ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

A LINKAGE ANALYSIS AND A GENOME-WIDE ASSOCIATION STUDY ON FAMILIAL MULTIPLE SCLEROSIS

M.Sc. THESIS

Elif EVEREST

Department of Molecular Biology-Genetics and Biotechnology

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521121135

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Thesis Advisor: Assoc. Prof. Dr. Eda TAHIR TURANLI

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Programı : Herhangi Program

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

AİLESEL MULTIPL SKLEROZ'DA BAĞLANTI ANALİZİ VE GENOM ÇAPI İLİŞKİLENDİRME ÇALIŞMASI

YÜKSEK LİSANS TEZİ

Elif EVEREST
521121135

Moleküler Biyoloji – Genetik ve Biyoteknoloji Bölümü
Moleküler Biyoloji – Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Doç. Dr. Eda TAHİR TURANLI

Anabilim Dalı : Herhangi Mühendislik, Bilim
Programı : Herhangi Program

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Elif Everest, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID 521121135, successfully defended the **thesis** entitled "**A LINKAGE ANALYSIS AND A GENOME-WIDE ASSOCIATION STUDY ON FAMILIAL MULTIPLE SCLEROSIS**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :   **Assoc. Prof. Eda TAHİR TURANLI**   ....
İstanbul Technical University

Jury Members :   **Prof. Dr. Aksel SİVA**   ....
İstanbul University

Jury Members :   **Assoc. Prof. Gizem DİNLER DOĞANAY**   ....
İstanbul Technical University

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To my beloved ones,

FOREWORD

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June 2015   Elif EVEREST

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ABBREVIATIONS

ADEM	: Acute Disseminated Encephalomyelitis
BBB	: Blood Brain Barrier
CCC	: Complement and Coagulation
CDMS	: Clinically Definite Multiple Sclerosis
CI	: Confidence Interval
CIS	: Clinically Isolated Syndrome
CNS	: Central Nervous System
CSF	: Cerebrospinal Fluid
DIS	: Dissemination in Space
DIT	: Dissemination in Time
DMT	: Disease Modifying Therapies
EAE	: Experimental Autoimmune Encephalomyelitis
EBNA-1	: EBV Nuclear Antigen 1
EBV	: Ebstein-Barr Virus
EDSS	: Expanded Disability Status Scale
FDR	: False Discovery Rate
GWAS	: Genome Wide Association Studies
HLA	: Human Leukocyte Antigen
IgG	: Immunoglobulin G
IFNAR1	: Interferon (Alpha, Beta, and Omega) Receptor 1
IFNAR2	: Interferon (Alpha, Beta, and Omega) Receptor 2
IL2RA	: Interleukin 2 Receptor Alpha
IL7RA	: Interleukin 7 Receptor Alpha
IMSGC	: International Multiple Sclerosis Genetics Consortium
INS	: Insulin
INS-IGF2	: Insulin - Insulin-Like Growth Factor 2 Read-Through Product
INSR	: Insulin Receptor
MAB21L1	: Mab-21-Like 1
MAF	: Minor Allele Frequency
MBP	: Myelin Basic Protein
MHC	: Major Histocompatibility Complex
MOG	: Myelin Oligodendrocyte Glycoprotein
MRI	: Magnetic Resonance Imaging
MS	: Multiple Sclerosis
MSIF	: Multiple Sclerosis International Federation
NAGM	: Normal Appearing Grey Matter
NAWM	: Normal Appearing White Matter
NK	: Natural Killer
NMO	: Neuromyelitis Optica
NPL	: Non-Parametric Linkage
OCB	: Oligoclonal Band

OR	**:**	Odd Ratio
PI3K	**:**	Phosphatidylinositol 3-Kinase
PBMC	**:**	Peripheral Blood Mononuclear Cell
PCR	**:**	Polymerase Chain Reaction
PLP	**:**	Myelin Proteolipid Protein
PML	**:**	Progressive Multifocal Leucoencephalopathy
PPMS	**:**	Primary Progressive Multiple Sclerosis
PRMS	**:**	Progressive Relapsing Multiple Sclerosis
RAS	**:**	Renin-Angiotensin System
RIS	**:**	Radiologically Isolated Syndrome
RRMS	**:**	Relapsing-Remitting Multiple Sclerosis
S1P	**:**	Sphingosine-1-Phosphate
SAMS	**:**	Single Attack Multiple Sclerosis
SAPMS	**:**	Single Attack Progressive Multiple Sclerosis
SNP	**:**	Single Nucleotide Polymorphisms
SPMS	**:**	Secondary Progressive Multiple Sclerosis
Treg	**:**	Regulatory T Cell
TUSNP	**:**	Turkish SNP Database
VDBP	**:**	Vitamin D Binding Protein
WHO	**:**	World Health Organization
WTCCC2	**:**	Welcome Trust Case Control Consortium

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A LINKAGE ANALYSIS AND A GENOME-WIDE ASSOCIATION STUDY ON FAMILIAL MULTIPLE SCLEROSIS

SUMMARY

Multiple Sclerosis (MS) is an immune-mediated, neuroinflammatory and neurodegenerative disorder affecting the central nervous system (CNS), and characterised by multifocal lesions in white and grey matter with demyelination, axonal transection, neuronal degeneration, gliosis, and perivenular inflammatory cell infiltrates. MS is a complex disease, which develops in genetically susceptible individuals under specific environmental influences. Early observations from classical genetic studies have shown that MS has a genetic background with a broad range of heritability estimates (25%-76%) reported by different studies. Early linkage analyses revealed a strong association of HLA-DRB1 locus of the class II human leukocyte antigen (HLA) region with MS. Subsequent linkage and candidate-gene based analyses have identified different HLA allele associations and a non-HLA association, interleukin 7 receptor alpha (IL7RA) gene. Further chip-based genome wide association studies (GWAS) have identified a total of 110 non-HLA associations, most of which are related to immune pathways, supporting the immune basis of MS. However, current knowledge on MS genetics can explain only about 27% of the predicted MS heritability, leaving much to be explored.

We have previously conducted a proteome study in our MS cohort that have been collected in Istanbul University, Cerrahpaşa Faculty of Medical, Neurology Department since 2007. The proteome study identified pathological pathways in MS including renin-angiotensin, aldostorene-regulated sodium reabsorption, complement-coagulation and notch signalling patways with potential biomarkers. In the current study, to our knowledge for the first time, we wanted to correlate genomic data from familial MS pedigrees and unrelated patient/control groups with the proteome data.

To this end, first we conducted a linkage analysis in MS patients and their affected and unaffected relatives. 10 multiplex MS families with 35 individuals were included in the analysis and SNP genotyping on the Illumina CytoSNP 300K array was performed for genomes of each individual. NPL scores were calculated for each of 3118 informative SNP markers spaced at an average of 1 cM intervals using SimWalk multipoint NPL analysis. Fine mapping of regions showing NPL scores higher than 1.7 was performed for each SNP markers spaced at every 0.2 cM, revealing that the most promising loci for linkage were mapped to 13q13.3 and 21q22.2, with NPL scores of 1.82 and 1.85, respectively. From the resulted loci, Interferon (Alpha, Beta, and Omega) Receptor 1 (IFNAR1) 18417, Interferon (Alpha, Beta, and Omega) Receptor 2 IFNAR2 11876 polymorphisms, and Mab-21-Like 1 (MAB21L1) CAG repeat number were selected as candidate genes for further analyses. Selected regions were amplified by polymerase chain reaction (PCR) and genotyped in 27 unrelated patients with MS and 10 healthy controls of Turkish

origin. Statistical analyses were performed to calculate genotype and allele frequencies, revealing a significant association of IFNAR2 11876 GG genotype with increased risk of MS (P = 0.027, OR 3.64 [95% CI 1.09 – 12.1]).

We further conducted a GWAS comprising of 11 unrelated MS cases that had been included in the proteomic analyses and in the linkage study, and 60 healthy controls of Turkish origin, revealing 14 SNPs with significant association ($P < 10^{-4}$), and 106 SNPs showing suggestive association with MS ($P < 10^{-3}$). Subsequently, chromosomal regions from the linkage analysis and SNPs from the GWAS were analysed in order to observe a correlation with the previous proteomic findings. One gene with significant (INS-IGF2, P = 4.39E-07), and eight genes with suggestive associations (PRKCE, MAPK9, RBPJL, ADAMTSL1, NR6A1, NOTCH2, IL1R1, NTN1) from the GWAS were found to involve in pathways those shown to be affected in MS subtypes, and there were three genes common between the GWAS and linkage results (CLDN14, RUNX1, LINC00598). When individual proteome data of each patient involved in the genetic analyses was observed, a total of 20 proteins having altered expression level in one or more patients were also found to have significant or suggestive association in the GWAS. Among them, CNTN5 had the only significantly associated SNP markers (P = 4.71E-05 and P = 7.79E-05). Using a multi-disciplinary approach that combined genetic, proteomic, and bioinformatic analyses, we identified several candidate genes, whose possible roles will be explored in our further studies.

AİLESEL MULTIPL SKLEROZ'DA BAĞLANTI ANALİZİ VE GENOM ÇAPI İLİŞKİLENDİRME ÇALIŞMASI

ÖZET

Multipl Skleroz (MS); merkezi sinir sistemini (MSS) etkileyen, immun-aracılı, nöro-inflamatuvar, nörodejeneratif bir hastalıktır. MS, hem ak hem gri maddede demiyelinizasyon ile görülen multifokal lezyonlar, aksonal transeksiyon, nöronal dejenerasyon, gliyozis ve perivenüler inflamatuvar hücre infiltratı ile karakterize edilir. Bu patolojik özelliklerin otoreaktif lenfositlerin kan beyin bariyerinden geçerek miyelin gibi MSS bileşenlerinin yıkımına sebep olmaları sonucu ortaya çıktığı düşünülmektedir. Bu proseste T hücrelerinin ana rolü oynadığı düşünülmekle birlikte, naif CD4$^+$ T yardımcı hücrelerinden farklılaşan Th1 ve Th17 hücrelerinin patogenezdeki rolleri önceki çalışmalarda gösterilmiştir. MS patolojisi; başta görsel, duyu, motor ve kognitif semptomlar olmak üzere geniş bir klinik tablo oluşturmaktadır. Hastalık, farklı klinik tipler olarak kendini göstermektedir. Temel olarak MS'in klinik belirtileri 20 ve 40 yaşları arasındaki genç yetişkinlerde yinelenen-düzelen MS (RRMS) formu olarak ortaya çıkmakla birlikte atak dönemleri arasında hastalarda tam ya da kısmi düzelmeler gözlenir. RRMS hastalarının birçoğu sonradan progresif MS formlarına dönüşmektedir. MS'in klinik spektrumu ayrıca presemptomatik fazlar içermektedir. Bunlardan biri olan klinik izole sendromda (KIS) hastalar tek bir atak geçirir ve ikinci bir atak ya da spesifik lezyon aktivitesi gösterdikleri takdirde klinik olarak kesin MS teşhisi konur. MS tanısı için Schumacher kriterleri başta olmak üzere Poser ve McDonald (2001, 2005 revizyonu, 2010 revizyonu) kriterleri geliştirilmiştir ve günümüzde tanıda kullanılan belli parametreler mevcuttur. Klinik muayenenin yanı sıra göz önünde bulundurulan parametreler; magnetik rezonans (MR) görüntüsünde MS-spesifik lezyonların ve beyin-omurilik sıvısında oligoklonal bantların varlığı ve tepkisel potansiyel ölçümleridir. Mevcut kriterler ile MS'in tanısı kolaylaştırılmış olsa da nöromiyelit optika (NMO) gibi MS'e benzerlik gösteren bir grup hastalığın mevcut olması kesin tanıyı zorlaştırabilmektedir. Aynı zamanda alt tipler arasındaki geçişlerin ya da hastaların tedavilere verecekleri tepkilerin tahmin edilebilmesinde de zorluklar yaşanmaktadır. Bu amaçla bir süredir hastaların vücut sıvılarında gerçekleştirilen proteomik analizlere yoğunlaşılmasıyla birlikte, henüz geliştirilmiş ve onaylanmış tanı kitleri bulunmamaktadır. Diğer taraftan, bu çalışmalar ile günümüze kadar birçok aday protein belirteci tanımlanmıştır.

MS, spesifik çevresel etkenler altında genetik olarak yatkın bireylerde ortaya çıkan kompleks bir hastalıktır. Klasik genetik çalışmalar sonucu MS'in 25%-76% arasında değişen kalıtılabilirlik hesaplamalarıyla birlikte genetik bir altyapısının olduğu gösterilmiştir. MS hastalarının birinci dereceden akrabası olmanın MS riskini 20-40 kat, yaklaşık 1/1000'den 1/25-50'ye arttırdığı gösterilmiştir. Ayrıca ikiz çalışmaları sonucu tek yumurta ikizlerinin MS için konkordans oranının çift yumurta ikizlerine göre daha fazla olduğu görülmüş, böylece gözlemlenen ailesel agregasyonun ortak çevredense ortak kalıtılan faktörlere daha çok bağlı olduğu ortaya çıkmıştır. MS'in

genetik temelinin ilk kez kanıtlandığı bağlantı analizleriyle 6.kromozomda bulunan majör histokompatibilite kompleks (MHC) bölgesinin MS riski ile ilişkili olduğu gösterilmiştir. Daha sonraki bağlantı analizleriyle ayrıntılı haritalama sonucu spesifik olarak sınıf II insan lökosit antijen (HLA) bölgesindeki HLA-DRB1 lokusunun MS ile güçlü bir asosiyasyon gösterdiği bulunmuştur. Takip eden bağlantı ve aday-gen temelli analizler ile birçok farklı HLA asosiyasyonuna ek olarak interlökin 7 reseptör alfa (IL7RA) geninin de MS ile ilişkisi gösterilmiştir. Sonrasında gerçekleştirilen çip temelli genom çapı ilişkilendirme çalışmaları (GWAS) ile 110 tane HLA olmayan asosiyasyon bulunmuştur. Bu genlerin birçoğu T hücre aktivasyonu ve lenfosit proliferasyonu gibi immün yolaklarda yer aldığından, MS'in immün temelini destekleyecek niteliktedir. Ayrıca genlerin üçte birinden fazlasının daha önce farklı otoimmün hastalıklarla ilişkisi gösterilmiştir. Fakat, birçoğunun MS ile fonksiyonel olarak ilişkisi henüz bilinmemektedir. Ayrıca MS ile ilişkisi gösterilmiş bütün HLA olmayan genler düşük-orta risk etkisine sahip yaygın varyantları teşkil etmektedir ve HLA asosiyasyonları ile birlikte MS'in tahmin edilen kalıtılabilirliğinin yalnızca 27%'si kadarını açıklamaktadır. Daha yüksek işlem hacmine sahip teknolojilerin geliştirilmesiyle, mevcut verilerin meta-analizleriyle ve disiplinler arası çalışmalarla MS genetiği ile ilgili bu büyük bilgi açığının ilerleyen zamanlarda doldurulması mümkündür.

Daha önce grubumuz, 2007'den beri İstanbul Üniversitesi, Cerrahpaşa Tıp Fakültesi, Nöroloji Bölümü'nde toplanan farklı alt tiplere sahip 179 MS hastasında ve 42 MS olmayan kontrolde proteomik bir çalışma gerçekleştirmiş ve toplamda 151 proteinin kontrollerle karşılaştırıldığında MS hastalarında ya da farklı MS alt tiplerinde ekspresyon seviyesinin değiştiğini saptamıştır. Çalışmada birçok potansiyel biyobelirteçle birlikte MS'teki patolojik yolaklar açığa çıkarılmıştır. Bu yolaklar renin-anjiyotensin, aldosteron-regüle sodyum geri emilim, komplement-koagülasyon ve notch sinyal yolaklarını içermektedir. Bu çalışmada ise, bildiğimiz kadarıyla şu ana kadar ilk kez, ailesel MS soyağaçlarından ve akraba olmayan hasta/kontrol gruplarından elde edilen genetik veriler, aynı bireylerin proteomik sonuçları ile karşılaştırılmıştır.

Bu amaç ışığında, öncelikle MS hastalarını ve sağlıklı/hasta akrabalarını içeren 28 aileden (42 MS hastası, 37 sağlıklı kontrol) etik kurul onayı ve her bir bireyden bilgilendirme onam formu alındıktan sonra kan örneği toplanmıştır. Kan örneklerinden DNA izole edildikten sonra bilgi verici nitelikteki 10 aile seçilerek bir bağlantı analizi gerçekleştirilmiştir. Öncelikle bu ailelerdeki 18 MS hastası ve 17 sağlıklı akrabada Illumina CytoSNP 300K array kullanılarak genom boyu SNP genotiplemesi yapılmıştır. Toplamda 300.000 SNP genotiplendirilmiş ve eleme kriterlerinin ardından 245.008 adet SNP çalışmalara dahil edilmiştir. Bağlantı analizi için 1 cM aralıklarla bulunan 3118 bilgi verici SNP, SimWalk multipoint non-parametric linkage (NPL) analiziyle taranmıştır. Genomda NPL skoru 1.7'den yüksek olan 13. (37.9 cM, en yakın SNP rs612701, NPL Z = 1.72, p = 0.019) ve 21. (41.82 cM, en yakın SNP rs2834861, NPL Z = 1.7, p = 0.019) kromozomlardaki birer bölge detaylı haritalama amacıyla sırasıyla 639 ve 831 SNP ile 0.2 cM aralıklarla taranmıştır. Bunun sonucunda, bağlantı için en umut verici lokusların sırasıyla 1.82 ve 1.85 NPL skorlarıyla 13q13.3 (34.11 cM, en yakın SNP rs1461965, NPL Z = 1.82, p = 0.015) ve 21q22.2 (45.08 cM, en yakın SNP rs11701543, NPL Z = 1.85, p = 0.014) olduğu belirlenmiştir. Bu lokuslardan Interferon (Alfa, Beta, and Omega) Receptor 1 (IFNAR1) 18417, Interferon (Alfa, Beta, and Omega) Receptor 2 IFNAR2 11876 polymorphisms ve Mab-21-Like 1 (MAB21L1) CAG tekrar sayıları

sonraki analizler için aday olarak seçilmiştir. Seçilen bölgeler Türk kökenli 27 akraba olmayan MS hastası ve 10 sağlıklı kontrolde polimeraz zincir reaksiyonu (PZR) ile çoğaltılıp genotiplenmiştir. Genotip ve alel frekanslarının hesaplanarak istatistiksel analizler yapılmış ve bunun sonucunda IFNAR2 11876 GG genotipinin MS riskiyle ilişkisi bulunmuştur (P = 0.027, OR 3.64 [95% CI 1.09 – 12.1]).

