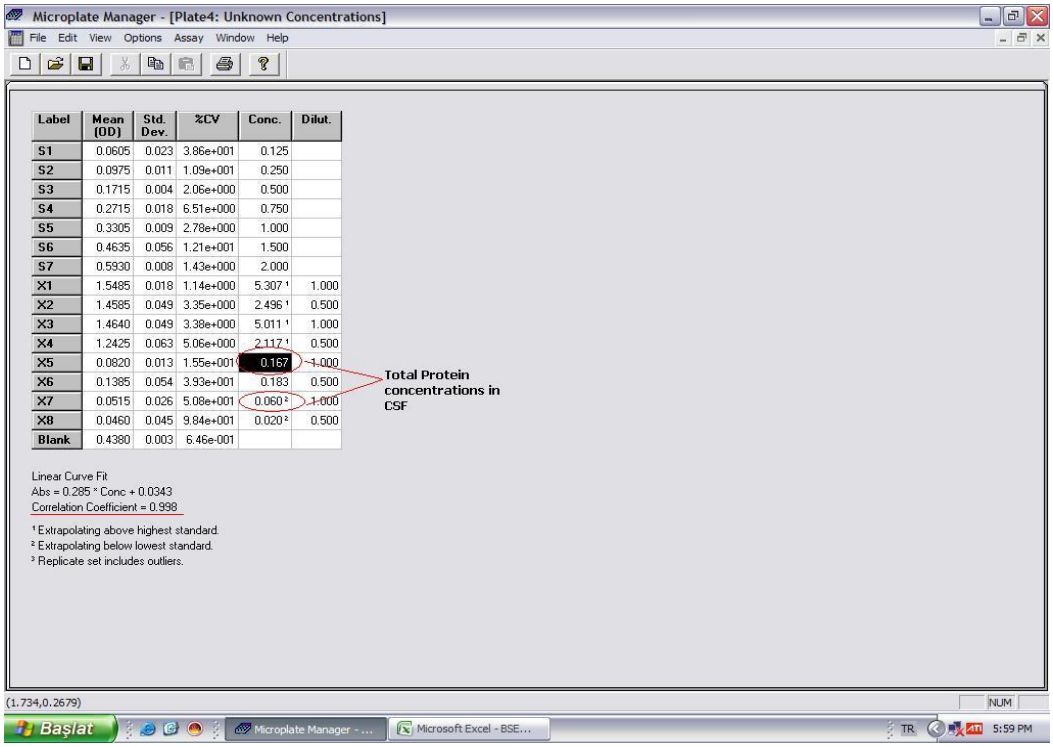

Figure 3.1: Total protein concentrations in CSF before protein precipitation

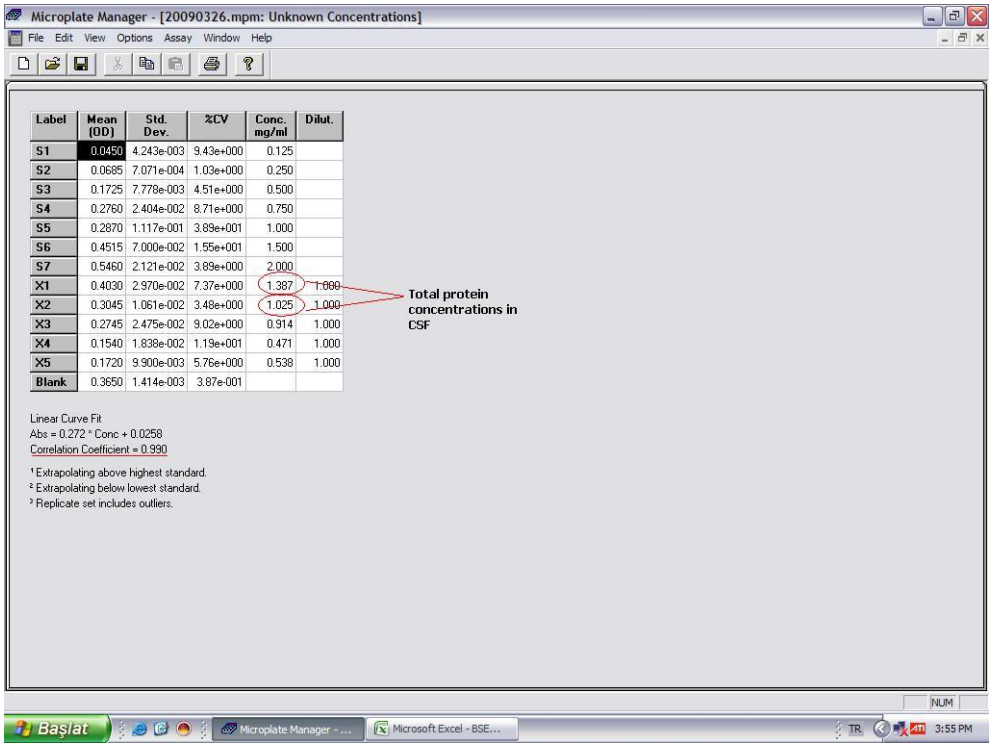

Figure 3.2: Total protein concentration in CSF after protein precipitation

Precipitation and measurement of total protein was applied to all samples and the mean value of CSFs' total protein among 72 patients was 0.993 and there were no significant difference in total protein concentration between different subtypes of MS.

Table 3.2: Total protein concentration of MS subtypes and control

Group	Mean Value
RRMS(n=31)	1.149
CIS(n=25)	1.042
PPMS(n=4)	1.033
SPMS(n=2)	0.748
Control(n=12)	1.134
Total(n=84)	1.040

3.3 SDS-PAGE

In the SDS – PAGE studies, separation of protein bands were optimized by using different concentration of gels and different amount of proteins. Also, albumin and

immunoglobulin G (IgG) content of CSF was eliminated by centrifugation and ultrafiltration. To separate the protein 2 different concentration of separating gel was used: For the proteins Tau (46 kDa) and GFAP (50 kDa), 12% gel, for the other proteins NF – L (68 kDa), MOG (28 kDa) and MBP (21.5 kDa) 7.5% gel was used. Understanding of localization of interested proteins were achieved by using FERMENTAS 14.1.3 protein marker. Loaded total protein values were always approximated to ~10mg/ml by using loading formula.

