INVESTIGATION OF THE RELATIONSHIP BETWEEN MULTIPLE SCLEROSIS AND *APOE*, *VDR*, *VDBP* GENE MUTATIONS

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to my family…

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ABSTRACT

INVESTIGATION OF THE RELATIONSHIP BETWEEN MULTIPLE SCLEROSIS AND A*POE*, *VDR*, *VDBP* GENE MUTATIONS

Multiple sclerosis (MS) is a neurodegenerative disease characterised by genetic and environmental factors, and clinical outcomes are getting worse by time. It is not known whether vitamin D or cholesterol and their genetic fundamentals are a marker of other susceptibility factors. Vitamin D participates in sustaining the function of immune response thus, it is aetiologically noticeable and likely to have potency for treatment of disease. Vitamin D binding protein (*VDBP*) and vitamin D receptor (*VDR*) specific polymorphisms are found to be related to such neurodegenerative diseases. In some cases, higher prevalence of different variants in apolipoprotein E (*ApoE*) which is the main gene known to be related to cholesterol metabolism have found in association with MS. Therefore, in this study, it was aimed to understand the associations between genetic and environmental factors which are thought to be related to vitamin D and cholesterol metabolism in MS. In this study, blood samples were taken from 51 patients who have already diagnosed as MS and 50 healthy individuals. Deoxyribonucleic acid (DNA) was isolated from each peripheral blood. DNA samples were carried out real-time polymerase chain reaction (RT-PCR) to determine rs4588 and rs7041 polymorphisms in *VDBP*, rs2228570 variant in *VDR* and $\varepsilon_1,\varepsilon_2,\varepsilon_3,\varepsilon_4$ variants of *ApoE*. Results were evaluated statistically. At the end of the study, rs2228570 (*Fok I*) and rs4588 mutations were found statistically significant in cases. In addition, the presence of G allele in rs2228570 was found statistically significant in patients. Higher total cholesterol, triglyceride and low density lipoprotein (LDL) level as well as lower high density lipoprotein (HDL) and serum vitamin D levels were found statistically significant in cases. A significant association between high triglyceride level and rs4588 mutation were detected in individuals. LDL level was found statistically low in individuals who have ε_2 genotype, in contrast, it was found high in individuals who have ε_3 variant. In conclusion; vitamin D, cholesterol metabolism and their related polymorphisms are likely to be an association with MS risk, though studies with larger cohorts are still needed.

ÖZET

MULTİPL SKLEROZ HASTALIĞI İLE *APOE*, *VDR* VE *VDBP* GEN MUTASYONLARI ARASINDAKİ İLİŞKİNİN İNCELENMESİ

Multipl skleroz (MS), genetik ve çevresel faktörler tarafından karakterize edilen nörodejeneratif bir hastalıktır ve hastalığın seyri zamanla kötüleşmektedir. D vitamininin veya kolesterolün genetik temellerinin, duyarlılık faktörlerinin bir göstergesi olup olmadığı veya etiyolojisinin önemli olup olmadığı bilinmemektedir. D vitamininin bağışıklık sisteminin normal işlevini sürdürmede önemli bir rol oynadığı, dolayısıyla hem etiyolojik hem de potansiyel olarak hastalık için tedavi edici etkisi olduğu düşünülmektedir. Vitamin D bağlayıcı protein (*VDBP*) ve vitamin D reseptörü (*VDR*) spesifik polimorfizmlerinin bu tür nörodejeneratif hastalıklarla ilişkili olduğu bulunmuştur. Bazı vakalarda, kolesterol metabolizmasıyla ilişkili olduğu bilinen ana faktör olan apolipoprotein E (*ApoE*) gen varyantlarının MS ile ilişkili olduğu saptanmıştır. Bu nedenle, bu çalışmada MS'li hastalarda vitamin D ve kolesterol metabolizması ile ilişkili olduğu düşünülen genetik ve çevresel faktörlerin araştırılması amaçlanmıştır. Çalışmada, MS tanısı almış 51 hastadan ve 50 sağlıklı bireyden kan örnekleri alındı. Öncelikle periferal kandan deoksiribonükleik asit (DNA) izolasyonu yapıldı. DNA örnekleri kullanılarak *VDBP*'de rs4588 ve rs7041 polimorfizmlerini, *VDR*'de rs2228570 ile *ApoE*'nin ε_1,ε_2,ε_3,ε_4 varyantlarını belirlemek için gerçek zamanlı polimeraz zincir reaksiyonu (RT-PZR) gerçekleştirildi. Sonuçlar istatistiksel yöntemler kullanılarak değerlendirildi. Çalışma sonunda; rs2228570 (*Fok I*) ve rs4588, hasta grubunda anlamlı düzeyde yüksek bulundu. Ek olarak hasta grubunda rs2228570 için G alleli varlığı istatistiksel olarak anlamlı düzeyde yüksek bulundu. Gruplar arasında yüksek total kolesterol, trigliserit ve düşük yoğunluklu lipoprotein (LDL) düzeyi ile birlikte düşük miktardaki yüksek yoğunluklu lipoprotein (HDL) ve serum hidroksi vitamin D (25(OH)D) düzeyleri istatistiksel olarak anlamlı bulundu. Bireylerde rs4588 mutasyonu ile yüksek trigliserit düzeyi arasında anlamlı ilişki saptandı. ε_2 genotipine sahip olma ile düşük LDL seviyesi ve ε_3 varyantını taşıma durumu ile yüksek LDL seviyesi arasında istatistiksel olarak anlamlılık tespit edildi. Sonuç olarak, daha büyük kohortları içeren çalışmalara ihtiyaç olsa da D vitamini, kolesterol metabolizması ve bu faktörlerle ilişkili polimorfizmlerin MS ile bağlantılı olabileceği düşünülmektedir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

ε_1	Epsilon 1
ε_2	Epsilon 2
ε_3	Epsilon 3
ε_4	Epsilon 4

$1\alpha,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
24-OHC	24-hydroxycholesterol
25(OH)D	25-hydroxyvitamin D
27-OHC	27-hydroxycholesterol
A	Adenine
Apa I	Restriction enzyme from *Acetobacter pasteurianus*
APCs	Antigen presenting cells
ApoA1	Apolipoprotein A 1
ApoA2	Apolipoprotein A 2
ApoB	Apolipoprotein B
ApoD	Apolipoprotein D
ApoE	Apolipoprotein E
Asp	Asparagine
ATG	Adenine-Thymine-Guanine
BBB	Blood brain barrier
Bsm I	Restriction enzyme from *Bacillus stearothermophilus*
C	Cytosine
CD127	Cluster of differentiation 127
CD20	Cluster of differentiation 20
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CIS	Clinically isolated syndrome
CNS	Central nervous system
CoA	Acetyl-coenzyme A

CRP	C reactive protein
CSF	Cerebrospinal fluid
CYP46A1	Cytochrome P450 family 46 subfamily A member
dH_2O	Distilled water
DIS	Dissemination in time
DIT	Dissemination in space
DNA	Deoxy ribonucleic acid
EBV	Epstein-Barr virus
EC	Endothelial cell
EDSS	Expanded disability status scale
FAM	6-carboxyfluorescein
Fok I	Restriction enzyme from *Flavobacterium okeanokoites*
FS	Functional systems
G	Guanine
Glu	Glutamine
GWAS	Genome-wide association studies
HDL	High density lipoprotein
HLA	Human leukocyte antigen
HLA-DRB1	Human leukocyte antigen, class II, DR beta 1
HLA-DRB1 *1501	Human leukocyte antigen, class II, DR beta 1 variant 1501
HMG-CoA reductase	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
IDL	Intermediate density cholesterol
IL2	Interleukin 2
IL2RA	Interleukin 2 receptor alpha
IL7RA	Interleukin 7 receptor alpha
ISR	International statistical review
LDL	Low density lipoprotein
Lys	Lysine
μL	Microliter
mg/dL	Miligram/Deciliter
MHC	Major histocompatibility complex
MRI	Magnetic resonance image
MS	Multiple sclerosis