Daha sonra, proteomik çalışmaya ve bağlantı analizine dahil edilmiş olan 11 akraba olmayan MS hastası ve 60 sağlıklı kontrol ile bir genom çapı ilişkilendirme çalışması (GWAS) gerçekleştirilmiştir. Çalışmanın sonucunda MS ile anlamlı derecede (P < 10^{-4}) ilişki gösteren 14, anlamlıya yakın (P < 10^{-3}) ilişki gösteren ise 106 SNP belirlenmiştir. Ardından, bağlantı analizi sonucu açığa çıkarılan kromozomal bölgeler ve GWAS sonucu bulunan SNP'ler analiz edilerek önceki proteom çalışması ile olası korelasyonlar incelenmiştir. GWAS sonucu MS ile anlamlı ölçüde ilişkili bulunan bir gen (INS-IGF2, P = 4.39E-07) ve anlamlıya yakın ölçüde ilişki gösteren sekiz genin (PRKCE, MAPK9, RBPJL, ADAMTSL1, NR6A1, NOTCH2, IL1R1, NTN1) MS alt tiplerinde etkilenmiş olduğu belirlenen yolaklarda rol aldığı gözlenmiştir. Ek olarak, GWAS ve bağlantı analizi için ortak olan üç gen (CLDN14, RUNX1, LINC00598) olduğu görülmüştür. Genetik analizlerde yer alan bireylerin tek tek proteom verisi incelendiğinde ise bir ya da daha fazla hastada ekspresyon seviyesi değişmiş 20 proteini kodlayan genin, GWAS sonucunda anlamlı ya da anlamlıya yakın ilişki gösteren SNP'i içerdiği belirlenmiştir. Bu genler arasından tek anlamlı SNP ilişkisi gösteren genin CNTN5 olduğu görülmüştür (P = 4.71E-05 and P = 7.79E-05). Bu çalışmada disiplinler arası bir yaklaşım izleyerek genetik, proteomik ve biyoinformatik analizler ile birçok aday gen belirlemiş bulunmaktayız. Bu genlerin MS'teki rolleri ileriki çalışmalarda araştırılacaktır.

1. INTRODUCTION

1.1 History of Multiple Sclerosis:

Multiple Sclerosis (MS) is an immune-mediated, neuroinflammatory and neurodegenerative disorder affecting the central nervous system (CNS), first identified by Jean-Martin Charcot in 1868. By the late 18th century, physicians divided the condition into different groups like rheumatic disease, constitutional weakness, paraplegia, which were also classified as active/passive, functional/organic or "the pox" based. Although all cases experiencing weakness were started to diagnosed as paraplegia and as paraplexia if paralysis was complete by Robey Dunglison, after a few decades, more specific examination of the brain and spinal cord at autopsy led to separation of a number of disorders that had been previously grouped together. Jean-Martin Charcot and his colleague Edme Vulpian noted a pattern in which young adults having tremor and paralysis had grey plaques scattered throughout the brain, brain stem, and spinal cord. They separated this type of pattern from paralysis agitans described by James Parkinson in 1817, and named the condition as "*sclérose en plaque disseminée*" which we now know as MS. Since then, there have been still efforts to further identify a clear-edged subdivision pattern of MS by clinical symptoms, disease course, prognosis, magnetic resonance imaging (MRI) characteristics, biological markers, and other pathological findings. Thus, it still remains a question whether MS is a single disease or a group of syndromes [1].
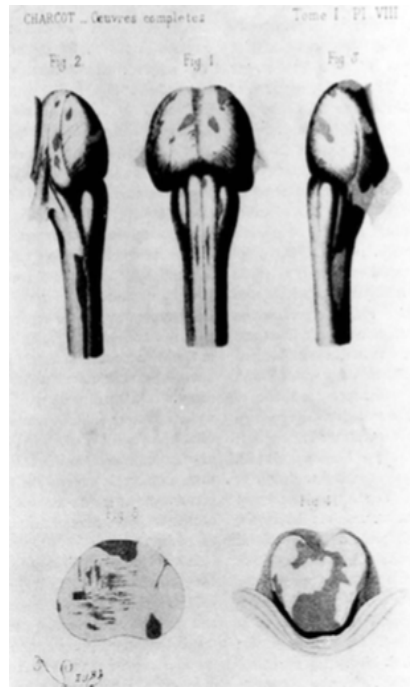
Figure 1.1: Views of the pons showing MS lesions on the exterior and cross sections of the medulla and pons by Charchot (Bournville,1892).

1.2 Clinical Manifestations and Disease Course

MS pathology, in which chronic inflammation of the CNS leads to demyelination and axonal/neuronal damage, results in several neurological manifestations (Table 1.1). Generally, symptoms can reflect MS lesions' location, extension, and severity of tissue damage; with best correlation between spinal cord lesions and progressive disability. However, besides that this correlation is only approximate, there is pathology in both white and grey matter that MRI cannot detect [2,3].

Table 1.1: Typical clinical manifestations of MS [4,5,6]

Visual symptoms	Impaired acuity, impaired colour vision, visual field defect, double vision, oscillopsia, phospenes
Motor system	Weakness, spasticity, dysmetria, tremor
Sensory system	Sensory loss, neuropathic pain, imbalance
Vestibular symptoms	Vertigo, imbalance
Bulbar symptoms	Dysarthria, swallowing dysfunction
Bowel/bladder symptoms	Constipation, urgency, incontinence, hesitancy, frequent urinary tract infections
Sexual dysfunction	Decreased libido, erectile dysfunction, anorgasmia
Cognitive impairment	Poor concentration or attention, slowed thinking, poor memory, impaired executive function

Mood disorders	Depression, anxiety, affective release
Fatigue	Handicap, motor, and systemic fatigue, heat intolerance
Pain	Chronic neuropathic pain, paresthesias, dysesthesias, neuralgic pain, Lhermitte's phenomenon, pseudoradiculopathy, spasticity-based pain, paroxysmal motor phenomena-based pain, bladder spasms, back or joint pain from immobility, compression fractures
Paroxysmal symptoms	Epileptic seizures, paroxysmal dystonia, hemifacial spasms, Lhermitte's phenomenon, Uthoff's phenomenon

Typically, clinical manifestations of MS first develop in young adults between the ages of 20 and 40, as a relapsing-remitting (RR) course in most of the cases, majority of whom later converting into a progressive course. RRMS is defined as serial exacerbations varying in neurological manifestations and severity between patients and each relapse of a patient, with partial or complete recovery. In time, RRMS patients converting into a progressive disease phase, in which there is relapse-free gradual worsening of disability, are diagnosed as secondary-progressive MS (SPMS). When gradual worsening of disease disability starts from the onset, it is called primary-progressive MS (PPMS), which is seen in a minority of the cases. Rarely, patients with gradual worsening at the onset also experience relapses, known as progressive-relapsing MS (PRMS) (Figure 1.2). There are also two definitions describing clinical severity of the disease: benign and malignant forms. Benign MS refers to patients who remain fully functional after 15 years from disease onset, whereas malignant MS patients experience a rapid progressive course with extensive disability or death after a shorter period from the onset [7].
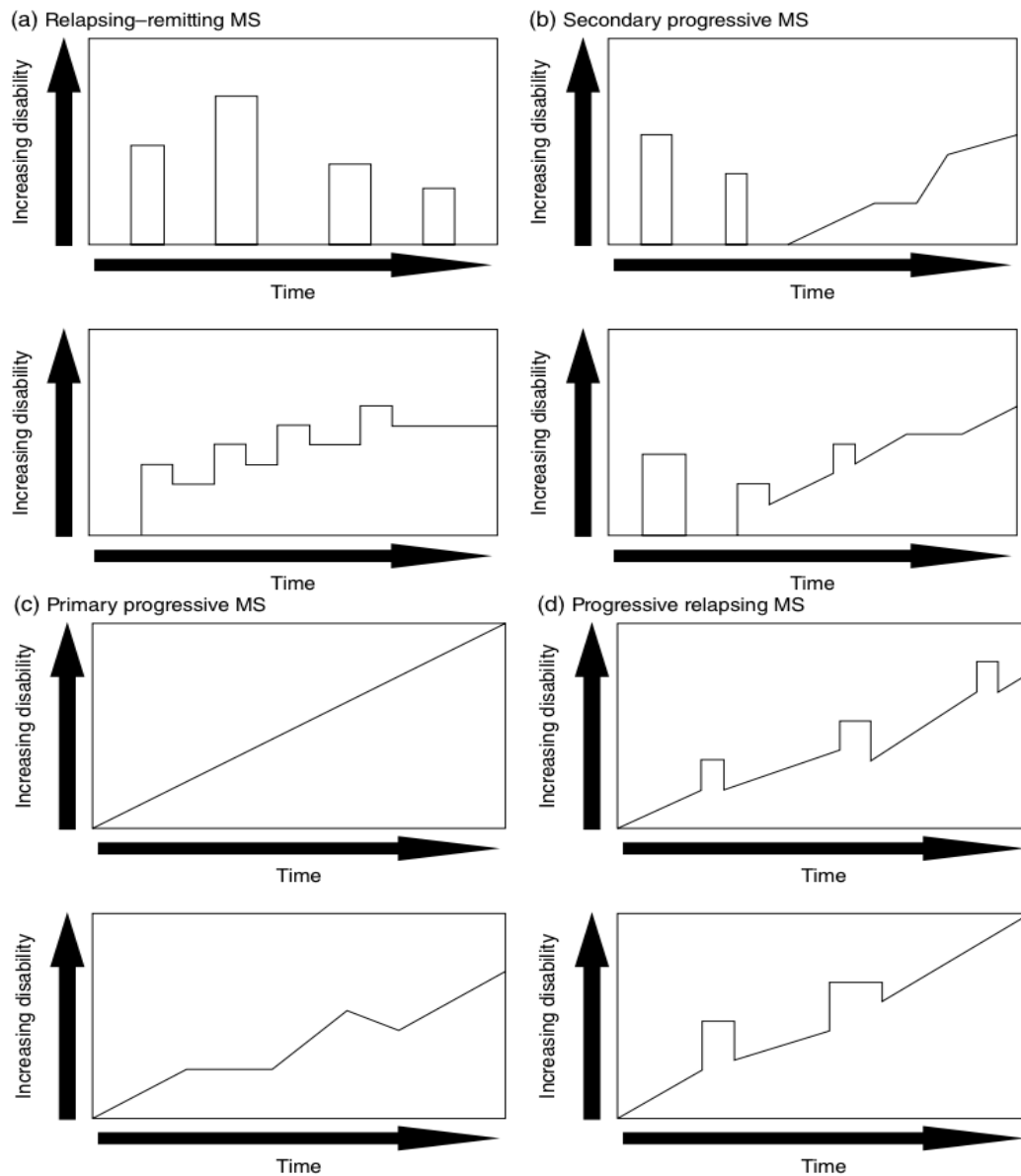
Figure 1.2: Typical disease course of MS in (a) RRMS, (b) SPMS, (c) PPMS, and (d) PRMS [7].

The spectrum of MS also includes presymptomatic phases with unpredictable duration before its initial clinical exhibition, one of which is radiologically isolated syndrome (RIS) characterised by MRI findings suggestive of MS, with no clinical manifestation. This clinically silent phase of the disease converts into symptomatic MS types in about one-third of the patients with RIS in five years [8]. When MRI finding compatible with MS is together with a single attack, the condition has been called clinically isolated syndrome (CIS), which may then convert into RRMS, also termed clinically definite MS (CDMS), or single-attack progressive MS (SAPMS) [9,10]. However, according to the last revised McDonald diagnostic criteria [11], CIS

refers to a single attack with only attack-related lesion on MRI, showing no other lesion activity and rest of the cases are defined as single-attack MS (SAMS). However, these terms are still new and needed to be well defined and sharper edged to allow both clinicians and researchers a mutual understanding. Therefore, study designs and outcomes can be consensus among clinical trials of current and new therapies, assisting clinicians in determining which treatment strategy should be used for each patient.

1.3 Diagnosis, Prognosis, and Treatment Strategies

1.3.1 Diagnosis of multiple sclerosis

Since MS can cause a variety of symptoms referable to different regions of brain and spinal cord, initial differential diagnosis can be challenging. Although diagnostic criteria for MS have still been evolving, current criteria rely on the main principles established in the middle 20th century: (1) dissemination in space (DIS) and time (DIT), implying that more than one CNS region should be affected and there should be more than one time point during the disease course, and (2) exclusion of other possible conditions [12]. The first diagnostic criteria for MS, Schumacher criteria, required two relapses separate in time and space, and exclusion of all other conditions [13]. In 1983, Poser Criteria [14] were proposed, necessitating the use of also paraclinical findings (evoked potentials and CSF analyses) in addition to demonstration of neurologic abnormalities, to support the diagnosis. Currently, abnormal intrathecal immunoglobulin (IgG) synthesis (increased IgG index, synthesis rate, and/or oligoclonal bands (OCB)), and evoked potentials demonstrating subclinical involvement in CNS sensory pathways are still typical examinations in MS diagnosis [15]. Later on, MRI findings demonstrating CNS lesions have begun to be used to meet the need for evidence of DIT and DIS. In 2001 [16], McDonald Criteria were proposed based on this principle, later revised in 2005 [17] and 2010 [11], establishing a diagnostic scheme with clarified definitions and requirements, simplified categories, and effective MRI interpretations. The last revision allows additional incorporation of MRI data in the assessment of DIT and DIS, therefore MRI findings can now be sufficient to make the diagnosis even after one clinical attack in certain conditions (Table 1.2).

Table 1.2: International criteria for MS diagnosis [11, 16, 17].

Clinical presentation	Additional data needed for MS diagnosis
≥2 relapses; objective clinical evidence of ≥2 lesions or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior relapse	None
≥2 relapses; objective clinical evidence of 1 lesion	DIS, demonstrated by MRI or a further clinical relapse
1 relapse; objective clinical evidence of ≥2 lesions	DIT, demonstrated by MRI or a further clinical relapse
1 relapse; objective clinical evidence of 1 lesion (CIS)	DIS, demonstrated by MRI or a second clinical relapse, and DIT, demonstrated by MRI or a second clinical relapse
Insidious neurologic progression suggestive of MS	1 year of disease progression plus two out of three of the following: - Evidence for DIS in the brain, demonstrated by MRI (≥1 T2 lesions) - Evidence for DIS in the spinal cord, demonstrated by MRI (≥2 T2 lesions) - Positive CSF

Although current criteria for MS allow more reliable diagnosis, there are a number of disorders that may mimic MS, mainly disseminated encephalomyelitis (ADEM) and neuromyelitis optica (NMO) [18]. Differentiation between MS related disorders is crucial in management of the conditions, since a treatment strategy of one can cause deterioration of another's course [19]. Therefore, in addition to current knowledge, unique clinical and neurological features, as well as immunological biomarkers should be defined to distinguish MS from MS-related disorders to tailor treatment in each individual patient. Accurate clinical and radiological features and biomarkers are needed also for predicting course of the disease, to assess whether patients will experience disability progression or have a more benign course [20].

1.3.2 Prognosis of multiple sclerosis

Period between experiencing the first symptoms and walking with a cane has been shown to take 15-30 years, reported to be longer in recent years probably due to the positive effects of the disease-modifying therapies (DMT). In case of PPMS, age of onset is 10 years longer than that of RRMS, however progression is more rapid [18].

Predicting disease course for CIS patients whether there will be a second attack, meeting the criteria for clinically definite MS diagnosis, can be challenging. However, there are certain clinical and epidemiologic features that have been used to assess the risk of conversion from CIS to CDMS, of which non-white race, age less than 30 years, involvement of less functional systems at first disease presentation, motor symptoms, smoking, increased EBV antigen titer, and high Expanded Disability Status Scale (EDSS) scores at baseline were shown to affect the conversion risk [20-24]. Moreover, MRI data shows that while conversion risk from CIS to CDMS is around 20% in patients without brain lesion, this risk is much higher (60-80%) in those with asymptomatic brain lesions [25-29]. In addition, in CIS patients with optic neuritis, the risk of developing CDMS in 15 years is 50%, and 72% in case there are one or more lesions on MRI. Conversion from RRMS to SPMS is also difficult to predict and define, since relapses may continue even if progressive phase initiates. In about 60-80% of the cases with relapsing forms, the course becomes chronic and progressive [20, 30]. To improve both diagnosis and prognosis, serum and CSF protein biomarkers have been investigated, even though currently there is no validated biomarker set in use other than CSF IgG OCBs [31]. Current promising candidate biomarkers may help in the determination of the most effective treatment strategy for each individual patient in the future.