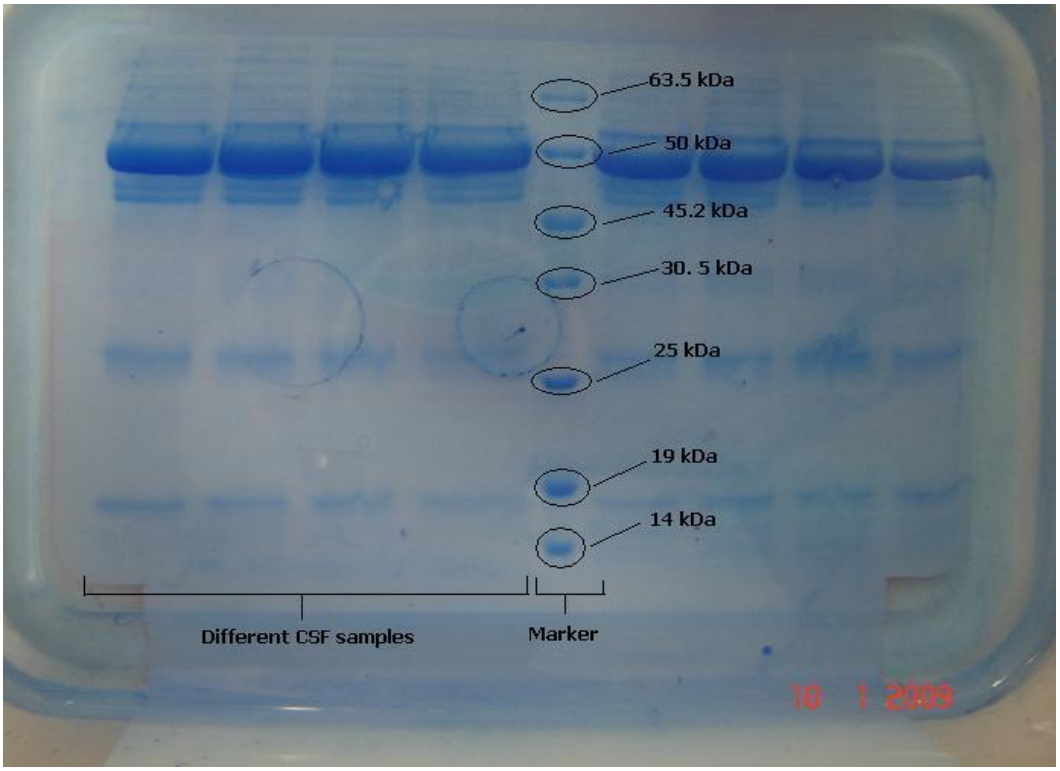

Figure 3.3: SDS – PAGE of CSF samples

3.4 Western Blot

When the transfer of proteins from gel to membrane is completed, Ponceau S staining solution was applied to confirm whether transfer was occurred or not. After protein bands have seen, destaining solution is applied to continue with western blot. Ponceau S staining has showed that transfer conditions for the proteins are optimized as overnight at 4^0C with transfer buffer mentioned in section 2.6.

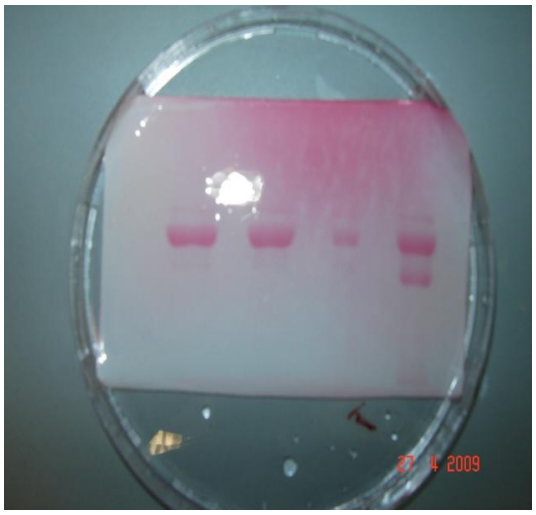

Figure 3.4: Ponceau S staining

Figure 3.5: Destaining of Ponceau S

Application of antibodies and substrate buffers revealed presence of proteins of interests.

Figure 3.6: GFAP and Tau proteins

Figure 3.7: NF–L, MOG, and MBP

3.5 Densitometry scans of membranes

Scanning's of all membranes were done in the same settings:

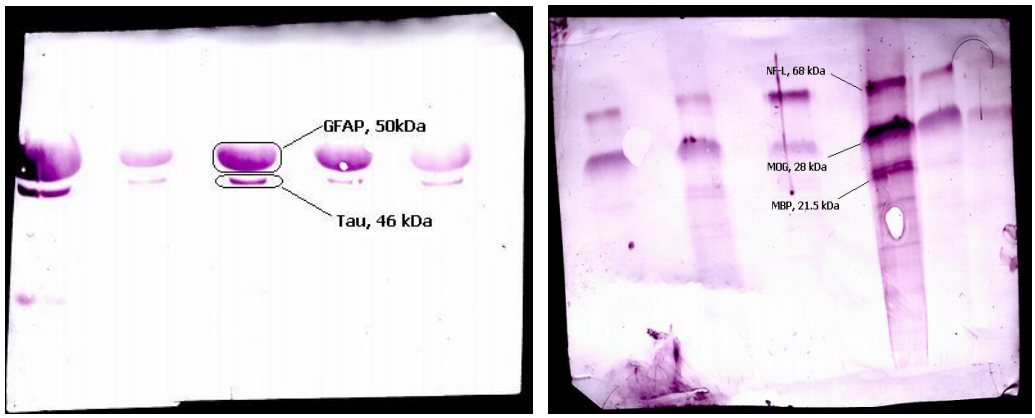

Figure 3.8: Scaning of mebranes

3.6 Analysis of protein bands with *ImageJ*

Analyses of the protein bands with *ImageJ* software gives numerical values indicating that number 255 is the most whiteness value and 0 is the most blackness value. That means, when the density and thickness of bands increases, number given by the programme is less. Thus to understand the linearity, reverse value of analyses is calculated by using formula (255 – given number). Also to make all analyses in a standart procedure, measured value is calculated, as loaded protein value is 10 mg/ml by using following formula [(255-given number)/ loaded protein concentration x 10]. All the values, for each protein are calculated according to given formula.

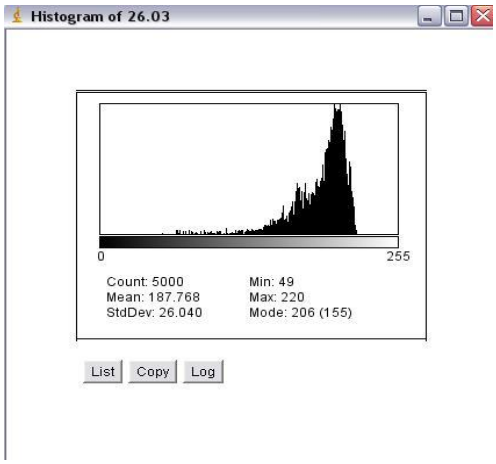

Figure 3.9: Analyses with *ImageJ*

For example;

Measured mean value is: 187.768

Absolute mean value is: 255 – 187.768= 67.232

Standart mean values is 67.232/10.32*10=65.147

Table 3.3: Standart protein concentration values of RRMS patients (n=31)