MSIF	Multiple Sclerosis International Federation
ng/mL	Nanogram/Mililiter
nmol/L	Nanomol/Liter
PCR	Polymerase chain reaction
pg/mL	Picogram/Mililiter
PPMS	Primary progressive multiple sclerosis
PRMS	Progressive relapsing multiple sclerosis
PTH	Parathyroid hormone
RRMS	Relapsing remitting multiple sclerosis
RTPCR	Real time polymerase chain reaction
RXR	Retinoid X receptor
SD	Standard deviation
SNP	Single nucleotide polymorphism
SPMS	Secondary progressive multiple sclerosis
SPSS	Statistical package for the social science
T	Thymine
Tag I	Restriction enzyme from *Thermus aquaticus*
Th1	T helper 1
Th17	T helper 17
Thr	Threonine
Tregs	Regulatory T cells
USA	United States of America
UVB	Ultraviolet B
V(D)J	Variable (diversity) joining
VDBP	Vitamin D binding protein
VDR	Vitamin D receptor
VDREs	Vitamin D responsive elements
VIC	2-chloro-7phenyl-1,4-dichloro-6-carboxyfluorescein
VEP	Visual evoked potentials
VLDL	Very low density cholesterol
WHO	World Health Organisation

1. INTRODUCTION

1.1. MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a degenerative disease that affects the central nervous system (CNS), characterised by inflammation, chronic autoimmune demyelination, and primary or secondary injury in axons [1, 2]. It usually occurs in ages between 20 and 40, mostly in women with different aspects. Clinically, it concepts variable neurological disabilities in vision and hearing organs, limb weakness or gait abnormalities, bladder dysfunction [1, 3].

The great number of MS patients are mostly confronted with relapsing-remitting attacks or new or re-probable neurological outcomes. Types of MS can subdivide into four major groups regarding the signs and course of the disease.

In the light of the indicating clinical features, disease phases of MS have divided into four major groups. It is still in the dark whether these different phases express characteristic features of a specific disease or, also, whether some variants express different pathophysiologic mechanisms of different disease. Currently, the classifications are based on consensus and the clinical course of the disease.

1.1.1. Clinically Isolated Syndrome (CIS): Initial Phase

Since the clinical continuum of MS is extremely flexible, and eventually, some neurological abnormalities or disabilities may occur with time in those with MS. An acute or subacute neurological dysfunction associated with white-matter lesion is considered as clinical onset in 85 per cent of patients with MS. In this case, this is termed as a clinically isolated syndrome (CIS) characterised by demyelination in optic nerves, brainstem, or spinal cord [4]. Episodes must last for at least 24 hours in patients, and it is considered as MS characteristic feature but may not meet the fundamental standards to diagnose MS since whether CIS may or may not go on to develop MS. In this case, radiological scan techniques such as magnetic resonance image (MRI) are crucial. As an example, the presence of classical demyelination lesions in MRI results from spinal cord and brain has defined as a most important determinant of the occurrence of a second relapse in CIS

patients [5]. However, CIS is not included in initiate phase MS markers. In the 2013 revision defining the certain criteria for MS, it is recommended that CIS should be enclosed in different phenotypes of MS [6].

1.1.2. Relapsing-Remitting MS (RRMS)

Relapsing-remitting or partial remitting is one of the most seen neurologic loss of function in a patient with MS. This neurologic loss can be occurred fast, constantly observed over couple hours, or worse, over days and weeks. Clinical outcomes of the disease can last days, weeks, and even months then may go on decreased degrees. Within early diagnosis, relapses (also known as exacerbation, bout or attack) can eliminate, but in time, it becomes harder to recover since the course of the disease progresses. The frequency and the number of episodes vary considerably and have significant effects on the prognosis of the MS. If there are long gaps between attacks and speakable improvement is mentioned on, then it may be said that disease shows good progress in the level of recovery. RRMS mostly shows itself in younger women population, fortunately, it may be possible to cure via acute anti-inflammatory agents such as steroids or immune-modifying therapies [3, 7]. It is foreseen that 85 per cent of MS patients had already RRMS and 80 per cent of them have observed secondary progressive MS (SPMS) development [8, 9].

1.1.3. Secondary Progressive MS (SPMS)

Most RRMS patients later develop SPMS by age, and the rare or recurrent progressive disability characterises it [10]. The most common progression for SPMS is an axonal loss [11]. Unfortunately, there are no typical clinical criteria which allow clinicians to decide where it is converted from RRMS to SPMS. It is recommended that data come from clinical trials and patient’s own story with MS should be considered to determine the level of disease [6]. As the disease progresses, the number of relapses increases and disability worsen, type of disease is termed as primary progressive multiple sclerosis.

1.1.4. Primary Progressive MS (PPMS)

PPMS (nearly 10 per cent portion in total individuals with MS) is characterised by recurrent, gradual or non-progressive recurrence from the onset and typically occurs in a population of men with myelopathy progressing with age [10]. Patients with PPMS, even the disease symptoms occur over months to years, have no ability to remember the date of relapses. Disease progression varies greatly: Some patients experience significant disability for one to two years while others progress for more than several decades. Contrast to RRMS, individuals with PPMS are much older at their 40s to 60s. The most prominent and common progression is spinal cord dysfunction [7]. PPMS differs from RRMS in terms of its pathophysiology.

1.1.5. Progressive-Relapsing MS (PRMS)

By five per cent of MS population, progressive relapsing MS (PRMS) is the least frequent version of MS by far, which has seen as progressive neuron dysfunction and different acute relapses from the beginning [8, 12]. Few information is available about the clinical features and prognosis of PRMS since it is observed in rare ratio. Most of the time, it is hard to distinguish PRMS from PPMS considering their similar clinical features and some specialist may use the same terminology for both types [12].

1.2. DISEASE DIAGNOSIS

There is currently no single diagnostic test for MS due to its aetiology is not completely understood. On the strength of the development in radiological scans, especially MRI, as well as the advancement in the new disease-modifying drugs; after a few revisions, the current “McDonald Criteria (2010)” is improved allowing MS to be diagnosed earlier [13-15]. These criteria are based on the dissemination in space (DIS) and time (DIS) by MRI and clinical data [10] DIS can be demonstrated by a presence of at least 1 T2 hiperintensive lesion in 2 out of 4 typical locations: Periventricular, subcortical, infratentorial and/or in the spinal cord. Lesions in gadolinium-enhancing are not included in the current DIS critics. Regarding to second revision of criteria based on DIT, it may be

characterised by a presence of a new T2 hiperintensive lesion or a gadolinium-enhancing lesion in the next MRI scan or by a presence of both gadolinium-enhancing and non-enhancing lesions in the first MRI scan. The time of which MRI scan was taken is not critical for both case [14].