1.3.3 Treatment of multiple sclerosis

Although there is no cure for MS, disease-modifying therapies have been developed to alter the disease course since 1993. IFN-β and glatiramer, the first era immunmodulators, were shown to result in about 30% reduction in annual relapse rate in RRMS patients by placebo-controlled and double blind clinical trials [32]. However, long-term follow up of a pivotal IFN- β1a trial showed that a minority of patients receiving the drug worsened, emphasising the heterogeneity between individuals in MS [33]. Consistently, other studies have showed that treatment with all members of IFN-β drug class results in new or active MRI lesions shortly after beginning the treatment in a minority of MS patients, whilst others experience better outcomes [34]. Unfortunately, there is no well-established biomarker to foresee responders and non-responders to these therapies.

Further therapeutic development in MS has been achieved by the approvals of natalizumab in and fingolimod [35-37]. Natalizumab, a monoclonal antibody specific to integrin-α4 in leucocytes, was shown to result in reduced inflammation with more than 65% reduction in relapse rate during 2 years of treatment and at least 90% inhibition of new MRI lesion formation [38, 39]. However, a 1:1000 risk of developing progressive multifocal leucoencephalopathy (PML), a rare and often fatal brain infection, has been reported later on, although the initial safety profiles were favourable [40]. Fingolimod is a prodrug, later converted into sphingosine-1-phosphate (S1P) analogue, which downregulates S1P receptors on leucocytes and endothelium, therefore T lymphocytes are trapped in lymph nodes, which results in reduced inflammation. Fingolimod treatment has resulted in reduced disease and MRI activity, and 55-60% lower relapse rates [35, 37, 41]. However, there are some safety concerns, since fingolimod is not target specific, targeting also CNS astrocytes and oligodendrocytes, arterial and bronchial smooth muscle cells, and atrial myocytes [41]. Later on, other molecules have been developed in which two oral immunomodulators teriflunomid and dimethyl fumarate [42, 43], and a monoclonal antibody alemtuzumab [44] were approved and other agents such as laquinimod, daclizumab, ocrelizumab, and ofatumumab have still been under clinical testing [45-48].

In general, natalizumab seems to be the most effective therapy in MS treatment, followed by fingolimod and dimethyl fumarate, respectively. However, there is heterogeneity in treatment responses of the patients, caused by factors still largely unknown. Although there is no biomarker to predict treatment responses, a careful clinical assessment can be crucial in deciding the initial therapy. For instance, glatiramer acetate has a favourable safety profile on pregnant women, therefore female patients with mild presentation of the disease with no brainstem or spinal cord lesion can be treated with this drug. In addition to difficulties in prediction of therapy responses, the overall effects of current treatments in progressive MS seem to be poor [32]. Therefore, current and forthcoming therapies should be integrated with improving scientific knowledge in order to develop effective personalised treatment strategies for each phenotype of MS.

1.4 Biology of Multiple Sclerosis

1.4.1 Pathology of multiple sclerosis

Multifocal lesions in white matter and grey matter with demyelination, axonal transection, neuronal degeneration, gliosis, and perivenular inflammatory cell infiltrates are pathological characteristics of MS (Figure 1.3) [7].
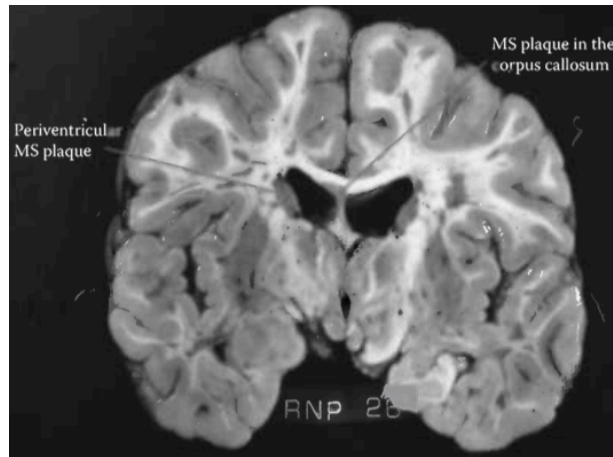
Figure 1.3: MS plaques in a cross-section of the brain (Courtesy of Michele Mass, MD, Portland VA Medical Center and Oregon Health & Sciences University)

Although currently it is largely accepted that inflammation occurs at all stages of the disease, whereas neurodegeneration on an inflammatory background, over the years, numerous number of studies have been reported contradictory findings on whether neurodegeneration or inflammation first occurs at the onset of MS [49-57]. Therefore, there are 2 models suggested for demyelination in MS pathology. Some studies show that myelin sheath is lost due to direct myelin damage (primary demyelination) followed by axonal damage (outside-in model). Alternatively, secondary demyelination occurs by axonal damage and neurodegeneration according to the inside-out model [58]. In either case, demyelination process involves tissue infiltration by T and B cells together with activated microglia, macrophages, and astrocytes eventually giving rise to chronic active lesion formation, followed by repopulation of oligodendrocyte progenitors at the damaged area providing remyelination. Remyelination correlates with elevated oligodendrocytes expressing myelin proteins, which is also supported by microglia and astrocytes [59]. However, remyelination capacity provides only transient recovery, and is shown to be higher in early lesions compared to those in chronic MS [60]. Absence of trophic support by

9

myelin during demyelination process in lesions eventually causes axonal damage and neurodegeneration. Axonal damage is also contributed by some compounds produced by microglia and macrophages [61]. Furthermore, it was shown that axons in normal appearing white matter (NAWM) also undergo neurodegeneration, which may be explained by mechanisms based on the inside-out model [62]. Similarly, both demyelinated regions and normal appearing grey matter (NAGM) face with axonal damage and neurodegeneration [58].

Although CNS inflammation is decreased giving place to more progressive neurodegenerative stage in long-standing disease, new MS lesions can continue to develop in the very late stages of the disease [63, 64]. Studies have suggested that there is a certain sequence of events generating MS lesions, which takes weeks to months to complete and can occur almost everywhere throughout the CNS (Figure 1.4). When there is a tight microglia cluster in an area of normal myelin without any visible abnormalities in the tissue, it is called a 'pre-active lesion'. If myelin begins to degenerate causing an inflammatory response, and macrophages are present among microglia; transition to an 'active lesion' is observed. Hypertrophic astrocytes are also seen in an active lesion, and lymphocytes present in the perivascular space, forming cuffs around the blood vessels. After a while, macrophages complete phagocytosis of myelin in the lesion, which becomes a 'chronic active lesion'. At this stage, macrophages leave the place, astrocyte number increases, and oligodendrocytes are diminished consistent with the myelin repair failure in late stages of MS. At last, an obvious low cellularity in a demyelinated, grey, sunken, sclerotic lesion with a few astrocytes indicates 'chronic inactive lesion', where neurodegeneration aspect takes full swing [58].
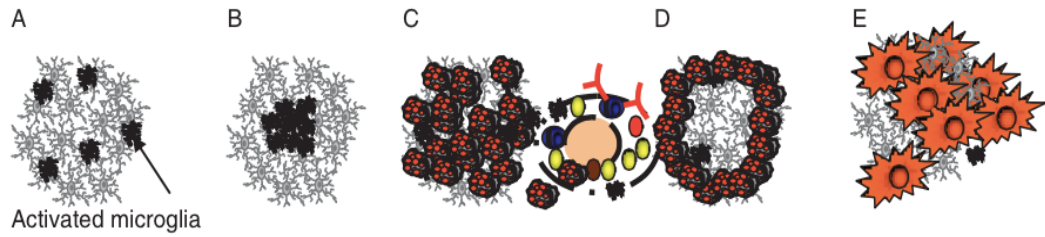
Figure 1.4: White matter lesion progression. NAWM with some activated microglia (A). A pre-active lesion in which microglia form a cluster (B). Macrophage recruitment from the blood, phagocytosis of myelin in the active lesion (C). Myelin is completely absent in the centre of the chronic active lesion (D). Hypertrophic astrocytes in the centre of the chronic inactive lesion – the gliotic scar (E) [58].

Although there was no reported extensive examination of grey matter MS pathology until the 1990s, 4 typical patterns were documented after immunohistochemical staining of myelin had become widely applied (Figure 1.5). A "type I lesion" is a clear roundish lesion including both white and grey matter without a boundary [58]. A "type II lesion" shows an entirely grey matter area of demyelination, whereas "type III lesions" cover demyelinated and non-inflammatory leptomeningeal layers whose distribution is very close to the cerebrospinal fluid (CSF), suggesting that CSF may be related to the this type of pathology. Lastly, a "type IV lesion" covers the entire cortical width, some of which may be developed as a consequence of a type II lesion enlargement first forming a type III, and eventually a type IV lesion [58]. Whilst an active white matter lesion shows it's described properties, demyelinated grey matter parts show no activity in terms of inflammation [65].
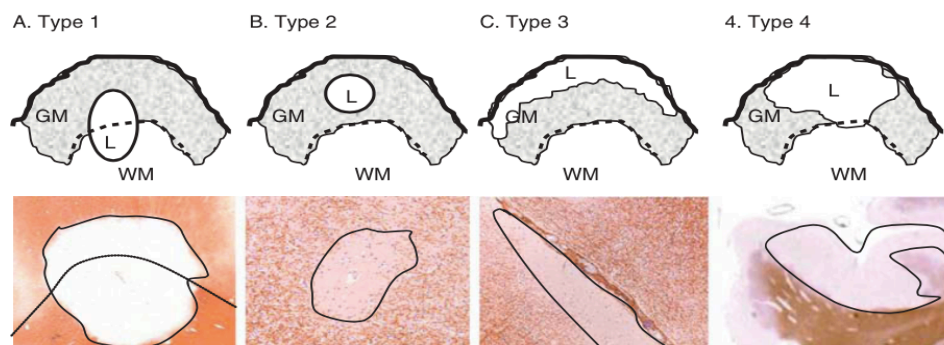
Figure 1.5: Grey matter lesions. GM, grey matter; WM, white matter; L, demyelinated lesions [58].

In addition to the brain lesion, extensive lesions involving both white and grey matter are seen in the spinal cord. Spinal cord lesions show little inflammatory activity and undergo extensive axonal damage leading to loss of up to 70% of existing axons [65].

1.4.2. Pathogenesis and physiopathology

Pathological characteristics of MS have been thought to result from migration of autoreactive lymphocytes across the blood brain barrier (BBB), causing destruction of self-target CNS components, such as myelin proteins [18, 66]. Studies on MS lesions showing heterogenous pathology have suggested that there are different mechanisms operating demyelination, indicating that MS may have more than one etiology, all of which result in the same pathological end points [68].

Genetic association findings revealing HLA class II genes, along with numerous other MS risk alleles in immune related loci [69], support the idea that MS is an autoimmune disease. HLA class II-bearing cells process and present antigens to $CD4^+$ T helper and $CD8^+$ effector T cells, and accordingly, reactive T cells to myelin proteins such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), myelin proteolipid protein (PLP) have been found in peripheral blood of MS patients [18]. However, the actual problem seems that regulatory lymphocytes fail to suppress these effector cells in MS pathogenesis resulting in their activation and proliferation, since autoreactive lymphocytes are also present in healthy individuals [70]. Indeed, it has been found that the difference between MBP-reactive T cells in MS patients and healthy individuals is that those in the patients express IL-2 receptor, a hallmark of activated T cells [71]. Moreover, autoimmunity may not be the primary mechanism, where self-antigen destruction may be the consequence of antigen spreading occurred by liberation of CNS components leading to secondary immune response. Thus, it still remains to be elucidated whether outside-in model, in which the pathologic circumstances begin outside and proceed into the CNS, or inside-out model suggesting abnormalities within the CNS recruit inflammatory cells from the peripheral blood, is the initial pathogenic mechanism promoting the disease. In each case, abnormal entry of leucocytes into the CNS through BBB is seen. Accordingly, altered expression levels of chemokines and respective receptors presumably causing increased leucocyte trafficking into the CNS have been detected

in peripheral blood, CSF, and CNS lesions of MS patients, also emphasising the effects of chemokine-based regulation of the disease pathogenesis [18].

In each possible disease mechanism, T cells play a major role in both mediating and regulating MS pathophysiology. Upon antigen stimulation, myelin reactive naïve CD4$^+$ T helper cells activate, expand, and differentiate into different T helper cell subsets [72]. Among these subsets, IFN-γ secreting pro-inflammatory Th1 cells have previously been thought to be responsible for mediating the disease [73]. Later on, IL-17 producing Th17 cells have also been shown to play a crucial role in EAE development. Moreover, after elevated IL-17 mRNA levels have been reported in CSF, PBMC, and MS lesions, Th17 numbers have also been shown to increase in MS [74, 75]. Relative contributions of Th1 and Th17 polarised cells to MS have still been investigated, yet both types seem to mediate disease pathogenesis. In addition, myelin reactive CD8$^+$ effector T cells have been detected in MS, and higher frequency of CD8$^+$ T cells has been found in MS lesions [76, 77]. These cells have been thought to contribute to demyelination and axonal damage by expressing cytotoxic molecules such as perforin and granzyme B. In MS lesions, granzyme B expressing CD8$^+$ T cells have been found in close proximity to injured axons [78]. As mentioned above, altered frequency [79, 80] and function [81-83] of CD4$^+$ and CD8$^+$ regulatory T cells (Treg), which are responsible for self-specific T cell response suppression and peripheral tolerance maintenance [84, 85], have been reported in MS. Taken all together, deregulation of CD4$^+$ T and CD8$^+$ T cells result in a transition from physiological surveillance to a pathological immune response causing demyelination [86], and relative contribution of these cells may be one of the underlying reasons of disease heterogeneity.

Although pathogenic relevance is unknown, detection of increased IgG and oligoclonal bands in CSF has become a routine in MS diagnosis, indicating the role of B cells in the disease mechanism. Proinflammatory effects of B cells are thought to predominate over their anti-inflammatory effects in MS patients [87]. B cells may affect T cell activation by their antigen presentation function or they may directly contribute to formation of MS lesions. Indeed, autoantibodies without any evidence of high affinity pathogenic antibody have been previously reported in MS [88], and it has been shown that autoantibodies to myelin proteins may be a part of demyelination process [89]. Moreover, treatment with a monoclonal antibody,

rituximab, dramatically reduces inflammation by deleting B cells in RRMS, emphasising the role of B cells at least in relapsing forms of MS [90]. In addition, ectopic B cell follicles in the CNS of MS patients were detected, especially in those having progressive phases [91].

In addition to adaptive immune response, innate immune system has been shown to have roles in MS pathogenesis. In EAE, infiltrating monocytes were shown to trigger severe paralysis [92]. Moreover, conflicting studies revealing both positive and negative effects of natural killer (NK) cells have been reported [93]. Further investigation exploring roles of each immune cell and their interaction with each other and other components leading to MS pathogenesis is needed in order to improve our understanding of complex nature of the disease. In summary, upon migration of activated self-reactive T cells into the CSF and perivascular space, BBB permeability further increases, causing additional access of other inflammatory cells. As a result, severe disruption of BBB, and CNS damage occurs, which in turn result in formation of lesion, where axonal and myelin loss occur by cytotoxic $CD8^+$ T cells and toxic intermediates like nitric oxide and glutamate, causing clinical exacerbation of the disease. After the lesion acute phase is over, remyelination starts with more abundant remyelination in a minority of the patients (approximately 20%) [86]. In each case, demyelination eventually disrupts neuron signaling in affected regions, causing irreversible and permanent neuronal damage and disability as the disease progresses [94].

1.5 Epidemiology of Multiple Sclerosis

MS is the most common non-traumatic cause of the neurological disability in young adults, with age of onset between 20 and 40 years [95]. However, global knowledge on MS epidemiology has been scarce. To fill this gap, a joint study of the Multiple Sclerosis International Federation (MSIF) and the World Health Organisation (WHO) was published in 2008 [96], as the first *Atlas of MS* with an update in 2013 [97]. According to the *Atlas of MS 2013*, the estimated total number of people affected by MS is increased from 2.1 million (30/100.000) in 2008, to 2.3 million (33/100.000) in 2013. The increase in prevalence can be attributed to increased incidence in some countries, as well as improved diagnosis and reporting of MS, and increased survival of both MS patients and general population [98].
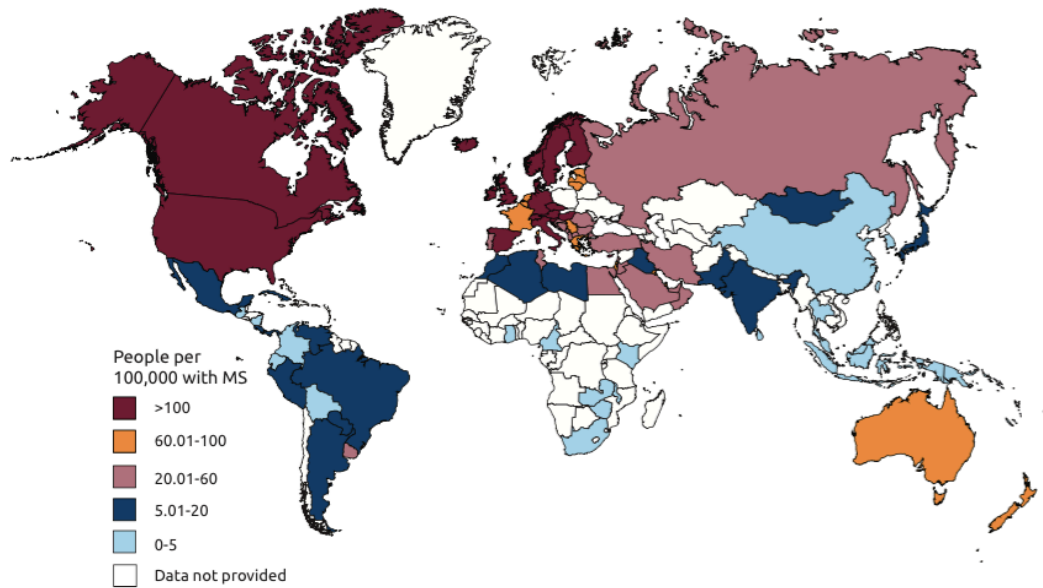
Figure 1.6: Global prevalence of MS in 2013 [97].