Samples	Tau	GFAP	NFL	MOG	MBP
MS-ND-1-001/BOS1	48.068	23.844		31.762	
MS-ND-1-002/BOS1	46.952	35.813		33.929	
MS-ND-1-003/BOS1	30.117	14.242		44.008	
MS-ND-1-006/BOS1*	88.205	15.666	34.308	42.443	9.730
MS-ND-1-007/BOS1	106.934	6.295	29.359	48.005	17.392
MS-ND-1-008/BOS1	35.327		75.083	35.715	17.898
MS-ND-1-009/BOS1	68.110	5.766	89.576	160.378	33.841
MS-ND-1-010/BOS1	118.786	7.648	83.002	77.860	16.372
MS-ND-1-011/BOS1	32.852	9.034	61.871	43.601	28.261
MS-ND-1-019/BOS1	47.992	4.539	134.278	46.294	
MS-ND-1-020/BOS1	25.075	11.517	58.171	27.683	
MS-ND-1-024/BOS1	89.188	32.747	16.131		
MS-ND-1-026/BOS1	29.270	4.962	48.084	25.631	
MS-ND-1-027/BOS1	22.808	13.413	32.198	51.316	
MS-ND-1-038/BOS1	79.704	61.436	88.225	34.658	14.497
MS-ND-1-040/BOS1	55.333	96.481	77.437	55.232	3.951
MS-ND-1-042/BOS1	45.140	14.390	146.341	65.273	79.697
MS-ND-1-044/BOS1	41.961	46.192	85.042	9.517	33.082
MS-ND-1-045/BOS1	16.914	61.972	94.394	47.463	6.536
MS-ND-1-049/BOS1	74.637	53.820	81.417	53.794	
MS-ND-1-050/BOS1	46.094	68.198	115.487	35.481	0.049
MS-ND-1-054/BOS1	66.011	39.879	66.532	41.252	
MS-ND-1-055/BOS1	69.038	60.065	162.708	29.650	66.860
MS-ND-1-056/BOS1	77.906	55.729	138.165	117.075	1.434
MS-ND-1-060/BOS1	62.980	32.873	59.567	46.541	
MS-ND-1-071/BOS1	84.528	21.904	68.104		
MS-ND-1-072/BOS1	30.027	44.703	85.043	40.108	
MS-ND-1-073/BOS1	61.480	32.856	81.951	50.976	
MS-ND-1-074/BOS1	37.347	22.290	79.110	46.600	
MS-ND-1-075/BOS1	33.631	29.464	72.822	54.992	
MS-ND-1-076/BOS1	35.073	33.827	74.696	56.039	

Table 3.4: Standart protein concentration values of CIS patients (n=25)

Samples	Tau	GFAP	NFL	MOG	MBP
MS-ND-1-004/BOS1	27.885	38.959		47.403	
MS-ND-1-005/BOS1	27.782	20.439		66.697	
MS-ND-1-012/BOS1	97.939	8.560	45.009	58.488	36.506
MS-ND-1-013/BOS1	102.232	25.303	59.946	52.851	30.013
MS-ND-1-014/BOS1	75.542	17.347	73.845	57.197	
MS-ND-1-015/BOS1	67.015	11.922	83.470	41.363	
MS-ND-1-016/BOS1	25.524	2.781	44.036		
MS-ND-1-017/BOS1	30.173	2.920	56.462	33.165	
MS-ND-1-021/BOS1*	23.547	8.361	45.021	26.318	
MS-ND-1-022/BOS1	59.890	25.059		58.808	
MS-ND-1-025/BOS1	31.300	21.557	66.539	39.618	
MS-ND-1-028/BOS1	24.431	7.158	293.613	124.489	
MS-ND-1-031/BOS1	73.674	15.795	56.430	62.634	
MS-ND-1-032/BOS1	50.803	25.189	74.209	53.400	
MS-ND-1-035/BOS1	61.470	15.176	78.749	61.288	
MS-ND-1-036/BOS1	58.821	60.659	31.447	66.874	0.948
MS-ND-1-037/BOS1	66.333	64.405	112.114	23.507	60.429
MS-ND-1-039/BOS1	21.120	4.226	104.694	32.456	146.780
MS-ND-1-041/BOS1	62.738	6.609	43.917	25.645	
MS-ND-1-043/BOS1	37.183		33.954	77.682	
MS-ND-1-047/BOS1	49.324	89.676	156.954	54.195	11.190
MS-ND-1-062/BOS1	33.371	34.267	67.448	35.608	
MS-ND-1-064/BOS1	23.048	19.191	66.466		
MS-ND-1-065/BOS1	58.602	48.998	147.700	62.562	5.702
MS-ND-1-067/BOS1	25.397	26.678	67.649		

Table 3.5: Standart protein concentration values of PPMS patients (n=4)

Samples	Tau	GFAP	NFL	MOG	MBP
MS-ND-1-030/BOS1	88.915	44.419	61.232	60.863	
MS-ND-1-033/BOS1	88.023	49.069	75.465	63.541	
MS-ND-1-034/BOS1	87.614	70.684	134.846	128.109	32.514
MS-ND-1-061/BOS1	65.702	62.070	70.209	76.707	

Table 3.6: Standart protein concentration values of SPMS patients (n=2)

Samples	Tau	GFAP	NFL	MOG	MBP
MS-ND-1-018/BOS1	91.345	42.846	47.697	61.849	
MS-ND-1-023/BOS1	137.977	41.077	160.511	86.432	

Table 3.7: Standart protein concentration values of Control samples (n=12)

Sample	Tau	GFAP	NFL	MOG	MBP
MS-ND-1-029/BOS1	20.823	6.632		24.460	
MS-ND-1-046/BOS1	31.758	8.931	20.159	12.305	16.478
MS-ND-1-048/BOS1	25.715	12.643	28.551	18.339	
MS-ND-1-051/BOS1	29.598	10.276	15.491	10.764	
MS-ND-1-057/BOS1	29.008	9.454	30.124	5.977	
MS-ND-1-058/BOS1	34.137	7.068	14.712	9.067	
MS-ND-1-059/BOS1	23.028	13.018	15.122	10.635	
MS-ND-1-063/BOS1	25.517	8.975	56.567	11.357	69.085
MS-ND-1-066/BOS1	15.693	1.815	48.727	21.619	73.205
MS-ND-1-068/BOS1	13.826	2.302	19.872	1.851	3.528
MS-ND-1-069/BOS1	27.118	14.219	5.124		3.916
MS-ND-1-070/BOS1	36.051	33.376	36.403	7.558	44.641

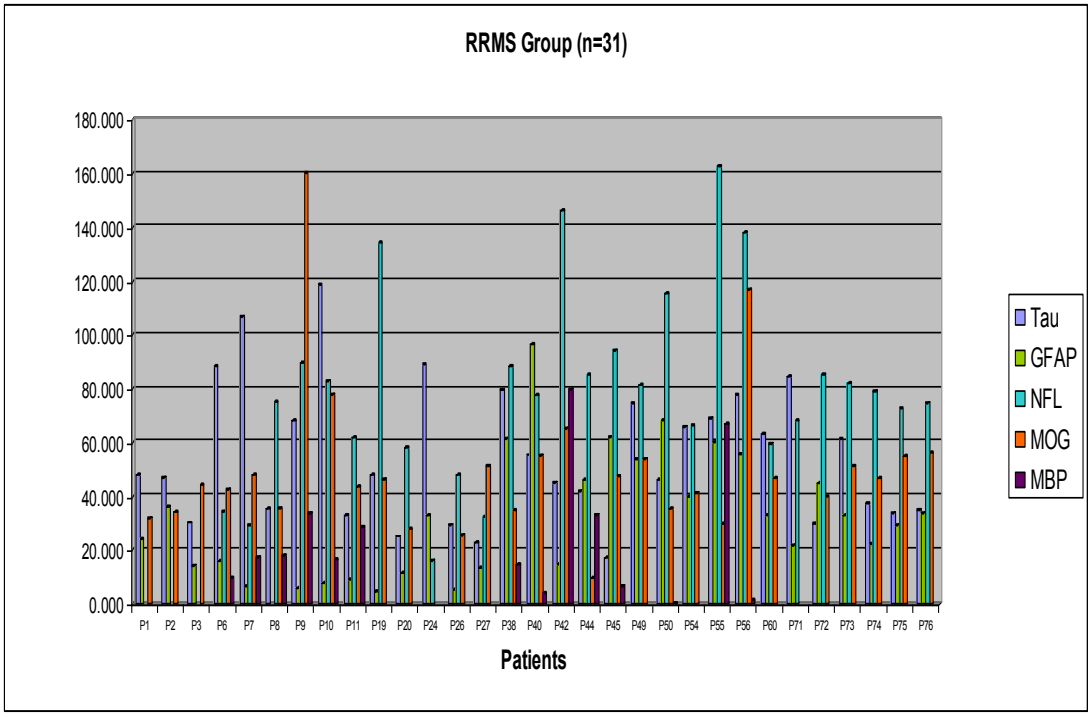

Figure 3.10: Distribution of proteins in RRMS patients (n=31)

Mean values of different proteins in the RRMS groups is shown in figure 3.10. MBP could be detected in 14 patients in lower concentrations.