Nowadays, its characteristic phenomena are based on the clinical outcomes (patient's story), supported by helpful tests such as neuroimaging (MRI), in some cases by cerebrospinal fluid (CSF) as well as visual evoked potentials (VEP) analysis alongside McDonald criteria that are used for the clinician to get an MS diagnosis of the individual [16]. Evoked potential tests can be useful as they can show functional disorders on afferent or efferent pathways that are not clinically examined. As similar, analysis of CSF could be very helpful to provide diagnose [3]. Also, disability status of a patient with MS is scored by expanded disability status scale (EDSS) which is outcome measurement scale developed by Kurtzke. Scale allows measuring the maximal function of each patient based on neurological examination. The scale consists of 10 steps and is prepared to have a space of 0.5 units between both steps. Steps from 1.0 to 4.5 refer to MS patients who are able to walk by themselves. These steps arise due to the disability measurements that occur in the eight functional systems (FS) [17].

Hemogram (total blood cell count), sedimentation blood test, C-reactive protein (CRP) test, thyroid-liver-kidney function test, vitamin D level measurements are among the other tests used for differential diagnosis of MS. Patient's smoke intake, the case of EBV infection can also be provided.

1.3. EPIDEMIOLOGY OF MS

Multiple sclerosis is known to be one of the most common neurodegenerative disorders. Unfortunately, in many countries, this neurologic disease is mostly occurred in young adults without causing trauma. Globally, population of MS is approximated as 2.3 million in 2013 [19, 20]. The disease is scattered globally: its prevalence ranges from less than five cases per 100,000 people in tropical regions or Asia and, hence, between 100-200 cases in 100,000 individuals in mild areas, especially in the region of Northern European which is highly populated (Figure 1.1) [21, 22].

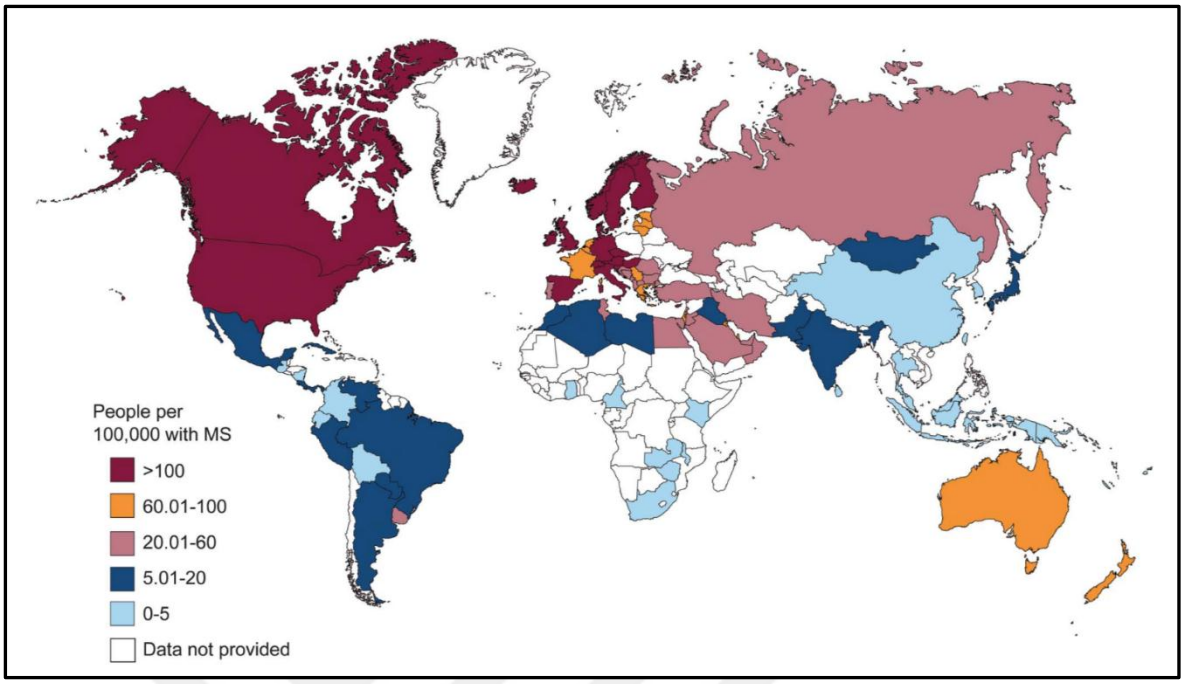

Figure 1.1. The geographical distribution of multiple sclerosis per 100,000 population. Taken from Browne *et al.* [19]

Data coming from study collaboration of World Health Organization (WHO) and Multiple Sclerosis International Federation (MSIF) indicate that there is a peak in the prevalence of MS since 2008 [19, 21, 23] The increase in MS prevalence reported between 2008 and 2013 can be attributed partly to survival (both MS and the general population is more prevalent) and increased incidence of MS in some countries, it can reflect in improvements in diagnosis (MRI techniques, increased numbers of neurologists). MS reporting may also link to the publications of epidemiological surveys [19].

It is known that patients with MS over than 65 years are more inclined to have PPMS (29 per cent), SPMS (26 per cent) rather than younger equivalents of whom have RRMS (57 per cent) [24]. This can be the result of that repairs, remyelination and other physiological functions become less efficient as the disease progresses. However, it should be noted that not every RRMS develop SPMS following disease courses or by aging [25]. Although aging is a critical factor for MS, the disease is not only presented in older elders. Typically, young adults between the ages of 20 and 50 appear at the peak of the age of 30, and childhood or old age occasionally arises [21].

Female gender has been considered as a potential influencer of MS, more often RRMS [10, 26]. Its frequency is higher in women than men (2:1 ratio) [21]. These differences demonstrate that genetic and hormonal triggers take part in in sensitisation, especially in the identification of genes on X and Y chromosomes. In support of this hypothesis, MS has been observed to be relatively stagnant during pregnancy, but with increasing frequency after birth. In the case of primary progressive MS, the subtype of a disease, seen in elderly individuals, the ratio of female to male has been found 1:1 [7].

1.4. CURRENT UNDERSTANDING OF THE DISEASE MECHANISM

Demyelination is a crucial pathological process which effects neuron fate. It is termed as the destruction of myelin sheath with preservation of axis cylinder continuity [27]. The distinguishing feature of the disease is the occurrence of sclerotic plaque displays the last phase of course characterised by inflammation, de- or re-myelination as well as axonal degeneration [10]. Even though the idea is squeezed, the arrangement and relationship of these different components continue to be completely unresolved. Myelin is produced by adult oligodendrocytes, each of them interacts with the short segments to axons 20-40 in the white matter pathways of the CNS [28]. MS is considered as one of the autoimmune diseases which inflammatory demyelination occurs in CNS with a variety of clinical presentations [29].

Some authorities thought that the disease process is the result of the increase of autoreactive lymphocyte migration throughout the blood-brain barrier (BBB**)**. The transition results in imperfections that allow these cells to develop an immune response. Lymphocytes of MS patients are ineffective in suppressing effector cells. These cells cannot effectively cause to decrease in stimulation due to overexpression of β-arrestin 1, an essential promoter of naïve and activated CD4 (cluster of differentiation 4) + T cell survival. When the local regulatory mechanisms broke down within the brain, this causes the inflammation controlled by perivascular CD8 (cluster of differentiation 8) + cell, triggers plaque accumulation in different parts of CNS. Studies have shown that the main role attributed to experimental allergic encephalomyelitis T-helper 1 (Th1) (interferon-γ secretory) cells is misrepresented. Inflammation is mediated by T cells which produces

interleukin-17 under the control of interleukin-23. Interleukin 17 and 22 allow T-helper 17 (Th17) cells to penetrate the brain-brain barrier, where Th17 cells can kill neurons [29].