As represented in Figure 1.6, prevalence of MS varies around the world, being highest in North America (140/100.000) and Europe (108/100.000), and lowest in Sub-Saharan Africa (2.1/100.000) and East Asia (2.2/100.000). Overall, with notable exceptions, MS exhibits a latitude gradient with high prevalence in northern parts of North America and Europe and in southern parts of Australia and New Zealand, and decreasing prevalence in regions closer to the equator. Moreover, the ratio of women to men with MS varies around the world, being twice as common among women in general, and considerably higher in some regions such as East Asia in which female-to-male ratio is 3 [97]. Epidemiological data on MS for Turkey have been reported for two metropolitan cities; Edirne [99] and Maltepe/Istanbul [100], with 33.9/100.000 prevalence and 2.55 female-to-male ratio and 101.6/100.000 prevalence and 1.56 female-to-male ratio, respectively.

1.6 Genetics and Environment in Multiple Sclerosis

1.6.1 Genetic epidemiology

Early observations from classical genetic studies have provided pivotal data suggesting that MS has a genetic component. Previous heritability estimates for MS range from 25% to 76%, while the largest population-based study recently conducted for MS, reported 64% heritability estimate with a 95% confidence interval (CI) of

0.36-0.76 [101] (Table 1.3). Family studies have shown that having a first-degree relative with MS increases the risk 20 to 40 times, from approximately 1/1000 to 1/25-50 [102-106]. Moreover, in second and third degree relatives of affected individuals were shown to have an increased recurrence risk compared to those who do not have any affected relatives [102, 106, 107]. Also a positive family history was reported for 20% of all patients with European descent [66]. In accordance with these observations, increased concordance rates in monozygotic twins compared to dizygotic twins (Table 1.3), indicate that observed familial aggregation is likely due to shared heritable factors rather than shared environment. In a recent meta-analysis comprising of previous twin studies, reported a 50% estimate of heritability, 21% shared environment component, and 29% unique environment component [108]. Moreover, a population-based adoption study conducted in Canada revealed that the frequency of MS among first-degree non-biological relatives living with the index case was not significantly different from that of general population [109]. All together, observed familial clustering in MS seems to be mainly due to genetic factors.

Table 1.3: Main twin studies conducted for MS

Population	Number of Concordant Monozygotes/Total (%)	Number of Concordant Dizygotes/Total (%)	Heritability (95% confidence intervals)
France [93]	1/7 (5.8%)	1/37 (2.7%)	0.25 (0-0.88)
Italy [110]	8/55 (15%)	6/150 (4%)	0.48 (0.06-0.86)
North America [111]	56/418 (13.4%)	19/380 (5%)	0.31 (0.13-0.49)
Canada [112]	37/146 (25%)	12/224 (5.4%)	0.53 (0.28-0.8)
UK [113]	11/44 (25%)	2.61 (3.3%)	0.66 (0.22-0.94)
Denmark [114]	5/37 (24%)	1/71 (3%)	0.76 (0.33-0.88)
Sweden [101]	12/78 (15.38%)	4/237 (1.69%)	0.64 (0.36-0.76)

Exceptions for the latitude gradient, also observations showing reduced prevalence of MS among African-Americans [115, 116] regardless of geographical location indicate that differences of genetic susceptibility factors for MS across different populations may be the answer of at least a part of the geographical distribution of MS. On the other hand, migration studies have reported that people, who migrate to

an area having different MS prevalence than that of their homeland, tend to adopt the MS risk of their new homeland, unless they migrate after their childhood years [117], which can be explained by environmental influences rather than genetic factors. Collectively, it is clear that there is an active interplay between genetic and environmental factors determining the distribution of MS in different populations, as well as determining development of MS.

1.6.2 Molecular genetics studies

1.6.2.1 Linkage and candidate gene-based analyses

The first direct finding for MS genetics came from linkage analyses conducted in 1972, revealing that major histocompatibility complex (MHC) region on chromosome 6p21 is associated with risk of MS [118, 119]. Later, this association was fine-mapped to specifically HLA-DRB1 locus of the class II human leukocyte antigen (HLA) region, showing the strongest effect for HLA-DRB1*1501 haplotype (heterozygosity conferring an odds ratio (OR) 2.7 and homozygosity of 6.7) [120]. Besides this, there are a number of HLA-DRB1 haplotypes both positively and negatively associated with risk of MS, whose relative frequencies varying between populations play important role in determining the susceptibility to MS (Figure 1.7) [121-125]. In addition to HLA-DRB1 alleles, long-suspected protective effect of class-I HLA loci was confirmed, revealing that this effect is mainly driven by HLA-A*02:01 allele [122, 126]. However, even the largest study included 730 multiplex families with 2692 individuals, subsequent linkage analyses have failed to reveal additional MS-associated non-HLA regions [127]. Moreover, a well-powered non-parametric linkage analysis performed by International Multiple Sclerosis Genetics Consortium (IMSGC) did not reveal any other associated loci outside the MHC region [128]. Alternative to and together with linkage analyses, a numerous number of candidate-gene based studies have been performed. However, the only non-HLA association was identified between the SNP rs6897932 in interleukin 7 receptor alpha (IL7RA) gene and MS by a study comprising of large case and control numbers [129].
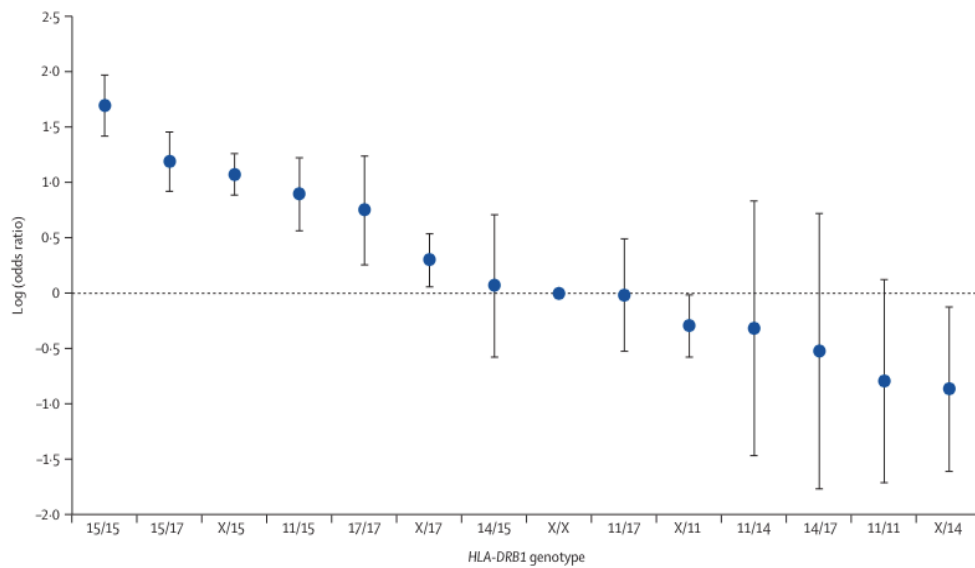
Figure 1.7: Log-transformed odds ratios for multiple sclerosis for combinations of HLA-DRB1 alleles. X, any non-disease associated allele [130],

Pioneer family based linkage analyses and candidate-gene based studies have left a large gap in explaining the genetic basis of MS, since HLA region together with IL7R association accounts for only a small part of the underlying genetic predisposition to MS [120].

1.6.2.2 Genome-wide association studies

After HapMap project had identified 'tag' SNPs within haplotype blocks that identify corresponding haplotypes, effective and cost-efficient Chip-based genome wide association studies (GWAS) have been performed, identifying common variations associated with complex disorders [131]. In an afford to fill the gap in MS genetics, IMSGC conducted the first GWAS using trios from UK and USA in 2007, revealing two risk loci: IL7RA and novel interleukin 2 receptor alpha (IL2RA) [132]. After that, growing number of replicable single nucleotide polymorphisms (SNPs) with minor allele frequencies (MAF > 0.5%) exerting small to moderate risk effects have been revealed by high-throughput genotyping technologies. To date, 14 GWAS have been completed (Table 1.4), revealing 110 non-HLA risk variants in 103 loci, also confirming the major role of HLA-DRB*1501 (Figure 1.8) [133].

Table 3.4: Genome-wide association studies conducted for MS

Population	Case/Control Number	SNP Number	Novel Associations
UK & USA [121]	931/Parents	334.923	➢ 29 loci ➢ Immunologically relevant genes – mostly T helper cell differentiation
UK [134]	975/1466	12.374	➢ 2 loci (ARTS1, IL23R)
Spain [135]	242/242	428.867	➢ No novel locus
Netherlands [136]	45/195	250.000	➢ 1 locus (KIF1B)
Mixed [137]	978/883	551.642	➢ No novel locus
USA [138]	860/1720	709.690	➢ 3 loci (CD6, IRF8, TNFRS1A)
Australia & New Zealand [139]	1618/3413	302.098	➢ 2 loci associated with other autoimmune diseases (SNPs on 12q13-14 and 20q13)
Sardinia/Italy [140]	882/872	555.335	➢ 1 locus (CBLB)
Germany [141]	590/825	300.000	➢ 2 loci (VAV2, ZNF433)
Finland [142]	68/136	297.343	➢ 1 locus (STAT3)
Mixed [132]	9772/17.376	474.806	➢ 2 loci (IL2RA, IL7RA)
Mixed [143]	1453/2176	906.600	➢ 3 loci (3p24.1, 9p24.1, 2p21)
Spain [144]	296/801	130.903	➢ No novel locus
Italy [145]	197/234	277.866	➢ No novel locus

Among these studies, those including fewer than 800 cases could not identify novel and replicable variations associated with MS, whereas each of the other studies added new loci to the growing list. The largest GWAS conducted by IMSGC and Welcome Trust Case Control Consortium (WTCCC2) identified 34 novel associations, and confirmed 23 previously associated variants. Most of these SNPs were mapped to close proximity to genes that are significantly over-expressed in immune-related pathways, such as T-cell activation and lymphocyte proliferation, emphasising the immune basis of MS. Moreover, more than a third of these SNPs have previously been implicated in other autoimmune diseases [134].
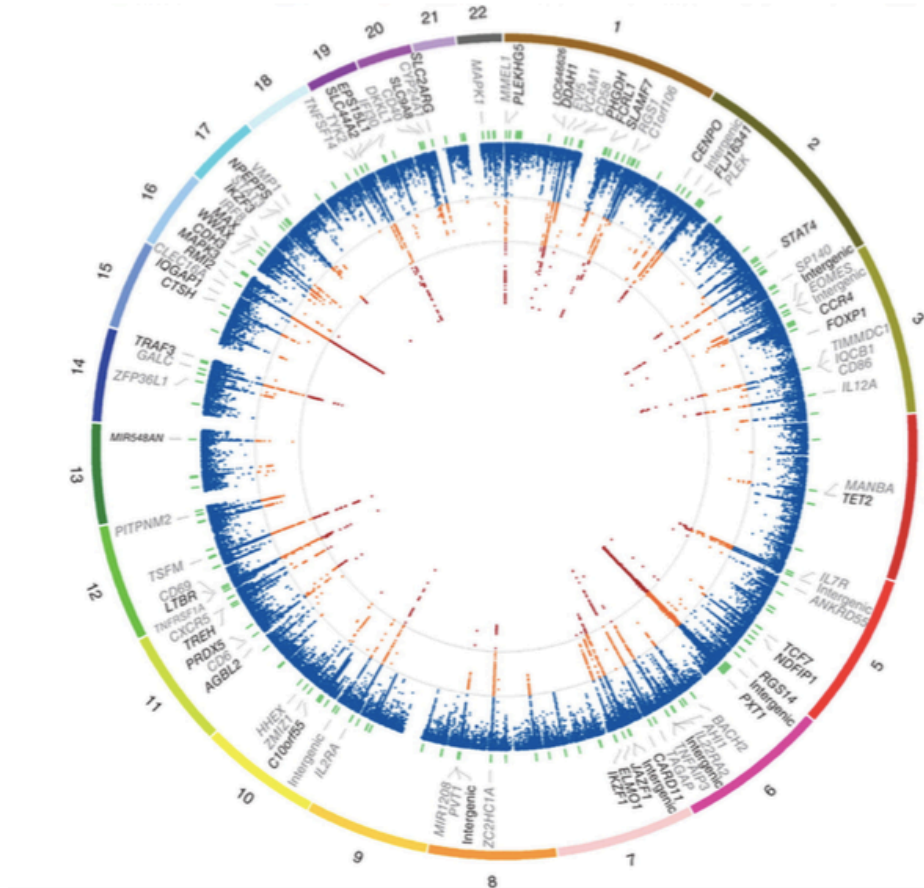
Figure 1.8: 2014 genetic atlas of MS. The most outer track indicates autosomal chromosomes, the second track shows the gene closest to the most associated SNP, the third track indicates the physical position of the 184 fine-mapping intervals, and the most inner track shows –log(p) for each SNP [69].

Among the 110 non-HLA associations identified in total, 15 of them are coding-variants, and 35 of them are in tight linkage disequilibrium with coding variants [146]. However, according to the PolyPhen and SIFT, prediction software programmes, only 7 of the 14 missense variations are possibly harmful [147, 148]. On the other hand, almost all of the 110 SNPs are suggestive of regulatory functions of corresponding regions, since HaploReg v2 tool showed that the SNPs coincide with chromatin features. 109 of 110 SNPs change at least one regulatory sequence motif or are in linkage disequilibrium with other variants that change these motifs [149]. In addition, cell-type-specific maps of active promoter regions for the SNPs significantly overlap in immune cells, but not any other cell types, adding further support to the immune based aetiology of MS [150]. However, the functional relevance of these variations is still largely unknown. Among the ones have been investigated, rs6897932 in IL7R, rs2104286 in IL2R, and rs1800693 in TNFRSF1A

genes were shown to increase the concentration of soluble form of the corresponding protein receptor, inhibiting signalling [132, 151, 152]. Also rs6677309 in CD58 was shown to cause reduced expression of CD58, thus resulting in regulatory T cell dysfunction [153]. In addition, rs34536443 in TYK2 results in reduced TYK2 activity and increased cytokine secretion from Th2 cells [154]. Further functional knowledge of these variants can provide crucial inside into aetiology of MS.

Although there are a substantial number of identified associations, all of the non-HLA associations are common variants with low-moderate risk, and together with HLA associations, they explain only about 27% of the predicted MS heritability [133]. Remaining "missing heritability" may be due to currently undetectable rarer variants (MAF<0.5%) with larger effects, common variants (MAF>5%) with smaller effects (1<OR<1.1), or unknown gene-gene interactions between known variants. Many association studies have been performed in order to reveal rare variants, which are population specific because of founder effects, resulting in inconsistent association findings across different populations [155]. Recently, exome sequencing studies in multiplex MS families have revealed several rare functional variants on CYP27B1 gene, which encodes for the enzyme that converts 25-hydroxyvitamin D3 into the active hormone 1,25 dihydroxyvitamin D3 [156] (Figure 1.9). Although this finding has not been replicated in any other cohorts yet, it supports the growing evidence for the role of vitamin D deficiency in MS susceptibility [157]. In addition, a rare variant in TYK2 was associated with the risk, but the finding needs to be confirmed [158]. However, resequencing of a number of candidate genes in more than 3000 MS patients suggested that rare variants with large effects are not common in autoimmune diseases, thus are unlikely to a large part of MS heritability [159]. Larger studies using high-throughput sequencing technologies or meta-analyses of existing data improving statistical power of current findings might shed light on remaining missing heritability of MS.

1.6.3 Environment and epigenetics

Genetic epidemiological studies indicating worldwide increase in MS incidence and female to male ratio, and migration studies showing adoption of the risk of the new region have led researchers to focus their interest on environmental factors that may influence MS susceptibility. Accumulating evidence supports several candidates,

most notably sunlight/vitamin D exposure and Ebstein-Barr virus (EBV). It has been shown that MS prevalence increases with increased latitude, which is correlated with sunlight exposure and vitamin D concentrations [160, 161], and the risk decreases with migration from high to low latitudes [162]. Among a body of evidence indicating association between exposure to sunlight and vitamin D with MS, the most direct evidence has come from a nested prospective case-control study, which included over 7 million individuals from US military and evaluated serum concentrations of 25-hydroxyvitamin D, a molecule enzymatically formed from inactive vitamin D (Figure 1.9). The study revealed that individuals with less than 63·3 nmol/L 25-hydroxyvitamin D (bottom quintile) had a 62% higher odd of MS than those in the top quintile (>99·2 nmol/L), indicating that serum 25hydroxyvitamin D concentration is a significant predictor of developing MS [157]. Accordingly, in a proteomic study of our group showed that vitamin digestion and absorption pathway is significantly affected in progressive MS (P = 1.73E-05), and VDBP (Vitamin D Binding Protein) level is decreased in CSF of CIS patients ($P <$ 0.001), indicating lower vitamin D metabolism in CIS group [163].
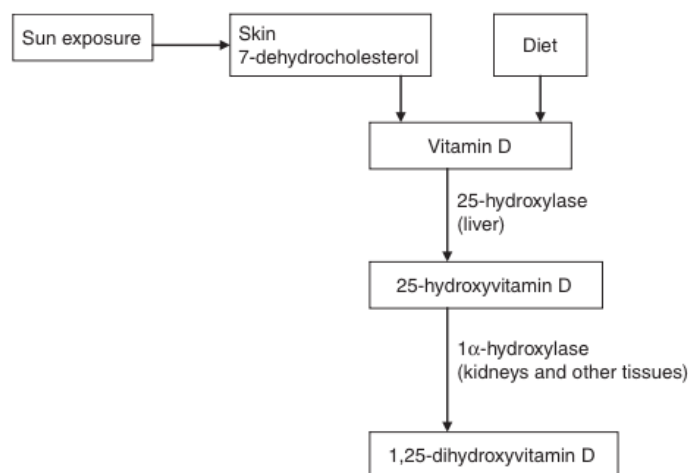
Figure 1.9: Origin and metabolism of vitamin D [164].