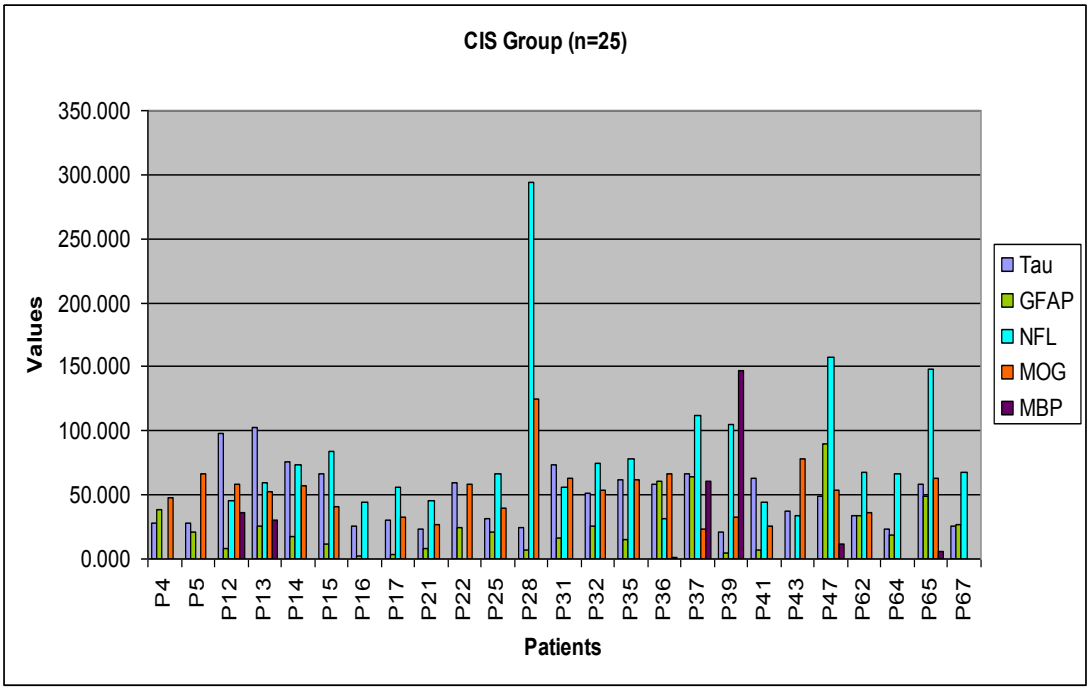

Figure 3.11: Distribution of proteins in CIS patients (n=25)

Mean values of different proteins in the CIS groups is shown in figure 3.11. MBP could be detected in 7 patients in lower concentrations.

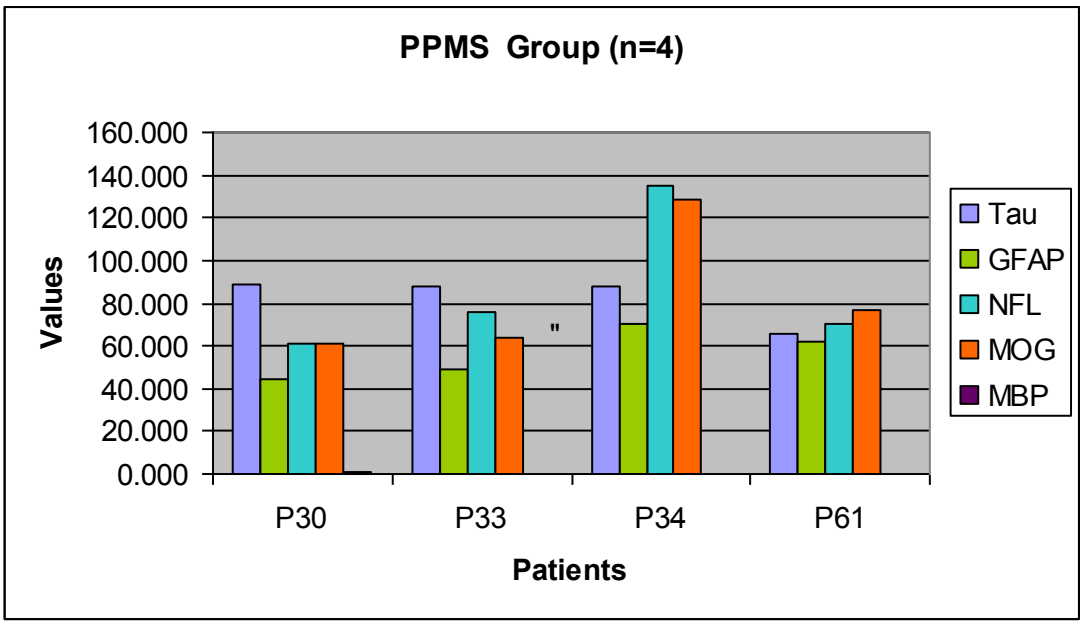

Figure 3.12: Distribution of proteins in PPMS patients (n=4)

Mean values of different proteins in the PPMS groups is shown in figure 3.12. MBP could be detected in only one patient.

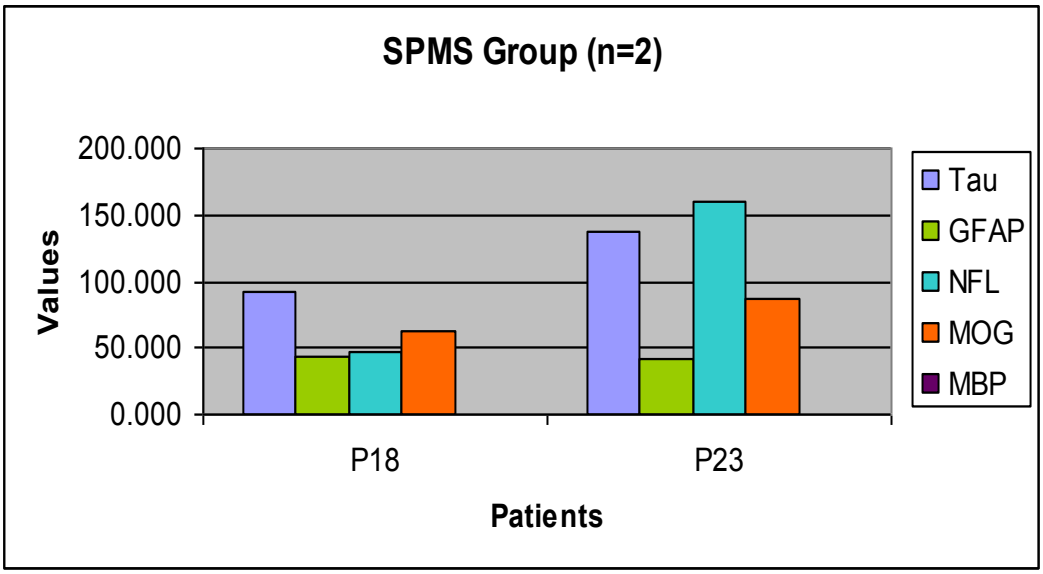

Figure 3.13: Distribution of proteins in SPMS patients (n=1)

Mean values of different proteins in the SPMS groups is shown in figure 3.13. MBP could not be detected.