An alternative hypothesis is that the first dysfunction occurs in the CNS and is not immunologic or inflammatory [30]. Following the initial damage, CNS antigens infiltrate into lymph nodes and this activates T and other immune cells, eventually they can pass through nervous system. Thus, regardless of the origin of the MS pathology, immune cell subpopulations are activated and migrate to the CNS. This is the distinctive features of the MS pathology: demyelination, oligodendrocyte destruction and damage in axons [31].

For activation of T cell, there should be an antigen occupation. (Figure 1.2). A pathogen is captured and decomposed by antigen presenting cells (APCs). Subsequently, APCs move towards to lymph nodes that has the pathogenic antigen associated with the major histocompatibility complex (MHC) on the surface of cells. Naive T cells within its receptors recognises the composition of antigen / MHC which is known as a first signal. A second is needed to activate, proliferate and differentiate T cells to effector cells [32].

B cells also have a critical role in the progression of MS disease considering that current monoclonal antibodies targeting the B cell antigen, known as CD20 (cluster of differentiation 20), are effective therapies in MS [32]. Oligoclonal bands which is responsible from the production of immunoglobulins in cerebrospinal fluid are commonly observed in MS patients. Many follicle-like structures are likely to place on the meninges of many MS patients. It is believed that B cells produce of antibodies targeting CNS, like the ones in the Ranvier node, are associated with several functions in cells such as antigen production and assist T cells [33].

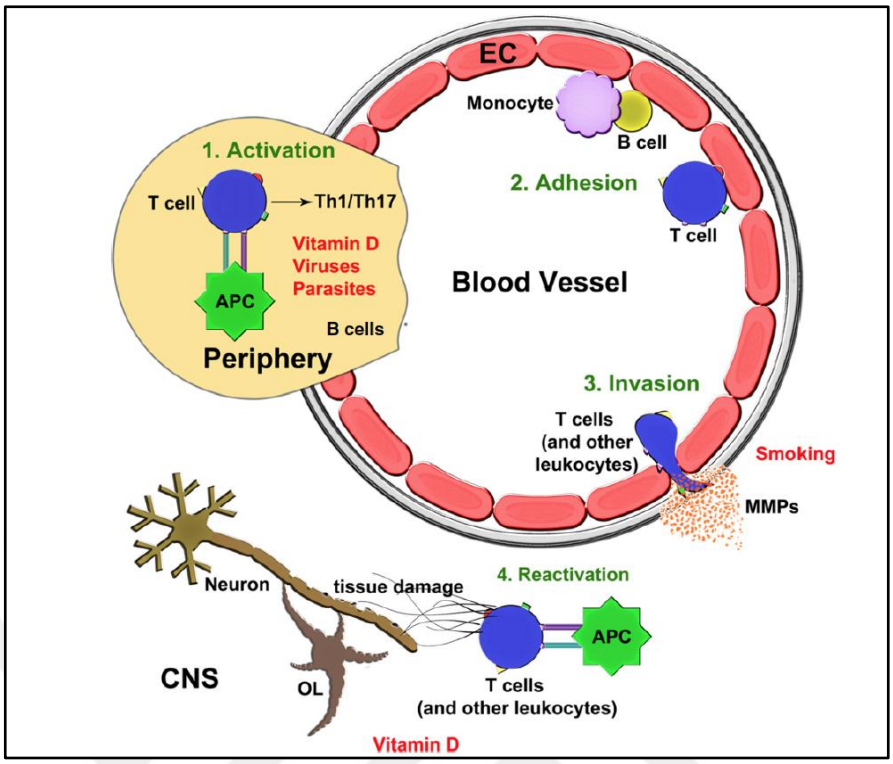

Figure 1.2. Steps in the immune-pathogenesis of MS. Taken from Koch *et al.* [32]

1.5. INFLUENCERS LINKED TO MS

Even though the cause of MS is still unknown, it seems clear that there is immune-mediated mechanism characterised by lesion of demyelination and gliosis in multiple locations within CNS white matter [3]. It is believed that both genetics and environmental factors play roles in the pathogenesis of MS considering it is a complex disease. Newly concepts also concluded that immune dysregulation can be added to those relative factors [9]. Current data are gathered from animal model studies and from genome-wide association studies (GWAS) and meta-analysis of different ethnic origins.

1.5.1. Genetics

Considering MS holds heterogeneity; it would be wrong to evaluate disease progression through a single gene. Several gene polymorphisms have been identified through MS disease. Here, it is presented the most commonly found associated genes according to results of large cohort studies.

The human leukocyte antigen (HLA) gene subset located on the 6p21.3 chromosome is the most potential loci of sensitivity for the MS for over 30 years. It is gently identifiable both by candidate gene relation as well as by whole-genome linkage applications. Primary signal comes from MHC, HLA class II, DR beta 1 (*HLA-DRB1*) gene [10, 22, 34, 35]. **1501* risk haplotype is predicted as the strongest MS risk factor in some ethnic groups, and its expression can be increased 1.6 times following stimulation with 1,25-dihydroxy vitamin D [36]. Larger data-set come from genome projects have identified new MS-related loci within HLA [21].

One of the recent additional genes that relate to MS is interleukin seven receptor alpha (*IL7RA*) which is also known as CD127 (cluster of differentiation 127) [22]. It is located on chromosome 5p13 and plays an important role in V(D)J recombination which is occurred in lymphocyte development/ cell survival and formation of the immune response [37]. rs6897932 polymorphism which is located on exon 6 of *IL7RA* was eighth most strongly associated with MS in several studies [22, 38, 39].

The other most related gene to MS is considered as interleukin two receptor alpha (*IL2RA*). *IL2RA* considered in several disease pathogeneses like diabetes. Gene particularly takes part in process responsible for differentiation of T helper cell, which is found to be related to the MS development [40]. The *IL2RA* gene has been illustrated suitability in MS progression. It was also found that a polymorphism locates in *IL2* promoter region was enhanced MS risk, but GWAS has shown that *IL2* polymorphisms do not hold statistically significance for MS [41].

1.5.2. Environmental Risk Factors

Studies indicated the effect of sunlight exposure and ultraviolet B (UVB) radiation on MS disease had been made since the beginning of 20th century. Case-control studies conducted regarding the information how individuals spend their time in open spaces and use indoors or outdoors as workspaces have provided results in support of this occasion [21]. The study, carried out on Tasmanian, indicated that the frequency of MS who had chance to sun bathing for an average of two or three hours per day between the ages of six and 15 had declined considerably [42]. The mortality rate due to MS has been shown to be negatively related to exposure to solar radiation, either residential or occupational [43]. In

another study, it was statistically supported that the possible risk of skin cancer related to sunlight found lower in individuals with MS, suggesting that exposure to sunlight may be a protective for MS. There was no correlation between other types of cancer and other neurodegenerative diseases in the study. The most important limitation of such studies is that exposure time to sunlight was severely reduced by MS patients who preferred to work indoors because of insomnia and physical disabilities, and this prevents secondary assessment [44].

It has been suggested that there are two common hypotheses in the aetiology of MS. Mostly acceptable one is poliomyelitis-hygiene hypothesis that states an interaction with the active substance throughout early childhood or infection protects from MS while late interaction causes the disease. As a second, Kurtzke's prevalence hypothesis, another theory based on the epidemics of the Faroe Islands after the World War II, suggests that MS originated from a more common pathogen in regions with high MS frequency [45]. Varicella, rubella, varicella zoster and mumps viruses are biologically acceptable infectious agents as MS pathogens, but epidemiological and laboratory studies showed that Epstein-Barr virus (EBV) plays an important role for MS. EBV, an agent of the ubiquitous herpesvirus, spreads widely and can cause persistent asymptomatic infection [22]. Studies have focused on EBV because expression of EBV antigens has found considerably higher levels in CSF [46].