MS risk among individuals infected with EBV during their childhood is about 10 times higher and individuals infected later in life is 2 to 3 times higher than that of non-infected individuals. In addition, among non-infected individuals, eventual infection with EBV causes a sharp increase in risk in about 5.6 years [165]. Along with this, higher serum antibody titers to EBV nuclear antigen 1 (EBNA-1) antigen have been reported in MS [166]. However, EBV infection is also common in healthy

population (> 90%), and whether EBV is a causal factor or MS predisposes to EBV infection is still not clear [167]. In addition to these, several other factors have been suggested in MS development, such as tobacco smoking. A recent meta-analysis has reported a pooled OR of 1.52 for ever versus never smoking in MS development [168]. Also, a dose dependent association to MS risk has been reported in earlier studies [169]. Moreover, other factors including infectious mononucleosis, levels of dietary fats and antioxidants, sex hormones, higher education level, hepatitis B vaccine, psychological stress may influence MS risk. However, further research is needed to establish the whole causative environmental mechanisms with accurate risk ratios and respective genetic interactions.

Together and compatible with the known environmental factors, altered methylation, histone acetylation, and other epigenetic statuses on several genes have been implicated in peripheral blood mononuclear cell (PBMC) or brain samples of MS patients compared to those of healthy individuals and in animal model of MS; experimental autoimmune encephalomyelitis (EAE) (Table 1.5).

Table 1.5: Epigenetic mechanisms implicated in MS pathogenesis

Factor	Epigenetic Alteration
MS Patients	
SHP-1	Increased methylation - Increased proinflammatory gene expression [170, 171]
HERV-W	Decreased methylation - Increased inflammation and reduced myelination capacity [172, 173]
PAD2	Decreased methylation - Increased myelin citrullination, myelin breakdown, and anti-myelin immunity [174]
PAD4	Decreased histone citrullination - Reduced myelin production [175]
miR-17, miR-20a	Decreased expression - Th1 and Th17 differentiation [176]
miR-155, miR-326, miR-34a	Increased expression - Decreased CD47 and increased macrophag [177]
miR-155, miR-338	Increased expression - Reduced neurosteroid production [178]
EAE studies	
T cell factor 1	Increased acetylation/ methylation - Th17 differentiation [179]
IFN-c/IL- 17A	Decreased methylation - Increased IFN-c and IL-17 production [180]
IFN-c/IL- 17A, IL-4/ Foxp3	Increased methylation, demethylation - Increased Th1 and decreased Th2 response [180]
IL-17A	Increased methylation - Decreased Th17 differentiation [181]
IRF1	Decreased acetylation - Increased Th17 differentiation [182]
Foxp3	Increased methylation - Decreased regulatory T cell activity [183]
miR-155	Increased expression - Increased Th17 differentiation [184]
miR-326	Increased expression - Increased Th17 differentiation [185]

In addition to the possible causal effects of epigenetic changes by mediating the influences of environmental factors, they also may at least partly explain the wide spectrum of clinical phenotypes of MS. All together, MS is a multifactorial disease caused by complex interactions of genetic, epigenetic, and environmental susceptibility factors, which have not been fully elucidated yet, leaving a gap in understanding how the genetic determinants of the disease result in the disease phenotype.

1.7 Biomarkers in Multiple Sclerosis

There is a lack of prognostic markers to help predicting disease outcome in terms of risks and time periods of the conversions as well as treatment responses, which are highly variable between individuals with MS. Currently, there is no validated biological marker either for diagnosis or prognosis of MS, even though many potential candidate biomarkers have been identified, including GFAP, MOG, tau, and NFL proteins [186, 187, 188]. In this context, a recent study of Avsar et al. performed proteomic analyses in CSF of a prospective cohort comprising of 179 patients, and 42 non-MS controls, revealing that a total of 151 proteins were differentially expressed in MS patients compared to controls, or in different clinical MS subtypes. In addition to several candidate biomarkers to be analysed in further studies, KEGG pathway analysis of the resulted proteins revealed that renin-angiotensin system (RAS) and complement and coagulation (CCC) pathway are affected in all disease subtypes, whilst aldosterone-regulated sodium reabsorption, renin-angiotensin, vitamin digestion and absorption, and notch signaling pathways are affected in a subtype specific manner [163]. Besides improving diagnostic and prognostic process in MS, identification of biomarker sets can also give information on both molecular mechanisms responsible for the notable phenotypic heterogeneity in MS and underlying disease mechanisms.

1.8 Candidate Genes Selected in the Study

IFNAR1 and IFNAR2 genes encode for type I membrane proteins each of which forms one of the two chains of interferons receptor alpha and beta. Both genes are known to involve in inflammation-related pathways including T cell activation,

cytokine-mediated signaling, response to interferon-alpha, and type I interferon signaling pathway, and highly expressed in leucocytes. IFNAR1 and IFNAR2 have been implicated in a number of inflammatory conditions, such as measles, hepatitis B and C infection, mumps, cerebritis, cerebral malaria, and multiple sclerosis. In a study of Levya et al., IFNAR1 18417 and IFNAR2 11876 polymorphisms were found to be associated with risk of MS in a cohort of 147 MS patients and 210 controls (p=0.001 and p=0.035, respectively) [189].

MAB21L1 encodes for a protein similar to a cell-fate determining protein expressed by MAB-21 in *Caenorhabditis elegans (C.elegans)*. The gene is highly expressed in the cerebellum. In 5' UTR region of MAB21L, there is trinucleotide CAG repeats with a normal range of 9-29, and expansion of this repeat was previously suggested to have a role in some neuropsychiatric and neurodegenerative conditions; including mental retardation, attention deficit/hyperactivity disorder, Huntington's disease, and Machado-Joseph disease [190, 191].

1.9 Aim of the Study

The aim of this study is to investigate genetic basis of MS and search for correlation between the results of previous proteomic study of our group and the genetic analyses involving the same MS cohort. In an effort to do these, after SNP genotyping on the Illumina CytoSNP 300K array in 35 individuals from 10 multiplex MS families across the genome, a non-parametric linkage (NPL) analysis was performed. Subsequent fine-mapping of two regions showing NPL scores higher than 1.7 revealed the most promising loci for linkage, from which Interferon (Alpha, Beta, and Omega) Receptor 1 (IFNAR1), Interferon (Alpha, Beta, and Omega) Receptor 2 IFNAR2, and Mab-21-Like 1 (MAB21L1) were selected as candidate genes for further analyses. Case-control studies was conducted including 27 unrelated patients with MS and 10 healthy controls of Turkish origin, and each individual was genotyped for CAG repeat number in MAB21L1, IFNAR1 18417, and IFNAR2 11876 variations. Then, a GWAS was conducted using 11 unrelated MS patients from the families and 60 healthy controls, revealing 14 SNPs with significant association ($P<10^{-4}$), additionally 106 SNPs showing suggestive association with MS ($P<10^{-3}$). Resulting chromosomal regions suggestive of linkage and significant/suggestive SNPs from the GWAS were analysed in order to observe

possible correlations with the proteome data. One gene with significant (INS-IGF2) and eight genes with suggestive association (PRKCE, MAPK9, RBPJL, ADAMTSL1, NR6A1, NOTCH2, IL1R1, NTN1) that found to involve in pathways those shown to be affected in MS subtypes, and three common genes between GWAS and linkage results (CLDN4, RUNX1, LINC00598) were detected. Additionally, individual proteome data of each patient involved in the genetic analyses was observed, and proteins having altered expression level in one or more patients, whose corresponding genes that showed significant or suggestive association in the GWAS were noted; including neuronal cell adhesion, titin-cap, and contactin. As the second objective of this study, we developed a database comprising of allelic variations previously associated with 17 common diseases in Turkish population, including MS, to provide population specific gathered information with pathway enrichment, meta-analysis, and other quick calculation tools.

2. MATERIALS and METHODS

2.1 Materials

2.1.1 Equipment

Equipment list is given in Appendix A.

2.1.2 Chemicals and buffers

Chemicals and buffers used in this study are shown in Appendix B.

2.2 Methods

2.2.1 Sample collection and preparation

28 families comprising of 42 MS patients and 37 healthy individuals were included in the study. All patients were diagnosed in Cerrahpaşa Faculty of Medicine Department of Neurology. Peripheral blood of each individual in the study groups was collected into 10-milliliter (ml) EDTA tubes and stored in -80°C. Each individual of the study group filled an informed consent form, given in Appendix C, prior to the sample collection, and ethics committee approval was taken. DNA isolation from each blood sample was performed using Roche DNA Isolation Kit for Mammalian Blood:

 - ➢ All solutions and blood samples were warmed to 15-25°C.
 - ➢ For 1 ml of blood, 3 ml of Red Blood Cell Lysis Buffer was added into a sterile 50 ml centrifuge tube and mixed.
 - ➢ Tubes were inverted for 10 minutes, and centrifuged at 875 x g for 10 minutes.

➢ Supernatants were discarded, and white cell containing pellets were thoroughly resuspended in residual supernatant at the bottom of the tube by vortexing.

➢ Last three steps were repeated to obtain completely red blood cell-free pellets.

➢ 0.5 ml of White Cell Lysis Buffer was added onto each pellet and tubes were vortexed thoroughly.

➢ Tubes were incubated at 37°C and vortexed in every 10 minutes until the solutions contained no undissolved particles.

➢ 260 µl of Protein Precipitation Solution was added into each tube and tubes were vortexed for 25 seconds.

➢ Solutions were transferred into sterile 2 ml ependorf tubes.

➢ Tubes were centrifuged at 12.000 x g for 10 minutes.

➢ DNA containing supernatants were poured into 50 ml centrifuge tubes containing 5 ml of absolute ethanol.

➢ DNA samples were collected and transferred into 1.5 ml ependorf tubes containing 200 ml of 70% ethanol.

➢ Tubes were centrifuged at 875 x g for 5 minutes, and supernatants were discarded.

➢ DNA pellets were allowed to air dry, and resuspended in 100 µl of TE Buffer by incubating at 65°C for 10 minutes and vortexing in every 2-3 minutes.

➢ Concentration and purity of isolated DNA samples were measured by Nanodrop at 260/280 nanometers (nm), and samples were stored in 4°C until use.

2.2.2 Linkage analysis and candidate gene selection

From 28 MS pedigrees, 10 families including one or more affected individuals and at least one first-degree relative were selected. DNA of 18 patients and 17 healthy relatives from the families was quantified by optical density, and SNP genotyping on the Illumina CytoSNP 300K array was performed for genomes of 35 individuals from the included families according to the manufacturer's protocol. A total of 300.000 SNP markers were sequenced in each individual, and SNPs that are on

chromosome Y, with call rates lower than 95% and minor allele frequency (MAF) lower than 0.01, and in strong linkage disequilibrium (r2>0.5) were excluded from the study. Multipoint non-parametric linkage analysis was performed, as implemented in SimWalk [192]. Uninformative SNPs were excluded yielding 245.008 informative SNPs, and Mendelian error rate of less than 1% was accepted. NPL results were calculated for each of 3118 informative SNP markers spaced at an average of 1 cM intervals, and expressed as NPL Z scores. Fine mapping of regions showing NPL scores higher than 1.7 was performed for each of 639 and 831 informative SNP markers spaced at every 0.2 cM for chromosome 13 and 21, respectively. NPL scores for each region were analysed, and chromosomal positions suggestive of linkage were determined. Analyses were performed using easyLINKAGE software v5.08 [193]. From the resulting loci, protein coding genes were listed and further observation was done based upon a literature review, by determining the relation of each gene to MS and other inflammatory/neurological conditions using MalaCards Human Disease Database, and unrelated genes were excluded. Among the selected genes, determination of possibe functional relevance with MS was done by considering biological proccesses and pathways in which each corresponding protein encoded by each gene involves (GeneCards The Human Gene Compendium, KEGG Pathway Database). As a result of the analyses, IFNAR1 18417 G/C and IFNAR2 11876 T/G polymorphisms, and MAB21L1 CAG repeat numbers were chosen for further investigation.

2.2.3 Analysis of IFNAR1, IFNAR2, and MAB21L1 variations

PCR reactions were carried out in a final volume of 50 µl, in a mixture of 5 µl 10X DreamTag Green Buffer and 0.5 µl DreamTag Polymerase (Thermo Scientific), 2 µl of each 10mM primer, 3 µl of 50 ng template DNA, 2 µl of 2 mM $MgCl_2$, 1.5 µl of 10 mM dNTP, and 34 µl of nuclease-free water. Primers designed for amplification of CAG repeat region in MAB21L1, IFNAR1 18417, and IFNAR2 11876 polymorphisms are given in Table 2.1.

Table 2.1: Primers for amplified regions and product size

Amplified Region	Primers	Product Size
IFNAR1 18417	Forward 5'-GCTCAGATTGGTCCTCCAGA-3' Reverse 5'-TTCCATGACGTAAGTAGTGCTG-3'	358
IFNAR2 11876	Forward 5'-GAGACCAGGCTCACTTGAATAA -3' Reverse 5'-CAGGGTGGTACTGGGTCCT-3'	360
MAB21L1 CAG repeat	Forward 5'-GCGGTTCTCTCACACAAGGA-3' Reverse 5'-GCCGCAACACTCAGAAATGG-3'	229

Amplification of the regions was performed for 27 unrelated MS patients' and 10 healthy individuals' genomic DNA. PCR conditions are given in Table 2.2.

Table 2.2: PCR conditions

Step	Temperature	Time	Cycle number
Initial denaturation	95°C	2 min	1
Denaturation	95°C	30 sec	35
Annealing	60°C (IFNAR1) 59.2^{o}C (IFNAR2) 62°C (MAB21L1)	30 sec	35
Extension	72°C	1 min	35
Final extension	72°C	7 min	1
Hold	4°C		

PCR products of IFNAR1 and IFNAR2 were loaded on a 1% agarose gel and run at 100V for 30 minutes. MAB21L1 products were loaded on a 4% agarose gel and run at 80V for 2 hours. Bands were identified under UV light and cut using a razor blade, placing into 1.5 ml ependorf tubes. PCR products were isolated from the gel pieces using High Pure PCR Product Purification Kit (Roche):

➢ Gel masses were determined by pre-weighting empty tubes, and then re-weighting with gel pieces in them.

➢ 300 µl of Binding Buffer was added for every 100 mg agarose gel slice into the tubes.

➢ Gels were completely dissolved to release the DNAs by incubating the suspensions at 56°C for 10 minutes and vortexing in every 2-3 minutes.

➢ 150 µl of isopropanol for every 100 mg agarose gel slice was added into the tubes and vortexed thoroughly.

➢ Entire contents of the tubes were added into High Pure Filter Tubes inserted into Collection Tubes.

➢ Tubes were centrifuged for 1 minute at maximum speed and flowthrough solutions were discarded.

➢ Filter Tubes were reconnected with the Collection Tubes and 500 µl of Wash Buffer was added to the upper reservoirs.

➢ Tubes were centrifuged for 1 minute at maximum speed and flowthrough solutions were discarded.

➢ Filter Tubes were reconnected with the Collection Tubes and 200 µl of Wash Buffer was added to the upper reservoirs.

➢ Filter tubes were recombined with clean 1.5 ml ependorf tubes.

➢ 50 µl of Elution Solution was added into upper reservoir of the each Filter Tube and tubes were centrifuged for 1 minute at maximum speed.Ependorf tubes containing the purified DNA samples were stored at $-20^{o}C$ until further use.

Purified PCR products were sequenced by Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit by MedSanTek. Sequences of corresponding regions were analysed using Geneious software programme to observe IFNAR1 18417 G/C and IFNAR2 11876 T/G polymorphisms, and MAB21L1 CAG repeat numbers in patients and controls. Genotypic and allelic frequencies of IFNAR1 18417 G/C and IFNAR2 11876 T/G and CAG repeat numbers were calculated, and those for patients were compared to both healthy controls included in the study and historic controls. Comparisons were done by Student's t test using GraphPad Prism 6 Software, and $P<0.05$ was considered significant.

2.2.4 Genome-wide association study

11 unrelated MS patients from the families, and 60 unrelated healthy individuals previously genotyped on the Illumina CytoSNP 300K array were included in the GWAS. SNPs that are on chromosome Y, with call rates lower than 95% and minor allele frequency (MAF) lower than 0.01, and in strong linkage disequilibrium ($r^2>0.5$) were excluded from the study. Individuals showing identity by descent (IBD) rate higher than 0.25 F (inbreeding coefficient) were excluded from the study. An additive model of genetic inheritance was assumed and correlation/trend test was applied. Bonferroni adjustment and false discovery rate (FDR) were performed for

multiple correction test. Illumina GenomeStudio v2011.1 Software was used to determine SNP associations based on allelic differences between patients and controls, and $P<5x10^{-4}$ was considered significant. The most significantly associated markers were determined.

2.2.5 Correlation between genetic and proteomic analyses

In order to observe whether there is a correlation between the linkage and the GWAS results with previous proteomic data of our group [163]; protein coding genes located on the suggestive linkage regions, genes possessing the SNPs with significant and suggestive associations in the GWAS, and genes encode for the proteins that found to be differentially expressed in MS subtypes were listed, and common genes were noted. Since CSF proteomic data of MS patients that involved in this study was available, each protein having altered expression level in each patient was individually compared with the linkage and the GWAS results, and overlapping genes were listed. Additionally, pathways in which proteins encoded by each gene revealed in the genetic analyses involve were noted (KEGG Pathway Database, GeneCards The Human Gene Compendium), and genes encoding for proteins that involve in the affected pathways implicated in the proteomic analyses were determined. The best-correlated genes were determined for further analyses.