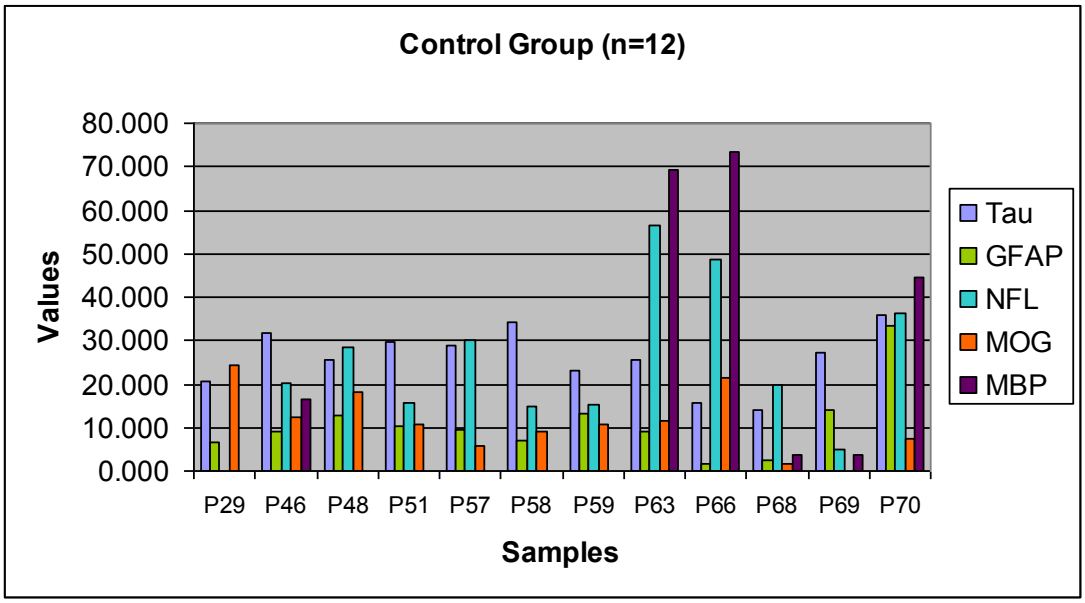

Figure 3.14: Distribution of proteins in control samples (n=12)

Mean values of different proteins in the RRMS groups is shown in figure 3.14. MBP could be detected in six samples.

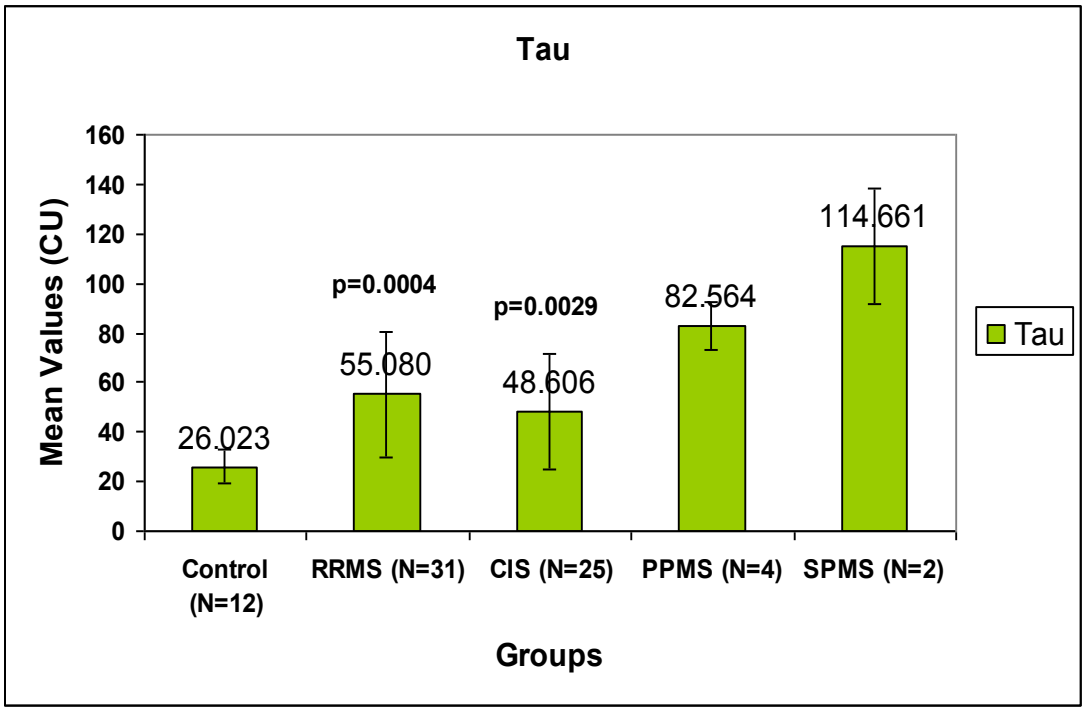

Figure 3.15: Mean values of Tau proteins among different groups

Distribution of Tau protein among different groups is shown in figure 3.15. Mean value of Tau concentration in RRMS (p=0.004) and CIS (p=0.0029) groups are significantly higher than control group. Other groups show a tendency to be higher concentration of Tau than control group. But the number of samples are not enough for statistical analyses.