When saliva exchange becomes more direct during adolescence, mononuclear infections closely related to MS are observed. Most episodes of MS epidemiology have resulted in paralysis with infection, thus they have parallel outcomes; in areas where MS is not frequent, younger people have the higher percentage in terms of infection with EBV, while, in regions where MS is more frequent, rate of EBV positivity cannot reach that high until puberty. In researchers' findings, the population with EBV seropositive is higher than 90 per cent and this suggests that early contact with EBV is likely to have protective effect for MS. This also smooths out the high incidence of MS with high socioeconomic status as well as the low frequency of MS in black people and individuals from Asia. It is believed that virus is the common agent that increases MS ratio in Australia from north to south [47]. However, studies have shown that about 93 per cent of MS patients do not have intrathecal anti-EBV antibody synthesis. Therefore, the relationship of EBV to adult MS should be investigated, and its role in pathogenesis should continue to be examined [48].

Physical and emotional stresses have been considered as potential influencer for MS. One of the important issues to consider is the definition of the stress and the time of impose. In one study, any relationship was excluded except for impact between cranial trauma and MS [49]. Results explaining effects of emotional stress in MS have found weak, however emotional stress effectors are likely to be informal factors. A Danish cohort study indicated the association between MS and child's death was highly significant [50]. In this study, most of the result and selection cohorts from other case-control studies were avoided. Yet, it is not possible to rule out that the death of a child is merely a clue rather than an informal factor, and that this may be related to differences in behaviour or in the environment [51]. A study reported that more MS patients were more stressed than controls, two years before the onset of illness [52]. Another study concluded that MS patients had more serious life events than controls in the early period of disease [53]. Nevertheless, studies show that stress is among MS enhancing factors that cannot be proven [21].

Smoking has been criticised as potential influencer for MS. In a study, evidences showed that smoking increases awareness of disease, it has been shown that the relative prevalence of MS in patients with smoking is higher than no-smokers [54]. Studies showed that passive smoking increases the risk of MS in child and adults [55, 56]. It also affects the inflammatory results in MS. In a study of patients with CIS, occurrence of clinically definite MS was higher for smokers than for non-smokers [57]. Smokers who had more lesions and partially lost their brain compared to non-smokers have been concluded in an imaging cross-sectional study [58]. Similar results have been obtained in another study which patients were taken under control approximately 2.8 years [59]. However, the relationship between cigarette and MS disease is still unclear. In a study, smoking has not been found in association with either the risk of secondary progression or the risk of reaching EDSS 4.0 or EDSS 6.0 [60]. Contrast to this, a study has shown a risky association between cigarette and secondary progressive course [61]. In a recent cohort study among large population, cigarette smoking has increased the risk of secondary progression, however no peak in EDSS scores [59]; in another study, cigarette went up the EDSS scores over a two-year follow-up period [62]. For this reason, although smoking appears to affect the early progression of disease, studies are still performed to determine the effects smoking on early and late MS terms.

While there are many environmental factors that may be associated with MS, there is not enough evidence unrelated to most MS gradients. These include organic solvents, dental amalgam, physical trauma, dietary fat, dietary antioxidants, high education status and oestrogen, which can alter the immune response. Apart from these, it is also possible to mention other environmental factors that may be associated with MS: tetanus toxoid inoculation, antibiotic use and antihistamines and increased uric acid in the blood [21].

1.6. VITAMIN D

Vitamin D was first shown in 1919 by Sir Edward Mellanby, who fed dogs with oat meal and kept them indoors, leading to the development of rickets [63]. In 1922, McCollum found that cod liver oil contained a vitamin that was effective in skeletal mineralization and healing of rickets, and then he named it "vitamin D" [64]. In the beginning of the 21st century, researchers thought that vitamin D might have crucial part in the development of MS [65]. It is conducted that such environmental factor plays a role in the susceptibility of MS progress rather than genetic factors [20].

1.6.1. Source & Metabolism

The active vitamin D in mammals in vivo presents by conversion of 7-dehydrocholesterol to vitamin D_3 [66, 67]. Vitamin D_3, a secosteroid hormone, is also known as cholecalciferol, and it is taken from two main sources: it is synthesized in the skin via UVB in daylight or taken in the diet (oily fish, milk, cereal and some orange juice, cheeses and vitamin supplements) [36]. Nonetheless, unlike the diet, it is possible to get about 25 times more vitamin D by exposuring to the sunlight for 20 minutes during summer [68, 69].

1,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) is an active form of vitamin D_3. 25-hydroxylase subsequently hydroxylate vitamin D_3 to $25(OH)D_3$ initially in the liver and then by 1-a hydroxylase, then vitamin D_3 forms $1,25(OH)_2D_3$ in kidneys, frequently [70]. This active form plays a critical role in bone, calcium and phosphate metabolism [36, 65, 71, 72].

Genomic actions in the active form of hormone are mediated by the vitamin D receptor (VDR), a transcription factor that binds $1\alpha,25(OH)_2D_3$, the active vitamin D metabolite [73, 74]. VDR is composed of three main domains: The N-terminal DNA binding domain with two zinc fingers that bind vitamin D responsive elements (VDREs) in the DNA sequence of vitamin D-mediated genes, the C-terminal ligand binding domain, and the hinge region which binds these two domains together [73]. In order to form a heterodimer structure to connect VDREs, VDR needs a heterodimeric partner retinoid X receptor (RXR) that releases corepressors and bring coactivator proteins together after transcription complex assembly [71, 74, 75].

VDREs can be obtained in the promoter region of many nuclear genes. It locates in the promoter region of the HLA DRB1*1501 allele. This takes attention because this specific allele and this region of the genome have been systematically linked to MS pathogenesis in Caucasian populations for a long time [76]. As recently recorded, vitamin D complex with cofactors binds to the VDRE, then organises the expression of a various number of genes which have been affected positively or negatively [3].

The effects of $1\alpha,25(OH)_2D_3$ and its analogues occur by binding to VDR. Comparison of the VDBP (vitamin D binding protein) binding domain and that of VDR structure shows that they are not similar. The vitamin D-binding site in the VDR is a closed receptor formed in the internal structure of the receptor, whereas the VDBP binding site locates at the surface of the molecule and partially in contiguity with the surrounding liquid compound [77]. The VDBP supports transport of all lipid soluble vitamin D analogues. A study concluded that analogues binding VDBP has lower efficiency than $1\alpha,25\text{-}(OH)_2D_3$. This transport to the target sites occurs if these analogues can find alternative plasma carrier proteins rather than VDBP. However, these analogues lead to changes from tissue to tissue and hepatic clearance which is likely to be on the natural substances of vitamin D. Recently, studies clear the importance of VBDP by showing the $1\alpha,25(OH)_2D_3$ clearance which occurs faster in the absence of VDBP within *VDBP* knockout mouse [78].

1.6.2. Vitamin D & MS Risk

MS is characterised by inflammation and axonal damage within the CNS. Its aetiology is not completely understood; however, it is now clear that an interplay between genetic and

environmental factors induces disease pathogenesis. One of the influencers which affects MS risk is UVB and it is particularly related to vitamin D metabolism. Studies conducted MS patients residing at increasing latitudes concluded that vitamin D may have protective effect on skins of individuals considering these people have long sun exposure time [78]. Studies revealed the antagonist relation between the concentration of 25-hydroxyvitamin D 25(OH)D and MS risk and the impact of vitamin D on disease activity [80-84].