2.2.6 Database construction

PubMed was searched for common disease gene association studies performed in Turkish population to develop the TUSNP, which comprises specific allelic variations that were previously associated with 17 common diseases in Turkish population. The list of diseases included in TUSNP is given in Table 3.1.

Table 2.3: Common diseases included in the study

Alzheimer's Disease	Macular degeneration
Amyotrophic lateral sclerosis	Multiple sclerosis
Atrial fibrillation	Myocardial infarction
Behçet's Syndrome	Osteoarthritis
Breast cancer	Psoriasis
Colorectal cancer	Rheumatoid arthritis
Exfoliation glaucoma	Systemic lupus erythematosus
Hypertension	Vitiligo
Lung cancer	

The data were collected from the studies published in Science Citation Index journals. Among the studies, only the ones showing significant risk or protective allele frequency differences between case and control groups were included, except for Behçet's Syndrome. *P* values were calculated using Chi-square and Fischer's exact test, and *P<0.05* was considered significant. Odd ratio (OR) was used to assess strengths of the relationships. In addition; pathway enrichment, meta-analysis, and other quick calculation tools were included in the TUSNP.

3. RESULTS

3.1 Linkage Analysis and Candidate Genes

SimWalk multipoint NPL analysis in 10 MS pedigrees excluded 324 uninformative SNP markers generating a genome-wide linkage analysis scanned for a total of 3118 informative SNPs. The analysis showed two chromosomal regions with NPL Z scores higher than 1.7 on chromosome 13 at 37.9 cM (nearest marker rs612701, NPL Z = 1.72, p = 0.019) and chromosome 21 at 41.82 cM (nearest marker rs2834861, NPL Z = 1.7, p = 0.019) (Figure 3.1). Subsequent fine mapping of the regions comprising the stated highest NPL scores (33.4 cM - 41.5 for chromosome 13 cM and 36 cM - 52.5 cM for chromosome 21) with 639 and 831 SNPs respectively, revealed that the highest linkage peaks were on 34.11 cM (nearest marker rs1461965, NPL Z = 1.82, p = 0.015) on chromosome 13 and 45.08 cM (nearest marker rs11701543, NPL Z = 1.85, p = 0.014) on chromosome 21 (Figure 3.2 and Figure 3.3), revealing suggestive evidence of linkage for chromosomal regions of 13q13.3 and 21q22.2 to MS.

Marker	CHR	cM	pNPLp	pNPLa	NPLp	NPLa	npLODp	npLODa
1. rs612701	13	37.90	0.0190	0.0190	1.7223	1.7223	0.6441	0.6441
2. rs2834861	21	41.82	0.0198	0.0198	1.7040	1.7040	0.6305	0.6305
3. rs7354757	21	42.83	0.0207	0.0207	1.6841	1.6841	0.6159	0.6159
4. rs2485773	13	39.17	0.0221	0.0221	1.6553	1.6553	0.5950	0.5950
5. rs9979015	21	40.80	0.0228	0.0228	1.6427	1.6427	0.5860	0.5860
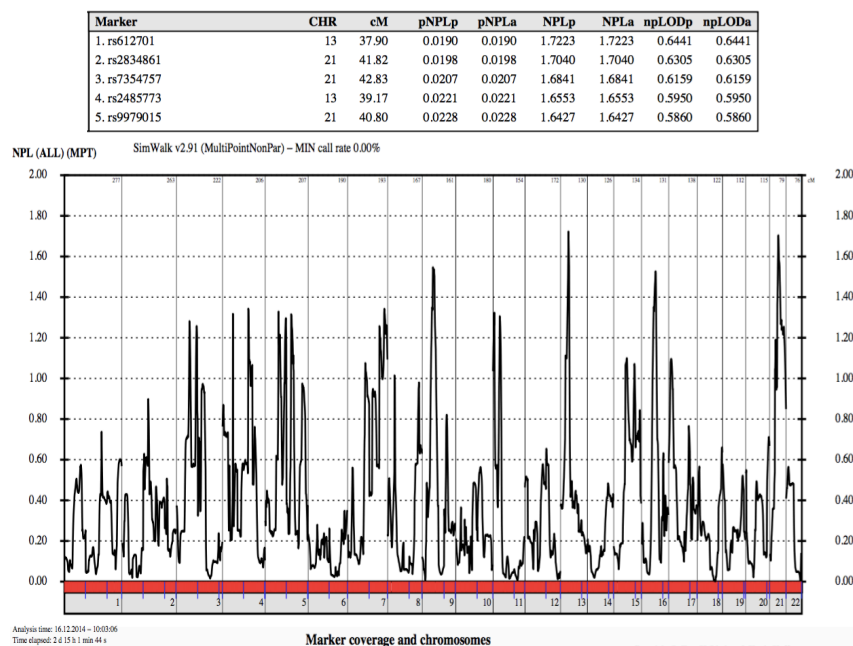
Figure 3.1 SimWalk Multipoint NPL Analysis showing the highest scores

Marker	CHR	cM	pNPLp	pNPLa	NPLp	NPLa	npLODp	npLODa
1. rs1461965	13	34.11	0.0150	0.0150	1.8240	1.8240	0.7224	0.7224
2. rs7989245	13	34.73	0.0151	0.0151	1.8222	1.8222	0.7210	0.7210
3. rs9542104	13	33.37	0.0152	0.0152	1.8182	1.8182	0.7179	0.7179
4. rs17053473	13	34.94	0.0152	0.0152	1.8174	1.8174	0.7172	0.7172
5. rs9315409	13	35.15	0.0153	0.0153	1.8155	1.8155	0.7157	0.7157
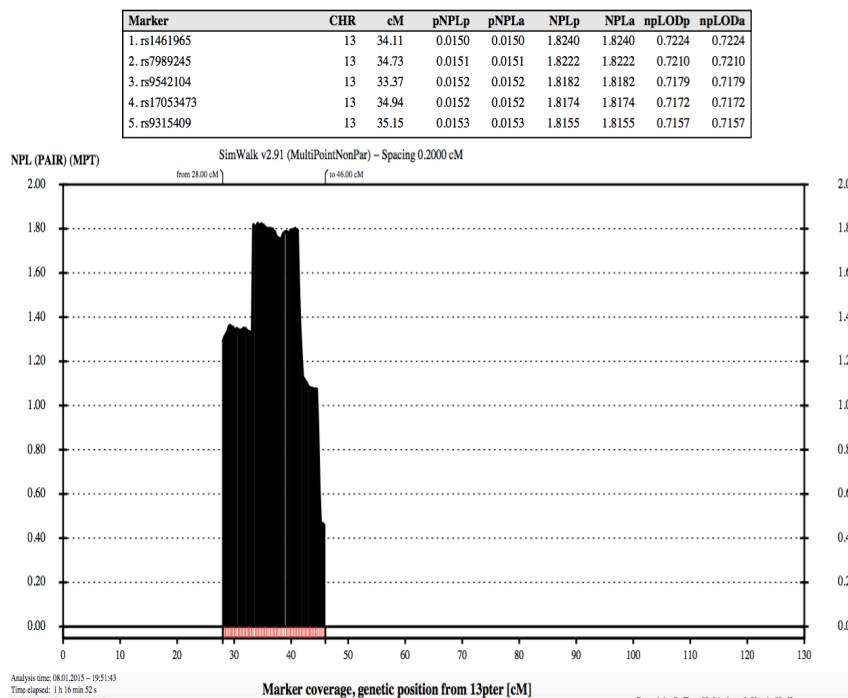
Figure 2.2: Fine mapping of chromosome 13 from 28 cM to 46 cM region

Marker	CHR	cM	pNPLp	pNPLa	NPLp	NPLa	npLODp	npLODa
1. rs11701543	21	45.08	0.0141	0.0141	1.8508	1.8508	0.7438	0.7438
2. rs2834046	21	37.05	0.0142	0.0142	1.8486	1.8486	0.7421	0.7421
3. rs2836096	21	46.98	0.0142	0.0142	1.8473	1.8473	0.7410	0.7410
4. rs2836400	21	47.21	0.0142	0.0142	1.8473	1.8473	0.7410	0.7410
5. rs28396271	21	37.26	0.0143	0.0143	1.8452	1.8452	0.7393	0.7393
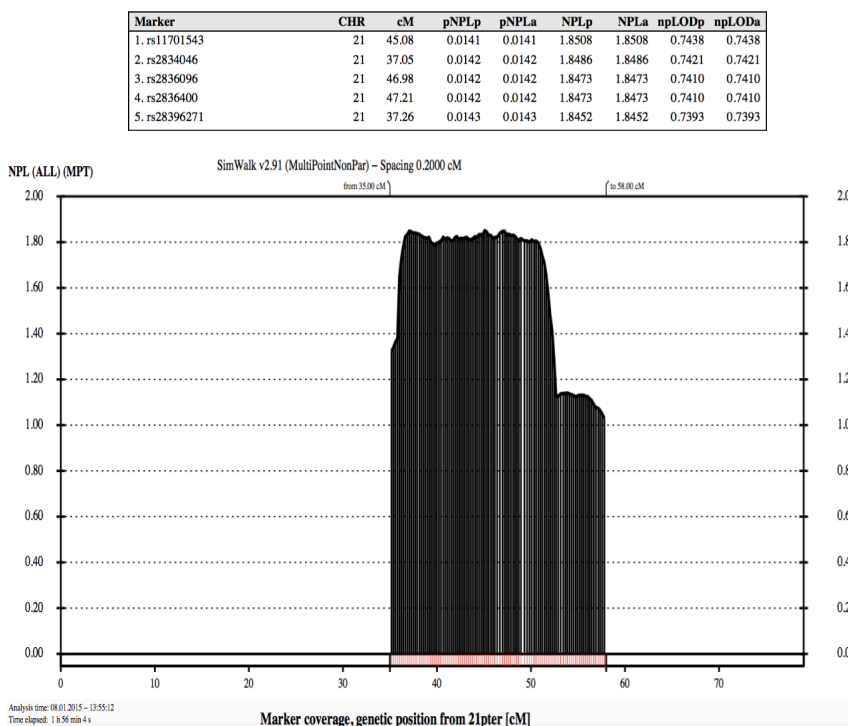
Figure 3.3: Fine mapping of chromosome 21 from 35 cM to 58 cM region

Within the regions of suggestive linkage, 24 protein coding genes in chromosome 13 and 58 protein coding genes in chromosome 21 were listed, and literature was reviewed in order to observe relation of the genes to MS and other neurological and/or inflammatory conditions. The search was resulted in 22 candidate genes (Figure 3.4).
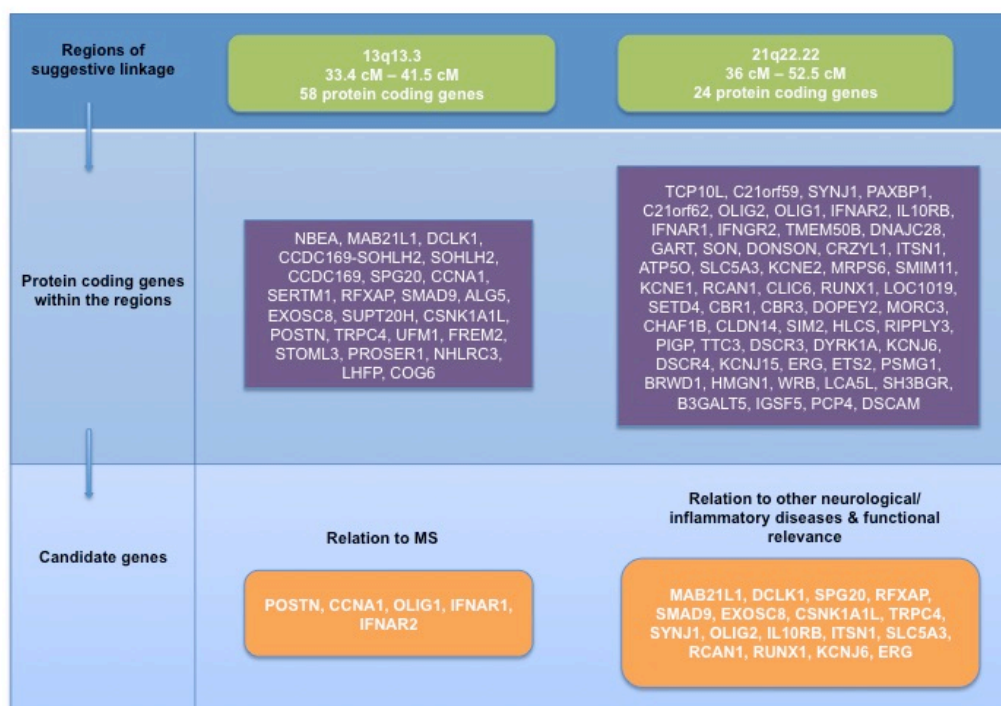
Figure 3.4: Selection of candidate genes

Among the candidate genes, IFNAR1 18417 and IFNAR2 11876 polymorphisms were found to be associated with risk of MS in a cohort of 147 MS patients and 210 controls by Leyva et al. (p=0.001 and p=0.035, respectively) [189]. In addition, increased number of highly polymorphic CAG repeat region in MAB21L1 gene was previously suggested to be related to a number of neurological conditions; including mental retardation, attention deficit/hyperactivity disorder, Huntington's disease, and Machado-Joseph disease [190, 191]. Therefore, IFNAR1 18417 and IFNAR2 11876 polymorphisms, and CAG repeat number in MAB21L1 gene were considered as strong candidates for increased risk of MS and selected for further investigation.

3.2 Analysis of IFNAR1, IFNAR2, and MAB21L1 variations

Sequences of the regions were analysed using Geneious R8 Software (Figure 3.5), genotype and allele frequencies of IFNAR1 18417 G/C, IFNAR2 11876 T/G, and

MAB21L1 CAG repeat numbers are given in Table 3.1, Table 3.2, and Table 3,3; respectively.
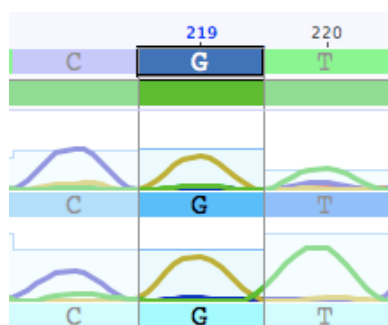
Figure 3.5 IFNAR2 sequence analysis in a patient homozygous for 11876 GG

Table 3.1: Genotype and allele distribution of IFNAR1 G18417C polymorphism in patients and controls

Gene	Patients N=25 (%)	Controls N=10 (%)	Controls Historic N=210 (%)	*P*-value
IFNAR1 rs2257167				
G18417G	16 (64)	6 (60)	151 (71.9)	
G18417C	8 (32)	4 (40)	54 (25.7)	
C18417C	1 (4)	0 (0)	5 (2.4)	0.54* 0.5**
Allele frequency				
G allele	40 (80)	16 (80)	356 (84.8)	
C allele	10 (20)	4 (20)	64 (15.2)	1* 0.38**

*Patients versus controls,**Patients versus historic controls

Table 3.2: Genotype and allele distribution of IFNAR2 T11876G polymorphism in patients and controls

Gene	Patients N=27 (%)	Controls N=10 (%)	Controls Historic N=210 (%)	P-value	OR; 95% CI
IFNAR2 rs1051393					
T11876T	11 (40.74)	4 (40)	104 (49.5)		
T11876G	11 (40.74)	4 (40)	93 (44.3)		
G11876G	5 (18.52)	2 (20)	13 (6.2)	0.92* 0.027**	3.64 [1.09-12.1]**
Allele frequency					
T allele	33 (61.1)	12 (60)	301 (71.7)		
G allele	21 (38.9)	8 (40)	119 (28.3)	0.93* 0.1**	

*Patients versus controls,**Patients versus historic controls

Table 3.3: MAB21L1 CAG trinucleotide repeat numbers in patients and controls

Gene	Patients (N = 27)	Controls (N = 10)	*P* value
MAB21L1 (mean CAG trinucleotide number)	31.88	30.4	0.3

Neither genotypic nor allelic difference did not reach to significance between cases and controls for IFNAR1 18417 G/C polymophism. However, IFNAR2 11876 GG genotype frequency was significantly higher in patients compared to historic controls (18.52% vs 6.2%, respectively, p = 0.027, OR 1.61 [95% CI 0.89 - 2.92]), even though there was no significant difference between allelic frequencies. Moreover, there was no CAG repeat number differences in MAB21L1 gene between the cases and controls (31.88 and 30.4, respectively).

3.3 Genome-Wide Association Study

Quality control of SNP markers was resulted in exclusion of 3.894 SNPs from chromosome Y, 11.638 SNPs having call rates lower than 95%, 118.727 SNPs in

linkage disequilibrium ($r^2>0.5$), and 31.136 SNPs with minor allele frequency lower than 0.01. Additionally, one of the two healthy controls were excluded from the study, since they showed IBD level higher than 0.25, indicating second degree relativity. The GWAS was performed with a total of 129.605 SNP markers in 11 MS patients and 59 healthy controls, revealing 14 SNPs showing significant association ($P<10^{-4}$). In addition, 106 SNPs showed higher frequencies in cases compared to controls, even though the differences did not achieve statistical significance ($P<10^{-3}$). SNPs having significant and suggestive associations are shown in Table 3.4.