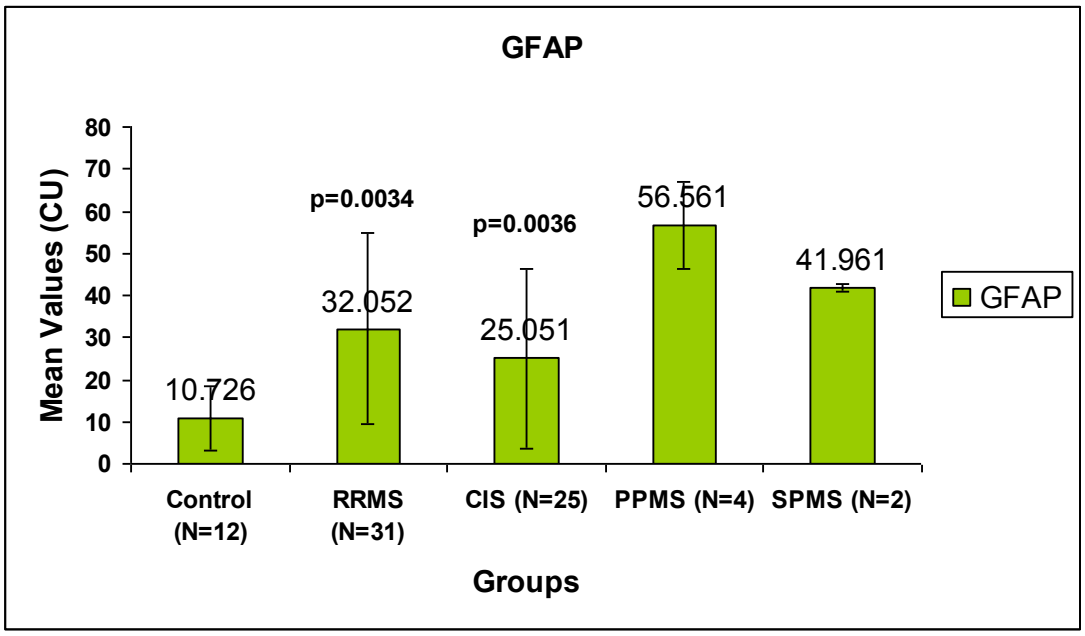

Figure 3.16: Mean values of GFAP proteins among different groups

Distribution of GFAP among different groups is shown in figure 3.16. Mean value of GFAP concentration in RRMS (p=0.0034) and CIS (p=0.0036) groups are significantly higher than control group. Other groups show a tendency to be higher concentration of GFAP than control group. But the number of samples are not enough for statistical analyses.

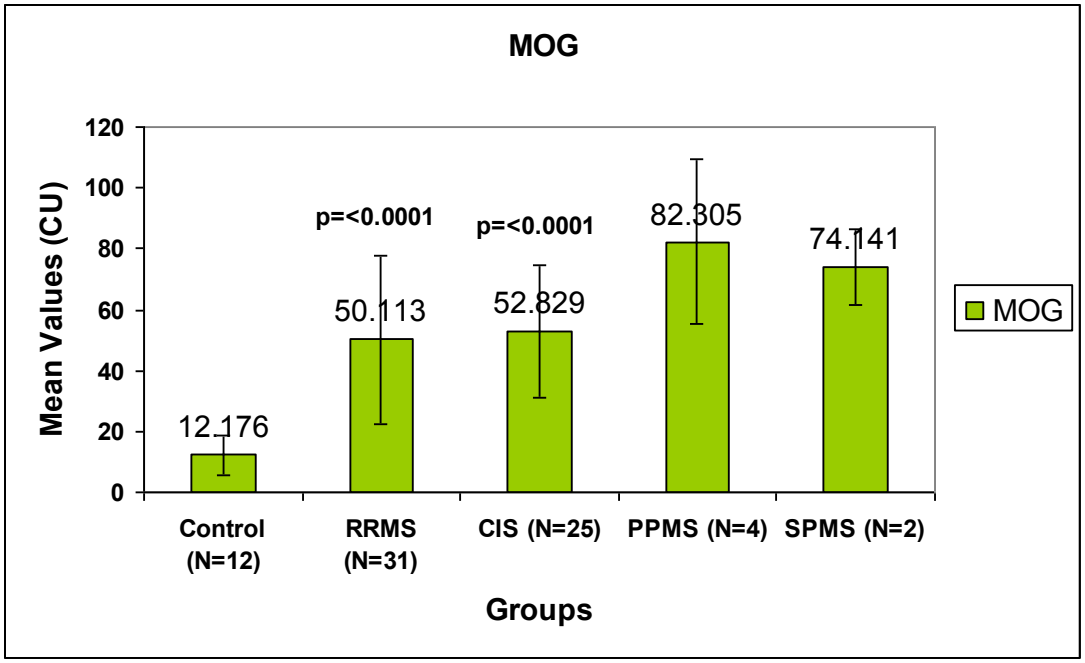

Figure 3.17: Mean values of MOG proteins among different groups

Distribution of MOG among different groups is shown in figure 3.17. Mean value of MOG concentration in RRMS (p=<0.0001) and CIS (p=<0.0001) groups are significantly higher than control group. Other groups show a tendency to be higher concentration of MOG than control group. But the number of samples are not enough for statistical analyses.

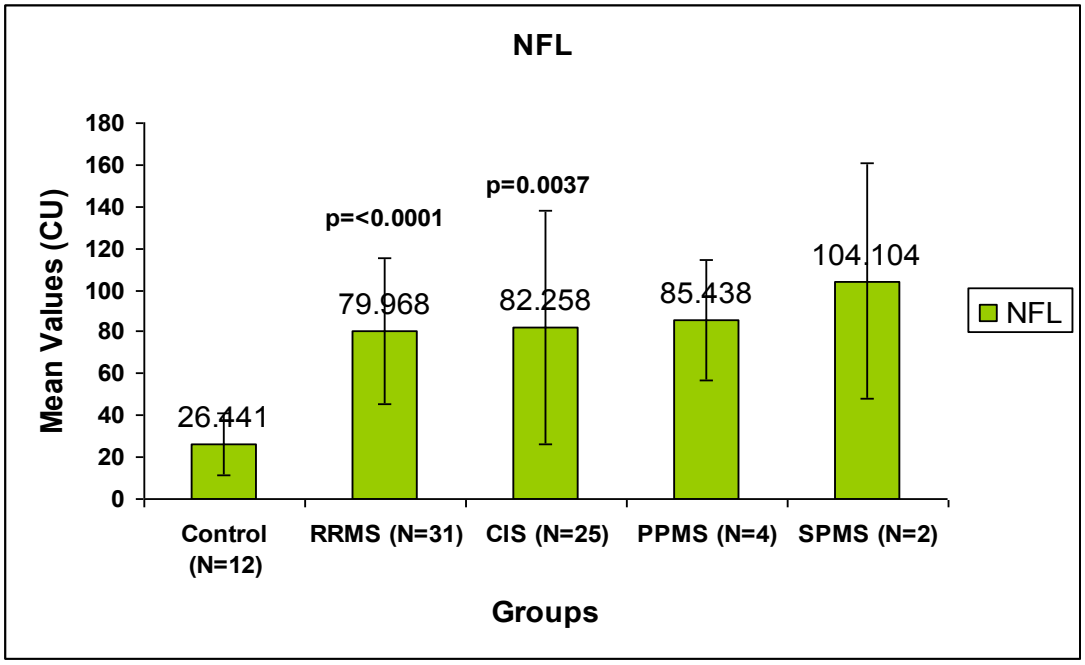

Figure 3.18: Mean values of NF – L proteins among different groups

Distribution of NFL protein among different groups is shown in figure 3.18. Mean value of NFL concentration in RRMS (p=<0.0001) and CIS (p=<0.0037) groups are significantly higher than control group. Other groups show a tendency to be higher concentration of NFL than control group. But the number of samples are not enough for statistical analyses.