Major sources for 25(OH)D intake are considered as sun exposure, vitamin supplement/diet and metabolism related genes (Figure 1.3). Also, the interaction between vitamin D and MS risk could be caused by latitude, smoking, or intake of other ingredients [85]. Considering genetic aspects and diet as well as vitamin D intake, and the relationship between 25(OH)D and MS can be examined by multiple effectors. Nevertheless, inter-individual variation at each factor 25(OH)D concentrations are only modest and therefore has a limited potential for prejudice. Though it is difficult to remove it from the confounding, it is generally assumed that the bias from the uncontrolled confounding is low, unless it is strongly related to both exposures and outcome, and there is no information to control it (Figure 1.3) [36, 85].

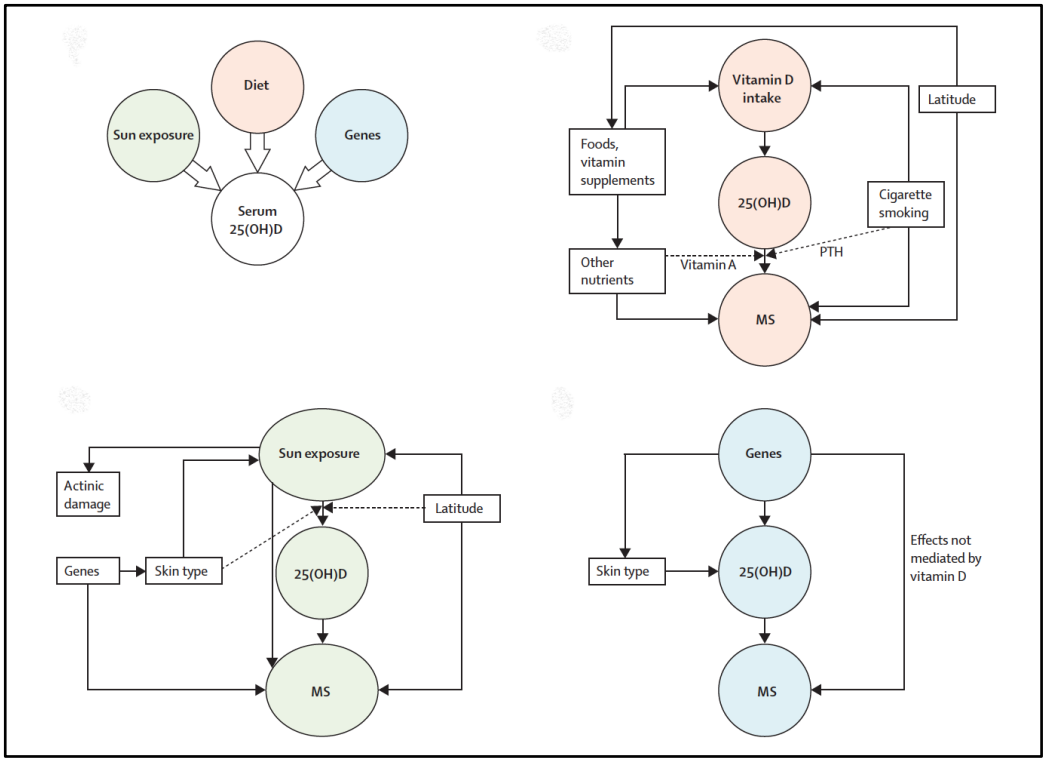

Figure 1.3. Possible influencers and the interactions between vitamin D and MS risk. Taken from Ascherio *et al.* [36]

Vitamin D and its critical role in calcium metabolism, and the bone preservation composite have been recognised for years [66, 86]. As a result, vitamin D has important immune regulatory role by inhibiting MHC class II and signal production of APCs then, regulates regulatory T cells (Tregs) [87, 88].

VDBP, an α2-globulin whose gene composed of 13 exons and 12 introns transports the active form of vitamin D to targets where it shows its function through VDR. [89, 90].

Two common single nucleotide polymorphisms (SNPs) of VDBP locate in exon 11 and they convert glutamic acid (Glu) to aspartic acid (Asp) in rs7041 (G>T) and threonine (Thr) amino acid to lysine (Lys) in rs4588 (C>A) [89, 91]. This causes to the formation of three common VDBP isoforms: Gc1f (rapid isoform; Asp416, Thr420), Gc1s (slow isoform; Glu416, Thr420) and Gc2 (Asp416, Lys420) which differ in amino acid sequencing and vitamin D binding affinity [88].

As it is written above, vitamin D regulates function through the ubiquitously expressed VDR. *VDR* locates in the 12q13 chromosome region and is known to be present in almost all cells within body [92]. Many studies related to *VDR*, four SNPs of *VDR* have possibly interaction with MS disease; three at the 3' end of the *VDR* gene (*Taq I, Bsm I, Apa I*) and one at the 5’ end (*Fok I*). The rs2228570 polymorphism observed in the second exon region of the gene is identified during translation of the ATG promoter region in the *VDR* complementary DNA. T>C polymorphism (ATG<ACG) was observed in the first translation initiation codon resulting with 3 amino acid protein shortening. This initial codon polymorphism is also referred to as *Fok I* polymorphism since it is defined using the *Fok I* restriction enzyme [93]. It should be kept in mind that the *Fok I* variant carry rs10735810 mutation, and then, these two together are named as rs22258570 [94]. The association between SNPs and MS is still under investigation, study characteristics and ethnicity impact their relationship [95].

1.7. APOLIPOPROTEIN E (APOE)

1.7.1. Mechanism of Action

Cholesterol is a fundamental compound of cellular and myelin membrane. It plays a specific role as cofactor for signal molecules and a compound that participates in the chemical reactions that produces another compound like steroid hormones, bile acids as well as vitamin D [96]. Its synthesis, transport and metabolism is highly complex. Synthesis of cholesterol is firstly triggered by transformation of acetyl-coenzyme A (CoA) to mevalonate through 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA-reductase). Mevalonate is then converted to lanosterol and then cholesterol is formed. Cholesterol is water-insoluble and it flows in the blood by binding to lipid transporters (lipoproteins) and it requires apolipoproteins which is consist of five different forms for formation. 95 per cent of low density lipoprotein (LDL), 60 per cent of intermediate density cholesterol (IDL) and 30 per cent of very low density cholesterol (VLDL) is composed of apoliporotein B (ApoB) [97]. They all are synthesized within periphery, however that cannot pass through BBB [98, 99]. High density lipoprotein (HDL) is mainly composed of ApoA1 and A2 and its minor components are termed as apolipoprotein E (ApoE) and apolipoprotein D (ApoD). The periphery, and, also the brain are the locations that HDL and its components are synthesized. And unlike the other types of lipoprotein, few amount of HDL can pass through BBB [100].

Cholesterol metabolism is mainly a result of oxidation and subsequent oxidative conversion by CYP46A1 to 24-hydroxycholesterol (24-OHC) in neuron cells, 27-hydroxycholesterol (27-OHC) controlled by the P450 enzyme CYP27A1 in out of CNS [101]. After conversion, 99 per cent of the 24-OHC is circulating, and one per cent of the 24-OHC goes to CSF. Both form is then transmuted to bile acids within liver. Also, HDL, ApoA1, ApoD and ApoE are used for transportation of excess products [100].