Table 3.4: SNPs showing significant and suggestive associations between cases and controls

Marker	Ref/alt allele	Chr.	Region	MAF (cases)	MAF (controls)	*P*
rs7873	T|C	11	INS-IGF2	0.36	0.03	4.39E-07
rs7092208	G|A	10	MGMT	0.32	0.04	9.66E-06
rs16990774	G|A	22	EFCAB6	0.23	0.02	2.25E-05
rs844959	G|T	X	SPANXC	0.27	0.01	3.11E-05
rs9388582	C|T	6	SOGA3	0.27	0.03	3.68E-05
rs4605385	G|A	2	SCN9A	0.41	0.08	3.75E-05
rs6737602	T|C	2	STK39	0.68	0.25	4.46E-05
rs7377981	C|T	4	WHSC1	0.14	0.00	4.69E-05
rs498973	C|T	17	PLXDC1	0.14	0.00	4.69E-05
rs11696329	C|T	20	ATP9A	0.14	0.00	4.69E-05
rs2656219	G|A	11	CNTN5	0.18	0.01	4.71E-05
rs12988176	C|T	2	STON1-GTF2A1L	0.27	0.03	6.68E-05
rs4474241	C|T	11	CNTN5	0.45	0.11	7.79E-05
rs13061453	G|A	3	CHCHD6	0.64	0.20	9.21E-05
Suggestive Associations						
rs2249115	C|A	21	CLDN14	0.50	0.14	1.05E-04
rs17058979	C|A	8	PNOC	0.23	0.03	1.25E-04
rs11790551	G|T	9	GNA14	0.23	0.03	1.25E-04
rs2834646	A|C	21	RUNX1	0.32	0.05	1.38E-04
rs7356599	C|T	5	MYO10	0.27	0.03	2.03E-04
rs2462915	C|T	14	BCL11B	0.32	0.07	2.25E-04
rs4980041	C|T	10	ZMIZ1	0.59	0.19	2.25E-04
rs9532539	G|A	13	LINC00598	0.82	0.36	2.42E-04
rs2272504	A|C	12	TRHDE	0.50	0.15	2.46E-04
rs10160702	C|T	11	KIRREL3	0.41	0.11	2.54E-04
rs5910394	C|T	X	DOCK11	0.82	0.31	2.54E-04
rs2376481	G|A	15	GABRG3	0.68	0.28	2.64E-04
rs7122320	G|A	11	CNTN5	0.59	0.20	3.03E-04
rs5994128	A|G	22	GAB4	0.59	0.20	3.03E-04
rs599462	A|G	11	BC041900	0.32	0.06	3.13E-04
rs6760801	G|A	2	CCDC85A	0.45	0.11	3.21E-04
rs4255775	T|G	16	KIFC3	0.22	0.03	3.27E-04
rs12123953	C|T	1	GIPC2	0.50	0.14	3.50E-04
rs4980047	G|A	10	ZMIZ1	0.50	0.16	3.65E-04
rs6869645	C|T	5	SLC6A3	0.18	0.02	3.70E-04
rs7850420	C|T	9	MAPKAP1	0.18	0.02	3.70E-04

rs12417295	C|T	11	ARHGAP42	0.18	0.02	3.70E-04
rs35206614	A|G	13	ATP7B	0.18	0.02	3.70E-04
rs34853349	A|G	14	LINC00617	0.18	0.02	3.70E-04
rs16984253	C|T	20	MYT1	0.18	0.02	3.70E-04
rs12485136	G|A	22	PNPLA5	0.18	0.02	3.70E-04
rs2875237	G|A	3	GRM7	0.27	0.05	3.72E-04
rs4973986	A|C	3	ULK4	0.27	0.05	3.72E-04
rs17311679	A|G	19	VRK3	0.27	0.05	3.72E-04
rs1774847	T|C	1	TMEM183A	0.77	0.36	3.85E-04
rs2295794	C|T	9	TLN1	0.09	0.48	3.89E-04
rs3008639	G|A	1	AIDA	0.18	0.02	4.21E-04
rs2414893	C|A	15	MEGF11	0.50	0.18	4.25E-04
rs12297776	C|T	12	ERGIC2	0.41	0.12	4.35E-04
rs507280	C|T	6	ROS1	0.36	0.10	4.55E-04
rs11190831	C|T	10	TLX1NB	0.36	0.10	4.55E-04
rs733126	C|T	11	USH1C	0.36	0.10	4.55E-04
rs2370473	G|A	2	BC039382	0.23	0.03	4.78E-04
rs16899078	C|T	5	CDH12	0.23	0.03	4.78E-04
rs502156	T|G	6	AK126334	0.23	0.03	4.78E-04
rs4751086	A|G	10	MGMT	0.23	0.03	4.78E-04
rs4766819	C|T	12	FBXW8	0.23	0.03	4.78E-04
rs7249176	A|G	19	LILRP2	0.23	0.03	4.78E-04
rs4611723	G|A	21	LOC339622	0.23	0.03	4.78E-04
rs4972701	C|T	2	BC046497	0.77	0.31	4.86E-04
rs8025178	G|A	15	TRPM1	0.64	0.23	4.91E-04
rs16971450	A|G	15	MTHFS	0.45	0.12	4.92E-04
rs17464525	G|A	1	AP4B1	0.27	0.04	5.19E-04
rs2034360	C|T	2	PRKCE	0.45	0.14	5.37E-04
rs17080136	T|C	5	MAPK9	0.45	0.14	5.37E-04
rs10976051	T|G	9	KDM4C	0.64	0.25	5.37E-04
rs626657	C|A	17	CACNB1	0.36	0.09	5.89E-04
rs7820617	T|C	8	TRIM55	0.27	0.03	6.56E-04
rs6577449	C|T	1	CAMTA1	0.23	0.03	7.05E-04
rs12712051	C|T	2	AFF3	0.23	0.03	7.05E-04
rs10853465	C|T	18	LINC00669	0.23	0.03	7.05E-04
rs6843239	C|T	4	SEC24D	0.18	0.56	7.36E-04
rs5918486	A|G	X	SRPX	0.55	0.16	7.48E-04
rs2147852	C|T	13	TBC1D4	0.50	0.18	7.53E-04
rs12870594	T|C	13	TEX26-AS1	0.32	0.06	7.83E-04
rs2133127	G|A	15	SNRPN	0.59	0.22	8.02E-04
rs2032701	G|A	3	MECOM	0.36	0.11	8.25E-04
rs2072793	G|A	20	RBPJL	0.77	0.38	8.31E-04
rs11003872	C|T	10	PCDH15	0.68	0.33	8.44E-04
rs6663477	A|G	1	VPS45	0.14	0.01	8.65E-04
rs7589234	T|C	2	AFF3	0.14	0.01	8.65E-04
rs1515495	A|G	3	TP63	0.14	0.01	8.65E-04
rs10045011	C|T	5	FYB	0.14	0.01	8.65E-04
rs799157	T|C	7	MLXIPL	0.14	0.01	8.65E-04
rs12771692	G|A	10	LOC399715	0.14	0.01	8.65E-04
rs4143858	A|G	11	CNTN5	0.14	0.01	8.65E-04
rs7957161	T|C	12	PLXNC1	0.14	0.01	8.65E-04
rs9565321	C|T	13	MYCBP2	0.14	0.01	8.65E-04
rs7233275	G|A	18	TMEM241	0.14	0.01	8.65E-04
rs11671231	C|T	19	LILRA5	0.14	0.01	8.65E-04
rs7143254	C|A	14	LRFN5	0.50	0.17	8.82E-04

rs11791292	T|G	9	ADAMTSL1	0.27	0.06	9.07E-04
rs3740829	T|C	11	ARHGAP32	0.27	0.06	9.07E-04
rs1330884	T|G	13	RNF219-AS1	0.14	0.51	9.26E-04
rs13440231	C|T	9	NR6A1	0.18	0.00	9.69E-04
rs2131905	C|T	1	AKNAD1	0.09	0.00	9.69E-04
rs17258606	T|C	1	NOTCH2	0.09	0.00	9.69E-04
rs12077365	T|C	1	OBSCN	0.09	0.00	9.69E-04
rs7581919	C|T	2	NBAS	0.09	0.00	9.69E-04
rs3917329	G|T	2	IL1R1	0.09	0.00	9.69E-04
rs3813247	G|T	2	TTN	0.09	0.00	9.69E-04
rs9835263	A|G	3	AK124857	0.09	0.00	9.69E-04
rs11098016	T|G	4	COL25A1	0.09	0.00	9.69E-04
rs1521026	G|A	5	CDH18	0.09	0.00	9.69E-04
rs7726659	A|G	5	ANKRD31	0.09	0.00	9.69E-04
rs6964977	A|G	7	WBSCR17	0.09	0.00	9.69E-04
rs10499905	C|T	7	SGCE	0.09	0.00	9.69E-04
rs10486860	C|T	7	DLX6-AS1	0.09	0.00	9.69E-04
rs6560837	C|T	10	DIP2C	0.09	0.00	9.69E-04
rs3816685	T|C	10	JMJD1C	0.09	0.00	9.69E-04
rs4769191	A|G	13	AK054845	0.09	0.00	9.69E-04
rs17093516	A|G	14	SYNE2	0.09	0.00	9.69E-04
rs2382868	T|C	16	GTF3C1	0.09	0.00	9.69E-04
rs35794595	G|T	17	NTN1	0.09	0.00	9.69E-04
rs414528	T|C	19	USE1	0.09	0.00	9.69E-04
rs4803831	G|T	19	OPA3	0.09	0.00	9.69E-04
rs6051643	G|A	20	DDRGK1	0.09	0.00	9.69E-04
rs12484542	C|T	22	CSNK1E	0.09	0.00	9.69E-04
rs5978925	C|T	X	KAL1	0.09	0.00	9.69E-04
rs1947442	G|A	4	CCSER1	0.41	0.11	9.91E-04
rs6753123	C|T	2	LOC284950	0.32	0.08	9.97E-04

Ref/alt allele, reference and alternative allele; Chr, chromosome; MAF, minor allele frequency.

3.4 Correlation between Genetic and Proteomic Analyses

Correlation analyses of the linkage, GWAS, and proteome data yielded in 9 overlapping genes in GWAS and proteomic results, and 3 overlapping genes in GWAS and linkage analysis (Table 3.5 and Table 3.6, respectively). Among the significant associations revealed in the GWAS, rs7873 located in Insulin - Insulin-Like Growth Factor 2 Read-Through Product (INS-IGF2) was the strongest association (4.39E-07), and in the proteomic analyses sodium reabsorption pathway and type II diabetes mellitus, both of which involve INS, found to be affected in MS subtypes. In addition, 8 genes showing suggestive association in GWAS were found to involve in common pathways revealed in the proteomic data, including renin-angiotensin system, NOD-like receptor signaling, and Notch signaling pathways. Moreover, other 3 genes revealed suggestive association in GWAS was also in regions of suggestive linkage showing NPL scores between 1.6 and 1.83.

Table 3.5: Correlation between linkage and GWAS data

Marker	Chr.	Region	NPL Score	*P* (GWAS)
rs2249115	21	CLDN14	1.83	1.05E-04
rs2834646	21	RUNX1	1.6-1.7	1.38E-04
rs9532539	13	LINC00598	1.7	2.42E-04

Table 3.6: Correlation between GWAS and CSF proteome data

Marker	Chr.	Region	Common Pathways	*P* (GWAS)
rs7873	**11**	**INS-IGF2**	**Sodium reabsorption, Type II diabetes**	**4.39E-07**
rs2034360	2	PRKCE	Renin-angiotensin, Type II diabetes	5.37E-04
rs17080136	5	MAPK9	NOD-like receptor signaling, Type II diabetes	5.37E-04
rs2072793	20	RBPJL	Notch signaling	8.31E-04
rs11791292	9	ADAMTSL1	Notch signaling	9.07E-04
rs13440231	9	NR6A1	Notch signaling	9.69E-04
rs17258606	1	NOTCH2	Notch signaling	9.69E-04
rs3917329	2	IL1R1	NOD-like receptor signaling	9.69E-04
rs35794595	17	NTN1	Notch signaling	9.69E-04

To analyse individual proteomic data of each patient, corresponding or possibly corresponding genes of proteins or protein fragments that found to have altered levels in MS patients were searched for associations resulted from the linkage and the GWAS. Besides the direct correlations; genes encoding for different forms of corresponding proteins, protein fragments, or subunits were also considered, which was resulted in a total of 20 correlations (Table 3.7). Among them, the only direct correlation was contactin protein, whose level had 1-4 fold change in MS patients, and accordingly, 2 significant and 2 suggestive SNP markers located on CNTN5 gene encoding for contactin 5 protein were identified in the GWAS (Table 3.8).

Table 3.7: Correlations between GWAS and individual CSF proteome data

Protein	Patient number with altered protein level	Fold change
Contactin	7	1-4
amyloid beta A4 protein	4	1-4
MAP9	4	1-4
Ig gamma 2 H chain, BUR [human, Peptide Mutant, 348 aa]	4	1-4
Ig heavy chain V region - human (fragment)	5	2-4
immunoglobulin heavy chain constant alpha 1 membrane bound isoform 2 [Homo sapiens]	2	1-3

Immunoglobulin kappa variable (IGKV1)	1	1-2
Immunoglobulin kappa constant (IGKC)	1	1-2
immunoglobulin light chain [Homo sapiens]	3	1-2
Igl chain VLJ	2	2-4
Complement C4d	2	1-2
putative myosin 15B	4	1-4
TMEM95 protein [Homo sapiens]	1	1-2
neuronal cell adhesion molecule	4	1-3
Titin-cap (telethonin) [Homo sapiens]	3	1-2
Fibroblast growth factor receptor substrate 3	1	2-3
Leucine rich alpha 2 glycoprotein	2	1-2
Integrin alpha-IIb	2	2-3
acylphosphatase 2, muscle type, isoform CRA_a [Homo sapiens]	3	1-4
Calcium/calmodulin-dependent protein kinase type II subunit beta	1	2-3

Table 3.8: Direct correlation between GWAS and individual CSF proteome data

Marker	Gene	Differentially Expressed Protein in MS Patients	Fold change	*P* (GWAS)
rs2656219	CNTN5	Contactin	1-4*	4.71E-05
rs474241	CNTN5	Contactin	1-4*	7.79E-05
rs7122320	CNTN5	Contactin	1-4*	3.03E-04
rs4143858	CNTN5	Contactin	1-4*	8.65E-04

 * 1-2 fold change in 2 patients, 2-3 fold change in 4 patients, and 3-4 fold change in 1 patient.

3.5 Database

Data were generated from previous association studies for 17 common diseases conducted for Turkish population, and the TUSNP database was developed. For each study, gene name, SNP ID number, sample size, risk/protective allele frequency in cases and controls, *P* value, OR (95% CI), and PubMed ID were represented. Link to the GeneCards Human Gene Compendium (http://www.genecards.org) was provided for each gene comprising the corresponding SNP. An example is given in Figure 3.6.

Figure 3.6: An example for the TUSNP data presentation

The data generated from MS studies are shown in Table 3.9.

Table 3.9: Association findings for MS in Turkish population

Disease	Sample Size	Gene	SNP-Risk/Protective Allele	Allele Freq. in Controls	Allele Freq. in Patients	P	OR	95% CI	Reference (PMID)
MS	103 MS cases, 101 controls	HLA-DRB1	1501	0.139	0.282	0.02	2.4	1.2-5.0	9328791
MS	103 MS cases, 101 controls	HLA-DRB1	04	0.178	0.34	0.01	2.3	1.2-4.6	9328791
RRMS	64 RRMS cases, 101 controls	HLA-DRB1	04	0.178	0.3593	0.01	2.6	1.3-5.3	9328791
MS	103 MS cases, 101 controls	HLA-DQA1	0101	0.307	0.155	0.02	0.4	0.2-0.8	9328791
MS	103 MS cases, 101 controls	HLA-DQA1	0103	0.188	0.068	0.02	0.3	0.1-0.7	9328791
MS	103 MS cases, 101 controls	HLA-DQA1	0301/2	0.208	0.339	0.05	1.9	1.0-3.6	9328791
MS	103 MS cases, 101 controls	HLA-DQB1	0501	0.237	0.097	0.01	0.3	0.2-0.8	9328791
MS	103 MS cases, 101 controls	HLA-DQB1	0602	0.099	0.262	0.005	3.2	1.4-7.1	9328791

MS	103 MS cases, 101 controls	HLA-DQB1	0302	0.148	0.291	0.02	2.3	1.2-4.7	9328791
MS	180 OCB-positive cases, 188 controls	HLA-B	8	0.058	0.155	0.003	2.97	1.09-8.08	19457560
MS	30 OCB-negative cases, 188 controls	HLA-B	8	0.058	0.167	0.002	3.25	1.21-8.75	19457560
MS	122 cases, 188 controls	HLA-B	8	0.058	0.131	<0.05	2.44	0.88-6.79	15301866
MS	122 cases, 188 controls	HLA-B	7	0.053	0.131	<0.05	2.69	0.94-7.69	15301866
MS	180 OCB-positive cases, 188 controls	HLA-DR	3	0.063	0.133	0.02	2.28	0.84-6.15	19457560
MS	30 OCB-negative cases, 188 controls	HLA-DR	3	0.063	0.133	0.02	2.28	0.84-6.15	19457560
MS	180 OCB-positive cases, 188 controls	HLA-DR	15	0.053	0.239	0	5.61	2.08-15.07	19457560
MS	30 OCB-negative cases, 188 controls	HLA-DR	15	0.053	0.1	0.001	1.98	0.66-5.91	19457560
MS	180 OCB-positive cases, 30 OCB-negative cases	HLA-DR	15	0.1 (OCB-negative)	0.239 (OCB-positive)	0.007	2.82	1.27-6.28	19457560
MS	122 cases, 188 controls	HLA-DR	2	0.042	0.278	<0.05	8.78	3.01-25.61	15301866
MS	53 MS cases, 66 controls	MEFV	rs61752717	0.0303	0.1037	0.02	3.7	0.99-13.74	20483145
MS	53 MS cases, 66	MEFV	rs3743930	0.0075	0.066	0.013	9.35	0.84-103.59	20483145

	controls								
MS	53 MS cases, 66 controls	MEFV	rs28940579	0	0.0471	0.004	10.93	0.59-201.73	20483145
MS	125 cases, 160 controls	IL4	Intron 3 VNTR-P1	0.063	0.14	0.002	2.44	1.38-4.41	23756167
MS	409 cases, 256 controls	IL1B	rs16944-2 (protective)	0.486	0.418	0.015	1,178	0.999-1.388	23594042
RRMS	277 cases, 256 controls	IL1B	rs16944-2 (protective)	0.486	0.408	0.011	2,211	1.261-3.877	23594042
MS	101 cases, 164 controls	IL8	rs18723 8-C	0.229	0.361	0.0013	1,909	1.298-2.808	24402877
RRMS	69 cases, 164 controls	IL8	rs18723 8-C	0.229	0.348	0.01	1,797	0.964-3.347	24402877
RRMS	120 RRMS cases, 120 controls	MIF	rs75562 2-C	0.247	0.415	<0.05	2.16	1.18-3.95	20233515
MS	17 male cases, 33 female cases	APOE	ε4 allele	0.015 (female cases)	0.147 (male cases)	0.007	11.31	2.05-62.24	22165672

 For the SNPs analysed in more than one study, the TUSNP automatically provides a pooled OR result by conducting meta-analysis. The website also includes other tools for pathway enrichment, Hardy Weinberg calculation, odds ratio and relative risk ratio assesment, Chi-square and Student's t-test, and power analysis provided in Utilities section. The TUSNP was designed to receive new data from users willing to submit their association findings regardless of statistical significance level, after a member of the editorial board overview and validate new submissions.