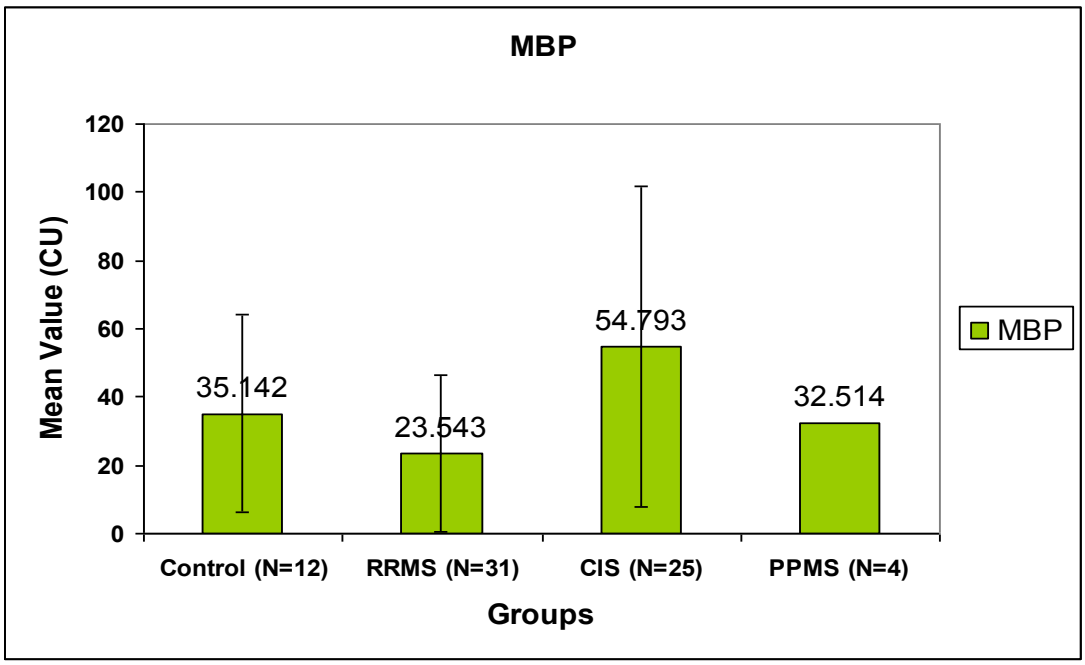

Figure 3.19: Mean values of MBP proteins among different groups

Distribution of MBP protein among different groups is shown in figure 3.19. Mean value of MBP in CIS group tends to be higher than the other groups as well as control group. On the other hand MBP concentration of RRMS has a tendency to be lower than control group.

3.7 *Student t Test*:

Statistical analyses were done by using *student t test* because of the less number of samples. Probability of hypothesis (p values) was evaluated for RRMS (n=31) and CIS (n=25) groups im compare to control sample values (n=12) due to fact that sample number of the other groups were not enough for statistical analyses. Meanings of *p*values are classified as:

- p: <0.05 : statistically significant
- p:<0.01 : statistically very significant
- p: <0.001 : statistically highly significant

4. DISCUSSION AND CONCLUSION

Tau proteins are microtubule associated proteins that are frequently found in neurons, especially in the axons and can be released to CSF. Tau proteins interact with tubulins and play important role in the stabilization and assembly of microtubules in the axons. Controlling microtubule stability is achieved by two features of Tau: isoforms and phosphorylation. By the phosphorylation characteristics of Tau, it can be used as a novel biomarker in Alzheimer's disease. The idea of to be a biomarker in MS for Tau protein, arised from the different isoforms of Tau and concentration of it's in the CSF. In a previous study among the 114 MS (84 RRMS and 30 progressive) patients, 79 inflammatory ONDs and 60 non – inflammatory ONDs controls, it is revealed that, for Tau protein even there is no difference between the MS groups, increased Tau level was observed (150%) in RRMS groups compared to controls [54]. In our study, mean values of CSF level of Tau proteins in RRMS (55.080 CU) (n=31) group and CIS group (48.606 CU) (n=25)were compared to the control group (26.023 CU) (n=12). It is revealed that, concentration of Tau level is statistically highly significant in RRMS group (p=0.0004) and CIS group (p=0.0029) as well as other groups and control groups. In PPMS and SPMS, sample numbers are not enough to make a statistical analyses in order to compare the level of Tau protein, but the graphics in figure 3.15 show that, PPMS and SPMS groups have a tendency of higher concentration of Tau than control group. As this results show that, Tau protein can be a biomarker of prognosis and diagnosis of MS depending on the its concentration in the CSF. Regardind the neurodegenration and Tau protein relation, it may say that severity of the disease can be correlated with the Tau protein concentration.

GFAP is an intermediate filament found in neurons and functional in maintaining the mechanical strength of astrocytes, structural integrity of cytoskeleton. It is involved in many cellular functioning processses such as functioning of the blood brain barrier. Also GFAP is believed to be involved in in the long termupkeep of normal CNS myelination. In a previous study, GFAP level of CSFs in PPMS and SPMS show significant lower level in respect to RRMS and also elevated CSF level in MS than

ONDs. And they correlated this results with the disability scale which means when the disability characteristics of the subtypes increases, the level of GFAP, in CSF increases [57]. In this study, it is observed that level of GFAP protein in CSF in RRMS (32.052 CU) (p=0.0034) and CIS (25.051 CU) (p=0.0036)groups showed statistically highly significant level of protein concentration according to the control group (10.726 CU). Other groups PPMS (56.561 CU) and SPMS (41.961 CU) have also higher level of GFAP concentration but they have not statistically enough number of samples to analyse. As previously indicated, GFAP is important in glial activation and damage. In regard to blood brain barrier damage and glial activation in the pathogenesis of MS, GFAP can play role as a glial and BBB damage biomarker and also concentration of GFAP may correlates with the disease disability scale.

MOG is a glycoprotein that is functional in the myelination of nerves, serves as adhesion molecule providing structural integrity and functions in the formation of OLG cells. MOG is a major protein thought to be related demyelinating diseases of CNS, especially MS. It is a target antigen that leads to autoimmune – mediated disease. In a previous study, it is observed that, anti-MOG frequencies varies %0 – 80 in MS group but higher than ONDs [57]. In this study, it is found that, level of MOG in RRMS (50.113 CU) (p<0.0001) and CIS (52.829) (p<0.0001) groups is almost same but they show statistically highly significant higher level of protein concentration than control group (12.176 CU). Other groups, PPMS (82.305 CU) and SPMS (74.141 CU) have also higher level of MOG protein concentration but they have not statistically enough number of samples to analyse. Regariding the findings in this study and previous findings, it can be said that concentration of MOG in CSF may help to understand the OLG damage and give an idea of myelination process in MS patients. Thus it may said that MOG can be a good biomarker of OLG damage and level of MOG in CSF may correlates with the disease severity [57].

NF – L is an intermediate filament protein, especially found in neurons. It is major cytoskeleton protein that is involved in the formation of axons. It may also play role in intracellular transport to axons and dendrides. In several studies, it is observed that, CSF level of NF – L in RR- and PPMS patients compared with healthy controls is increased in a significant level [56, 57]. In this study, concentration of NF – L protein in RRMS (79.968 CU) (p<0.0001) and CIS (82.258 CU) (p<0.0037) groups show statisticaly highly significant level of increase as protein concentration than control group (26.441 CU). By means of protein function, it can be said that, NF – L protein

concentration may correlates with the prognosis and disability scale of MS. Thus it NF – L protein can be good biomarker showing the axonal damage in the subtypes of MS [57].