ApoE is consisting of a 299-amino acid lipid carrier protein that has critic part in lipoprotein homeostasis and the lipoprotein transport system, and it is found in senile plaques vascular amyloid (β-amyloid), and neurofibrillary nodules. The *ApoE* gene encoding the ApoE protein is localized in the chromosomal region of 19q13.2 and contains four exons. The genetically three variants of alleles are termed as ε_2, ε_3 and ε_4. The ε_3 / ε_3

phenotype is the most common phenotype caused by these alleles [102]. ε_1 is a minor allelic form which is present in less than 0,1 per cent of the population. Allele variants can be distinguished by several conjunctions of the arginine and cysteine at 112 and 158 position, this affects mechanism of action and functional features of ApoE [103]. ApoE modulates CNS inflammation and influences repair following injury with allele-specific effects. ε_4 is associated with less effective downregulation of inflammatory cytokines in the brain than ε_3 at an earlier age in Alzheimer patients [35].

1.7.2. ApoE & MS Risk

The association between cholesterol and MS is firstly investigated in 1926 [104]. In those times, cholesterol was considered as "wasting of nerve structures" when it is found in CSF. In contrast, data were proved that ester formation of free cholesterol is relatively distinguishable features of demyelination in CNS [105].

A wide number of studies have concluded whether ApoE contributes to MS susceptibility or influences the disease course. Relations between MS disease and cholesterol, oxysterols and ApoE incidence and CSF levels have been reported [35, 103, 106, 107]. The ε_4 allele was also found to be overexpressed in PPMS patients (53,3 per cent) relative to healthy controls (8,9 per cent) or RRMS patients (24,4 per cent) in a Hungarian case-control study of 45 PPMS, 45 RRMS, and 45 healthy controls [108]. Contrast to this studies, in the United Kingdom, there was no interplay between *ApoE* ε_4 allele and PPMS in a case-control study of 50 PPMS patients and 159 healthy controls, and *ApoE* ε_4 and disease severity were not correlated [109].

2. MATERIALS

2.1. MATERIALS

- Ethanol, Absolute, Sigma Aldrich, St. Louis, U.S.A.
- MicroAmp Fast Optical 96-Well Reaction Plate (0.1 mL), Applied Biosystems ™, Foster City, CA, USA
- Optical Adhesive Covers, Applied Biosystems™, Foster City, CA, USA
- Proteinase K, QIAGEN GmBH, Hilden, Germany
- Sterile Filtered Pipette Tips 1000, 200, 10 µL, Capp, Nordhausen, Germany
- QIAamp DNA Blood Mini Kit, QIAGEN GmBH, Hilden, Germany
- Taqman® SNP Genotyping Assays, Applied Biosystems™, Foster City, CA, USA
- Taqman® Universal Master Mix, Applied Biosystems™, Foster City, CA, USA
- Water Nuclease Free, Thermo Scientific, Rockford, IL, USA

2.2. GENOTYPING ASSAYS

Each predesigned TaqMan SNP genotyping assay in this study was included two allele-specific TaqMan probes carrying attached fluorescent dyes and a primer pair to detect particular targets. Each Taqman SNPs were labelled with 2-chloro-7phenyl-1,4-dichloro-6-carboxyfluorescein (VIC) and 6-carboxyfluorescein (FAM) fluorescent dyes by manufacturer for the study (Table 2.1).

Table 2.1. Names of SNPs in the study

Name	Assay ID	Region
rs429358	C_3084793_20	GCGCGGACATGGAGGACGTG[C/T]GCGGCCGCCTGGTGCAGTAC
rs7412	C_904973_10	ATGCCGATGACCTGCAGAAG[C/T]GCCTGGCAGTGTACCAGGCC
rs4588	C_8278879_20	ATTGCCTGATGCCACACCCA[A/C/T]GGAACTGGCAAAGCTGGTAA
rs7041	C_3133594_30	CTAAAAGCAAAATTGCCTGA[A/G/T]GCCACACCCACGGAACTGGC
rs2228570	C_12060045_20	GCTTGCTGTTCTTACAGGGA[A/C/G/T]GGAGGCAATGGCGGCCAGCA

2.3. INSTRUMENTS

- Real-Time PCR System 7500 Fast Real-Time PCR System, Applied Biosystems™, Foster City, CA, USA
- Centrifuge, Spectrafuge 24D, Labnet International, Inc
- Heater, Bioer
- Nanodrop™ 2000/C Spectrophotometer, Thermo Scientific, Rockford, IL, USA
- Micro Pipettes (1000, 200, 20, 10 μL), Thermo Scientific, Rockford, IL, USA
- Vortex, IKA
- Water Bath

3. METHODS

3.1. CHARACTERISATION OF STUDY POPULATION

101 volunteers who have applied Erenköy Hospital for Psychiatric and Neurological Diseases, Neurology Polyclinic and Yeditepe University Hospital were recruited to the study. Fifty-one patients who were diagnosed as RRMS and fifty healthy individuals were enrolled as control group. Hemogram, sedimentation blood test, CRP test, thyroid-liver-kidney function test, vitamin D level measurements have been done. Patient's smoke intake and the case of EBV infection were exclusion criteria of study.

When the patients return to the hospital for follow-up purposes, they were included to the study once they have received the necessary information about the study, and two mL whole blood sample was obtained from each for molecular analysis.

Written permission was obtained from all participants, and study was performed by the human ethical committee of Yeditepe University approval.

3.2. GENE SELECTION AND EXPERIMENTAL DESIGN

Candidate genes selection was performed regarding to their potency which involves in either one or more of the following:

a) Pathogenicity of MS

b) Common processes of immunology and neurodegeneration.

Table 3.1 displays all the analysed genes and their SNP numbers along with their localization in human DNA. It should be considered that the current candidate genes are composed of genetic factors which are thought to be related to pathophysiology of MS indirectly.

Table 3.1. Names of the genes in the study

Name of Gene	Gene Location	SNP/Variant
VDBP (Vitamin D Binding Protein)	4q13.3 (13 exon)	rs4588
		rs7041
VDR (Vitamin D Receptor)	12q13.11 (10 exon)	rs2228570
ApoE (Apolipoprotein E)	19q13.2 (4 exon)	rs7412
		rs429358

Molecular analysis of the study was performed in Yeditepe University, Faculty of Medicine, Department of Medical Biology. Genomic DNA samples of 101 individuals were extracted from a whole blood sample using QIAamp DNA Blood Mini kit (Qiagen, GmBH, Hilden, Germany) regarding following instructions:

- Buffer AW1 and AW2 were supplied as a concentrated form in kit. Before first use, the appropriate amount of absolute ethanol was added into each as indicated on the bottles.
- 20 µL proteinase K was added into the bottoms of each 1.5 mL micro centrifuge tube.
- 200 µL whole blood sample was added to the tubes.
- Buffer AL as 200 µL was added to each sample. Sample were mixed by vortexing for 15 s.
- Samples were incubated at 56°C for 10 minutes in water bath. Then short term centrifuge at the highest speed was performed.
- 200 µL absolute ethanol was added to each sample, and the samples were mixed in vortex for 15 s. After mixing, briefly centrifugation of the 1.5 ml micro centrifuge tubes was done to eliminate drops from the inside of the lid.
- The mixtures were transferred to spin columns (in a two mL collection tubes) then, centrifuged at 8000 rpm for 1 minute.
- Spin columns were placed in clean collection tubes, and flow through of each was discarded.

- Buffer AW1 as an amount of 500 μL was added to the spin columns. Samples were centrifuged at 8000 rpm for one minute. The spin columns were placed in a clean collection tubes, and the flow through was discarded.
- 500 μL Buffer AW2 was added carefully to the spin columns. Samples were centrifuged at 14,800 rpm for three minutes.
- Spin columns were placed in a clean number-labelled 1.5 mL micro centrifuge tubes, and the filtrate were discarded.
- 200 μL elution buffer (AE) was added into each tube. Samples were incubated at room temperature (15–25°C) for one minute, and then centrifuged at 8000 rpm for one min.