4. DISCUSSION and CONCLUSION REMARKS

In this study; genetic basis of MS was explored, conducting a linkage study in MS pedigrees comprising of affected and unaffected individuals and a genome-wide association study using unrelated cases and healthy controls, and the results were compared to data of CSF proteome profiling that identified specific and shared pathways in MS clinical subtypes in the previous study of our group [163]. As a result, 2 suggestive linkage regions were identified, and among the selected candidate genes from the regions, associations of MAB21L1 CAG repeat number, IFNAR1 18417 G/C and IFNAR2 11876 T/G variations, and CAG repeat numbers in MAB21L1 were discovered by case-control studies, revealing a significant association of IFNAR2 11876 GG genotype with increased risk of MS (P = 0.027, OR 3.64 [95% CI 1.09 – 12.1]). Subsequent GWAS identified 14 SNPs with significant association (P<10^{-4}), additionally 106 SNPs showing suggestive association with MS (P<10^{-3}). When results were compared with each other and the proteome data, 9 genes with significant or suggestive associations from the GWAS were found to involve in pathways those shown to be activated in MS subtypes, and there were 3 genes common between GWAS and linkage results. Additionally, when individual proteome data of each patient involved in the genetic analyses was observed, a total of 20 proteins having altered expression level in one or more patients were also found to have significant or suggestive association in the GWAS.

IFNAR1 (interferon (alpha, beta and omega) receptor 1) and IFNAR2 (interferon (alpha, beta and omega) receptor 2) are genes that encode for type I membrane proteins each of which forms one of the two chains of a receptor for interferons alpha and beta. IFNAR1 and IFNAR2 are known to involve in a number of pathways including T cell activation, cytokine-mediated signaling, response to interferon-alpha, and type I interferon signaling pathway, and leucocytes are the cell type in which the highest expression levels for both of the genes occur. Given their inflammation based cellular roles, both genes have been implicated in a load of

inflammatory conditions, such as measles, hepatitis B and C infection, mumps, cerebritis, cerebral malaria, and multiple sclerosis. Previously, a number of cytokines associated with cerebral malaria have also been implicated in MS, suggesting a shared characteristics of inflammation that may be the answer of common susceptibility genes in both diseases, including IL10, IFNG, and TNF [194, 195, 196, 197]. In cerebral malaria, IFNAR1 18417 G/C polymorphism was described in reduced risk of developing *Plasmodium falciparum* infection, a complication seen in the disease, compared to those possessing at least one C allele [198, 199]. Subsequently, in a study by Levya et al., IFNAR1 18417 G/C and IFNAR2 11876 T/G polymorphisms were investigated in a cohort of 147 MS patients and 210 controls, revealing significant associations of IFNAR1 18417 CC genotype and C allele ($P = 0.001$ and $P = 0.0002$, respectively), and IFNAR2 11876 GG genotype and G allele ($P = 0.035$ and P = 0.046 ($P_{corr} = 0.055$), respectively) with risk of MS [189]. Taken into account that IFNAR1 and IFNAR2 are located on 21q22.1 which yielded one of the two highest NPL scores (1.85) in our linkage analysis, and as a result of the promising literature background, IFNAR1 18417 G/C and IFNAR2 11876 T/G polymorphisms were considered as strong candidates for increased risk of MS. Our case-control studies exploring IFNAR1 18417 and IFNAR2 11876 polymorphisms confirmed IFNAR2 11876 GG association ($P = 0.027$) revealing an odd ratio of 3.64 (95% CI 1.09 - 12.1), although the other associations did not reach to significance. In addition to IFNAR1 and IFNAR2 associations, MAB21L1 (Mab-21-Like 1) gene was yet another selected candidate gene located on 13q.1 which yielded the second highest NPL socre (1.82) in the linkage analysis. MAB21L1 encodes for a protein similar to a cell-fate determining protein expressed by MAB-21 gene in *Caenorhabditis elegans (C.elegans),* and highly expressed in the cerebellum. Whilst the normal range of the trinucleotide CAG repeat number in 5' UTR region of MAB21L is between 9 and 29, expansion of this repeat was previously suggested to have a role in a number of neuropsychiatric and neurodegenerative conditions; including mental retardation, attention deficit/hyperactivity disorder, Huntington's disease, and Machado-Joseph disease [190, 191]. However, when we compared the CAG repeat numbers in MAB21L1 between MS patients and controls, we did not observe any differences, suggesting that CAG repeat expansion in 5' UTR region of MAB21L1 does not affect susceptibility to MS. Based upon our linkage analysis and subsequent literature review; IFNAR1, IFNAR2, and MAB21L1 were considered as

promising candidates, and in addition to the investigated variations in this study, deep sequencing of the genes, as well as expression studies and functional analyses may further elucidate their roles in multiple sclerosis. On the other side, there are a number of other candidate genes resulted from our linkage analysis, some of which were previously implicated in MS or other inflammatory/neurological conditions. Among them, SNPs located in CLDN14, RUNX1, and LINC00598 genes showed suggestive association with MS in our GWAS results. Therefore, other candidate genes, particularly having correlation between the genetic analyses, should be carefully taken into consideration in further studies.

Correlation analyses between the GWAS results and the proteome data revealed that among the correlations, only rs7873 located on INS-IGF2 readthrough, an important paralog of INS, had significant association, and it was the most significant marker resulted from the GWAS (P = 4.39E-07). INS (insulin) gene encodes for pro-insulin, which is then post-translationally cleaved into covalently bound A chain and B chain peptides and C peptide of insulin. The main role of insulin binding to it's receptor INSR (insulin receptor) is stimulation of glucose uptake through PI3K (phosphatidylinositol 3-kinase), decreasing glucose concentration in blood. Therefore, many INS variations have been previously associated with type II diabetes mellitus, which is characterised by insulin resistance, thus high blood glucose levels due to insulin signaling defects. Insulin signaling also stimulates a number of other pathways one of which is aldosterone-regulated sodium reabsorption pathway that found to be activated in MS subtypes together with type II diabetes mellitus in the study of Avsar et al. The study showed that PI3K subunits (PIK3C1, PIK3C2, and PIK3R2), and INSR were upregulated in CSF of CIS patients compared to controls, revealing that sodium reabsorption pathway was significantly affected in CIS patients (P = 1,78E-04). Long before, it was shown that increased salt (sodium chloride; NaCl) concentrations, thus elevated hypertonicity could induce immune system activation as an external trigger [200, 201]. Accordingly, in a recent study of Kleinewietfeld et al., increased NaCl concentration dramatically augmented the induction of murine and human Th17 cells [202], which is a type of T helper cells that had been found to be critical for development of EAE [203]. This Th17 promoting effect of high-salt concentration for naïve CD4 cells was through phosphorylation of p38/MAPK, which then activates NFAT5 and SGK1, both of

which are components of aldosterone-regulated sodium reabsorption pathway, and downstream components of INSR signaling [202]. Therefore, variations in INS gene may be responsible for alterations in insulin receptor signaling cascade in CIS patients, causing increased Na+ uptake through SGK1 and conversion of naïve CD4 cells into Th17 cells. In this context, possible effects of sodium reabsorption pathway activation in MS will be explored in our further studies. Along with INS, other 8 genes suggestive of association from the GWAS that found to involve in affected pathways in MS subtypes, as well as correlations observed in individual proteomic data of the patients involved in the GWAS should be investigated in further studies.

In recent years, a considerable effort has been made for the discovery of genetic determinants of MS susceptibility by high-throughput technologies. However, only about 27% of the predicted heritability of MS has been elucidated yet. Remaining missing heritability is most likely due to undetected variants with small effects that cannot be uncovered by the detecting power of current technologies. Yet another challenge is that increasing number of data from genetic studies remain to be linked to functional understanding of MS pathogenesis. Therefore, we used multi-disciplinary approaches combining genetic and proteomic analyses with currently available bioinformatic tools in order to fit the findings into a biological frame by further studies. To further overcome the challenges in deciphering MS complexity, novel bioinformatic tools and statistical methods with higher power should be developed, all MS risk loci identified by GWA studies should be fine-mapped or directly sequenced, and resulted candidate genes should be validated by functional assays. Moreover, to improve functional assessment methods, novel *in vivo* animal models possessing human genes would be advantageous, making models mimic MS more closely than that of current mouse models.

As the second part of the study, we established the database TUSNP, the Turkish SNP Database, which represent the allelic SNP variants found to be associated with risk of 17 common diseases in Turkish population, including multiple sclerosis. By developing the TUSNP, we aimed to provide a comprehensive gathered information on allelic variants related to common disorders specifically in Turkish population, as association findings are mostly inconsistent across the populations. The TUSNP also provides users pathway enrichment, meta-analysis and other quick calculation tools, allowing further quick review of related associations. We aim to further extend our

database in a way to include a broader spectrum of common disorders. Although, the database includes all association studies only for Behcet's Syndrome currently, further updates will include reported association findings failed to reach a statistical significance for all disorders included in the TUSNP, in order to provide reliable meta-analyses and extended information on each common disease.

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APPENDICES

APPENDIX A: Equipment

APPENDIX B: Chemicals and Buffers

APPENDIX C: Informed Consent Form

APPENDIX A

MicroCL 17 Centrifuge (Thermo Scientific)

Allegra 25R Centrifuge (Beckman Coulter)

Vortex (Labnet)

Nanodrop (Thermo Scientific)

T100 Thermal Cycler (Bio-Rad)

Agarose gel electrophoresis system (Wealtec)

Weighing scale (Precisa)

Heat block (Techne)

Refrigerator (+4°C - Vestel)

Freezer (-20°C - Arcelik)

Freezer (-80°C - Nuve)

APPENDIX B

Red Blood Cell Lysis Buffer (Roche)

White Cell Lysis Buffer (Roche)

Protein Precipitation Solution (Roche)

Ethanol (Sigma-Aldrich)

DreamTag Green Buffer (Thermo Scientific)

DreamTag Polymerase (Thermo Scientific)

$MgCl_2$ (Thermo Scientific)

dNTP (Thermo Scientific)

Nuclease-free water (Medifar)

Agarose (Invitrogen)

DNA ladder (Thermo Scientific)

Binding Buffer (Roche)

Wash Buffer (Roche)

Elution Solution (Roche)

Isopropanol (AppliChem)

APPENDIX C

1.   Davet edildiğiniz "*A LINKAGE ANALYSIS AND A GENOME-WIDE ASSOCIATION STUDY ON FAMILIAL MULTIPLE SCLEROSIS*" isimli çalışma bir araştırma projesidir.

2. Çalışmanın amacı, MS hastalığının genetiğinin araştırılmasıdır.

3. Araştırmada gönüllülere uygulanacak herhangi bir tedavi bulunmamaktadır.

4. Araştırmaya katılmayı kabul ederseniz görevlendirilmiş bir hekim tarafından muayene edileceksiniz ve klinik bulgularınız kaydedilecektir. Muayene sonucunda doktorunuz uygun görürse çalışmaya alınacaksınız. Katılan gönüllülerin kollarından 10 ml (1 tüp) kan alınacaktır.

5. Sizden alınan kanlardan DNA izole edilerek genotipleme yapılacaktır.

6. Kan alınması sırasında oluşabilecek riskler: 1) İğne batmasına bağlı olarak acı duyabilirsiniz. 2) Düşük bir ihtimal olarak iğne batması sonucu kanamanın uzaması ve enfeksiyon riski bulunmaktadır.

7. Araştırmada gönüllü açısından hedeflenen herhangi bir klinik yarar bulunmamaktadır.

8. Bu çalışmaya katılmanız için sizden herhangi bir ücret talep edilmemektedir. Bunun yanı sıra size bir ödeme yapılmayacaktır.

9. Bağlı bulunduğunuz Sosyal Güvenlik Kurumuna (SGK) herhangi bir ücret ödemeniz gerekmemektedir.

10. Araştırmaya katılımınız sizin isteğinize bağlıdır ve istediğiniz zaman araştırmaya katılmayı reddedebilir veya araştırmadan çekilebilirsiniz.

11. Sizinle ilgili tüm kimlik bilgileri, klinik bulgular ve elde edilecek deneysel bulgular gizli kalacak ve kimseyle paylaşılmayacaktır. Araştırma konularının yayınlanmasında dahi kimlik bilgileriniz kimseyle paylaşılmayacaktır.

12. Araştırma konusuyla ilgili ve araştırmaya katılmaya devam etme isteğiniz etkileyebilecek yeni bilgiler edinildiğinde gönüllü veya yasal temsilcisi bilgilendirilecektir.

13. Sizden bir defaya mahsus olarak kan alınması planlanmaktadır.

14. Elde edilen kan örnekleri, MS'in etyopatogenezinin aydınlatılmasına katkıda bulunmak için kullanılacaktır.

15. Çalışmada bulunan analizler İstanbul Teknik Üniversitesi Moleküler Biyoloji ve Genetik Bölümü laboratuvarlarında gerçekleştirilecektir. Örnekler hiçbir şekilde yurtdışına gönderilmeyecektir.

Yukarıda gönüllü kişiye araştırmaya katılmadan önce verilmesi gereken bilgileri gösteren metni okudum. Yukarıda konusu ve amacı belirtilen araştırma ile ilgili yazılı ve sözlü açıklamalar yapıldı. Karar vermem için yeterli süre tanındı. Bu koşullarla söz konusu araştırmaya kendi rızamla katılmayı kabul ediyorum. Araştırma kapsamında alınan kan örneklerinin

o *Sadece yukarıda bahsi geçen araştırmada kullanılmasına izin veriyorum.*

o *İleride yapılması planlanan tüm araştırmalarda kullanılmasına izin veriyorum.*

o *Hiçbir koşulda kullanılmasına izin vermiyorum.*

Bu formun imzalı bir kopyası bana verilecektir.

Gönüllünün,

Adı-Soyadı:

Adresi:

Tel-Faks:

Tarih ve İmza:

Açıklamaları yapan araştırıcının,

Adı-Soyadı:

Görevi:

Adresi:

Tel-Faks:

Tarih ve İmza:

Bir sorunla karşılaşıldığında aşağıdaki numaralardan proje sorumlularına ulaşabilirsiniz;

Prof. Dr. Aksel SİVA: 0532 615 8781

Doç. Dr. Eda TAHİR TURANLI: 0532 524 6521

CURRICULUM VITAE

Name Surname: Elif Everest

Place and Date of Birth: Mersin / 12.10.1989

E-Mail: everest@itu.edu.tr

EDUCATION:

B.Sc.: Istanbul Technical University, Faculty of Science and Letters, Molecular Biology and Genetics Department (2007-2012)

PROFESSIONAL EXPERIENCE AND REWARDS:

Research assistant, Istanbul Technical University Faculty of Science and Letters, Molecular Biology and Genetics Department (2014 – present)

Internship in Yale University School of Medicine, Neurogenetics Lab of Neurosurgery Department (2012)

Internship in Yeditepe University, Genetics Diagnosis Centre, Department of Molecular Genetics/Cytology (2010)

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

▪ **Everest E.,** Avsar T., Siva A., Uygunoglu U., Tutuncu M., Saip S., Karacan I., and Turanli ET.: Suggestive linkage to chromosomal regions 13q13.3 and 21q22.2 in families with Multiple Sclerosis. *The European Human Genetics Conference*, June 06-09, 2015 Glasgow, Scotland, United Kingdom.

▪ **Everest E.,** Özgen EC., Kaya B., Saraç ÖS., and Turanli ET.: Turkish population-specific single nucleotide polymorphisms related to common disorders and a meta-analysis of association between HLA-B51 and Behçet's Syndrome in Turkey. *11. Ulusal Tıbbi Genetik Kongresi*, September 24-27, 2014 İstanbul, Turkey.

▪ Erdem G., Erözenci A., **Everest E.,** and Turanli ET.: Methylation status of alternatively spliced forms of MEFV transcripts determine differential protein interaction. *11. Ulusal Tıbbi Genetik Kongresi*, 24-27 Eylül 2014, September 24-27, 2014 İstanbul, Turkey.

▪ **Everest E.,** Karacan I., Sayın E., Bodur V., and Turanli ET.: Sık Görülen Hastalıkların Yatkınlık Genlerinde Popülasyon Spesifik Alel Frekansları Analizleri. *10. Ulusal Tıbbi Genetik Kongresi*, December 20-23, 2012 Bursa, Turkey.