MBP is another main functional protein in the myelination process of nerves in the CNS. Various forms of MBP with splice forms and post translational modifications are found in CSF and CNS space. In a previous study, it is observed that HLA DRB1*1501 allele positive MS patients showed high affinity to bind the MBP and their CSF MBP concentration was higher than OND controls [56]. In this study, 14 patients in RRMS groups, 7 patients in CIS group, one patient in PPMS group not in SPMS group and 6 control samples have MBP in their CSF samples. Also results did not showed any significant data. Non significant data can be aroused from several causes like, higher level of standart deviation caused by less number of samples, non – suitable antigen – antibody interaction caused by several isoforms of MBP protein in CSF. There may be another variant of MBP which is more abundant in CSF. Addition to this MBP isoform, other MBP forms should be studied and their differences should be understanded.

To sum up, this is the first study evaluating, five different, novel, candidate biomarkers in four main subtypes of MS patients and control samples. Moreover, quantitative approaches with those proteins by using western blot technique is another improvement that we applied for evaluating the level of proteins in that subtypes. Another feature of this study is, first clinical isolated syndrome study, comparing the level of same proteins with the MS subtypes. At the end, four of five proteins showed significant findings about their level in different groups. Even statistical findings are showing the significant results, sample numbers of groups are still not enough. Especially in prograssive forms (PPMS and SPMS) sample numbers should be increased as well as in control group. But on the other hand, sample collection is continuing and number of samples will be increased.

At the another part of the project, serum samples of the same patients and control samples will be analyzed and a quantitative comparison will be done between the serum and CSF level of those proteins. Also serum and CSF level of those protein comparison will also be the first study evaluating different proteins, in different groups and in different sample types. Actually this study is a preliminary study of future projects, including biomarker investigation study by using proteome researchs and methylome analyses of MS patients and subgroups. Also, these data can be

integrated into another project: Mathematical and animal modeling of MS to understand the pathological and immune mechanisms in MS.

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APPENDICES

APPENDIX A : Laboratory Equipment

APPENDIX B : Chemicals, Enzymes and Markers

APPENDIX C : Solutions and Buffers

APPENDIX A

LABORATORY EQUIPMENT

Balances	Schimadzu
	Precisa
	Eppendorf
Centrifuges	Allegra 25R Centrifuge Beckman
	Coulter
	Sigma
Minicentrifuge	Beckman coulter
Electrophoresis Equipments	Stratagene
Gel Documentation System	BIO-RAD
Mini-Vertical Gel System	BioWorld
Orbital Shaker	Forma
Thermomixer	Eppendorf
Microplate Reader	Biorad
pH meter	Mettler Toledo MP220
Vortex	Kermanlar
Pipettes	Thermo Finnpipette 1000 µl
	10 µL, 200 µl
Laminar Air Flow Cabin	Thermo Scientific
Fridge and Freezers (+4°C, -20°C, -80°C)	Heraeus Sepatech, Bosch, Sanyo, Arcelik
Water Distillation System	Millipore
Autoclave	
Ice Machine	Arçelik
Power Supply	Thermo Scientific
Densitometry	BioRad

APPENDIX B

CHEMICALS

Acrylamide	Merck
Antibodies for Western Blot	Santa Cruz Biotechnology: sc-32274 Tau (46) sc-33673 GFAP (2E1) sc-25652 NF-L (H-70) sc-66968 MOG (H-72) sc-25665 MBP (FL-304)
B-Mercaptoethanol	Sigma
Bis-Acrylamide	Merck
Bromophenol Blue	Sigma
Bovine Serum Albumine	Sigma Aldrich
DTT (Dithiothreitol)	Fermentas
Ethanol	Merck Riedel-de Haën
Glacial Acetic Acid	Merck
Glycerol	Sigma
Glycine	Merck
HCl	Merck
Isopropanol	Fluka
KCl	Carlo Erba
$MgCl_2$	Merck
Methanol	Fluka
NaCl	Carlo Erba
NaOH	Riedel-de Haën
Nitro blue tetrazolium 5-Bromo 4-chloro-3- indosyl phosphate (NBT/BCIP)	Roche
Nitrocellulose Membrabe	Santa Cruz:

	sc-3724 0.45 µm Pore sc-3718 0.22 µm Pore
Ponceau S	Riedel-de Haën
Sodium dodecyl sulfate (SDS)	Merck
TEMED	Merck
Tris Base	Merck Sigma
Tween 20	Merck
2-mercapto-ethanol	Merck

MARKERS

FERMENTAS 1.4.1 Protein Marker

APPENDIX C

SOLUTIONS and BUFFERS

Protein Precipitation Buffers

Trichloro Acetic Acid (TCA) Solution:

TCA solution dilution of final concentration 2.2g/ml was prepared with $dH_2O$and powder TCA stock.

SDS-PAGE Buffers

SDS Sample Buffer (4X)

	Final Concentarion
Tris-HCl, 0.5M, pH:6.8	0.25 M
SDS	8%
2-mercaptoethanol (10mM)	10%
Glycerol	30%
Bromophenol Blue	0.002%

SDS Running Buffer (1X)

	Final Concentration
Tris-HCl 0.5M	0.025 M
Glycine	0.192 M
SDS	0.1%

Comassie Brilliant Blue (CBB) Stain Dye

	Final Concentration
CBB	0.1 %
MeOH	50 %
Acetic Acid	10%

CBB Destain Dye

	Final Concentration
MeOH	10%
Acetic Acid	10%

Seperating Gel (2V)

	7.5 %	**12%**
dH_2O	4.85ml	3.3 ml
Acrylamide – Bis- (30% - 0.8%)	2.45 ml	4 ml
1.5M Tris HCl, pH:6.8	2.5 ml	2.5 ml
10% SDS	0.1 ml	0.1 ml
10% APS (100 mg/ml)	0.1 ml	0.1 ml
TEMED	8 µl	8µl

Stacking Gel (2V)	**4%**
dH_2O	2.7 ml
Acrylamide – Bis- (30% - 0.8%)	0.67 ml
1.5M Tris HCl, pH:6.8	0.5 ml
10% SDS	0.04 ml
10% APS (100 mg/ml)	0.04 ml
TEMED	4µl

Bradford Standarts

Standart 0: 30 ml dH_2O

Standart 1: 0.125 mg/ml BSA + 30 ml dH_2O

Standart 2: 0.25 mg/ml BSA + 30 ml dH_2O

Standart 3: 0.5 mg/ml BSA + 30 ml dH_2O

Standart 4: 0.75 mg/ml BSA + 30 ml dH_2O

Standart 5: 1 mg/ml BSA + 30 ml dH_2O

Standart 6: 1.5 mg/ml BSA + 30 ml dH_2O

Standart 7: 2 mg/ml BSA + 30 ml dH_2O

Western Blot Buffers

Towbin Transfer Buffer

		Final Concentration
Tris Base	3g	0.025 M
Glycine	14.4g	0.192 M
Methanol	200 ml	20%
dH_2O	added to 1 lt.	

TBS

		Final Concentration
NaCl	8.76g	0.15 M
Tris	12.1g	0.01 M
dH_2O	added to 1 lt	

pH is adjusted to 8.0

TTBS Wash Buffer (0.1%v/v)

TBS	990 ml
Tween 20	10 ml

Blocking Buffer

		Final Concentration
BSA	3g	3%
dH_2O	added to 100 ml	

NBT/BCIP Substrate Buffer

		Final Concentration
Tris – HCl, pH: 9.5	3g	0.1M
$NaCl_2$	1.461g	0.1M
$MgCl_{2.}\ 6H_2O$	2.541	0.1M
dH_2O	added to 250 ml	

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