DNA samples from each patient were subsequently quantified by using Nanodrop spectrophotometer. Samples with a DNA concentration of 10 ng/μL and above were included in the study. Samples were stored at -20°C until use. Polymerase chain reaction (PCR) samples were prepared for each SNP as follows in Table 3.2. Reaction mix was prepared and gently pipetting. Mixture was dispended into PCR tubes.

Table 3.2. Preparation of PCR reaction samples for each SNP

Compounds	**/well**	**101X (Number of Samples)**
Master Mix	5 μL	505 μL
dH_2O	3.75 μL	378.75 μL
Assay (Taqman)	0.25 μL	25.25 μL
Template DNA	1 μL*	-

*Template DNA was added separately to each well after reaction mix was distributed into each reaction wells.

10 μL PCR reactions were used for amplification of each genomic DNA under the following cycling conditions: holding stage at 60°C for 10 minutes, initial denaturation at 95°C for 10 minutes followed by 40 cycles for 15 seconds at 95°C and for one minute at 60°C and final extension at 60°C for one minute.

Samples of reaction were conducted by 7500 Fast Real-Time PCR System (Applied Biosystems™, Foster City, CA, USA) according to the following instructions.

- In order to create new experiment in software of the PCR device, CDs that is given by manufacturer must be used.
- New Experiment >> Assay Name >> Color selection for VIC and FAM >> OKEY
- Assignment of SNP for each sample >> Concentration of dye (2X) >> Check volume of wells (10 µL) >> Run Experiment (Approximately 90 minutes)

The software of system presented allelic discrimination of each polymorphism (Figure 3.1). Each dot represented individuals who were involved in the study. Blue dots were used to describe having homozygous variant while red dots were used for determining wild type genotype and green dots were for heterozygous genotype.

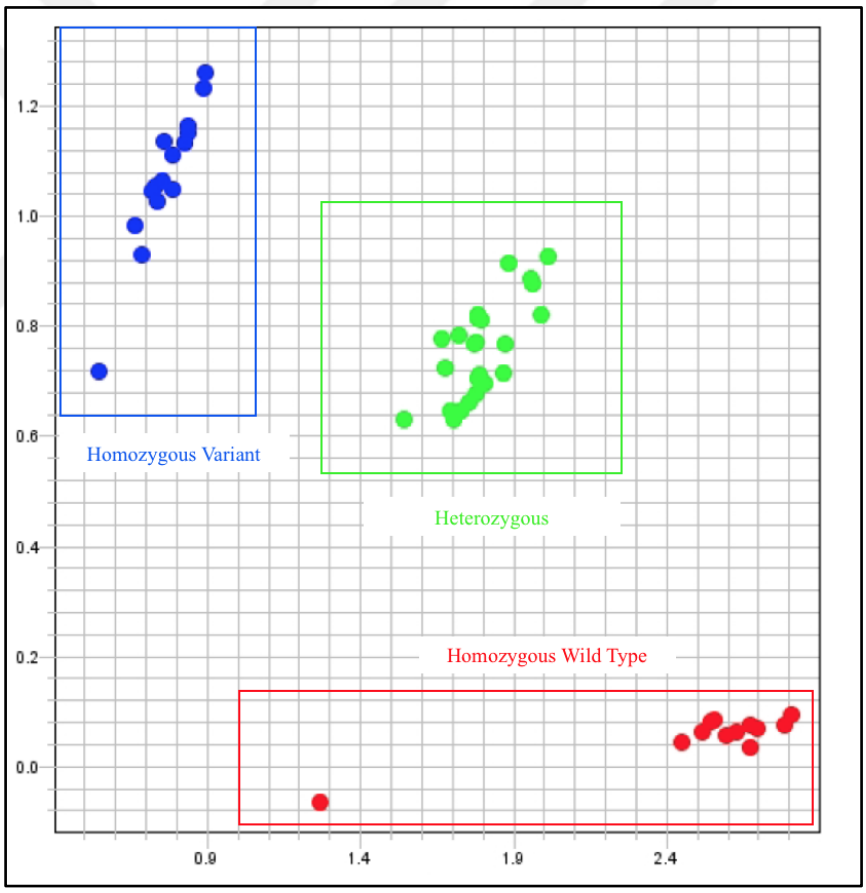

Figure 3.1. Representative image of allelic discrimination.

3.3. STATISTICAL ANALYSIS

The data were analysed on a computer using the Statistical Packages of Social Sciences (SPSS) version 25.0 software. The Kolmogorov-Smirnov test was performed to normalise the data. Descriptive statistics are shown as mean ± standard deviation (SD) for continuous variables, as frequency and percentage for categorical variables. The independent two-sample student T-test was used to compare the normally distributed data of two independent groups. The chi-square test or Fisher's exact test was used for the analysis of the difference between the categorical variables. The Kruskal-Wallis test was used for comparison of variables among groups that did not fit normal distribution, and the Mann-Whitney U test was used to compare pairwise. The results were interpreted after performing Bonferroni correction. The contingency coefficient was calculated to determine whether there was a relationship between mutations. A $p<0,05$ was considered as statistically significant.

4. RESULTS

4.1. BASELINE CHARACTERISTICS

Table 4.1 shows the demographic characteristics of the study population. When the characteristics were compared between groups; higher total cholesterol, triglyceride and LDL level as well as lower HDL and serum 25(OH)D levels were found statistically significant in cases (p<0,05).

Table 4.1. The characteristics features of the study population

Baseline Characteristics	**Groups (number of participants)**		**p values**
	Controls (n=50)	**Cases (n=51)**	
Age (years)	33,46 ± 7,04	36,63 ± 11,94	0,108
Total cholesterol (mg/dL)	174,52 ± 32,99	190,49 ± 43,38	**0,040***
Triglyceride (mg/dL)	69,05 ± 25,70	110,24 ± 49,42	**<0,001****
HDL (mg/dL)	64,04 ± 19,01	48,71 ± 12,66	**<0,001****
LDL (mg/dL)	100,63 ± 28,82	120,20 ± 39,44	**0,005***
Serum 25(OH)D (ng/mL)	22,25 ± 6,94	15,65 ± 9,08	**<0,001****
Vitamin B12 (pg/mL)	407,04 ± 56,98	405,39 ± 157,46	0,944
Folic Acid (ng/mL)	7,40 ± 1,49	7,28 ± 3,19	0,814
EDSS (points)	-	1,03±0,9	

HDL: high density lipoprotein, LDL: low density lipoprotein, EDSS: expanded disability status scale. mean±SD

*p<0,05 **p<0,001

4.2. ALLELIC DISCRIMINATION OF VDR & VDBP MUTATIONS

Table 4.2 shows allelic frequency analysis of *VDR* and *VDBP* genes in cases and controls. When groups were compared with each other, the presence of G allele in rs2228570 (*Fok I*) was found statistically significant in cases (p=0,004).

Table 4.2. Allele frequency of *VDR* and *VDBP* in study groups

Polymorphism and allele variation	**Groups**		**p values**
	Controls (n=50)	**Cases (n=51)**	
VDBP			
rs4588			
C	81 (81,0%)	76 (74,5%)	0,268
A	19 (19,0%)	26 (25,5%)	
rs7041			
G	56 (56,0%)	54 (52,9%)	0,663
T	44 (44,0%)	48 (47,1%)	
VDR			
rs2228570			
A	50 (50,0%)	31 (30,4%)	**0,004***
G	**50 (50,0%)**	**71 (69,6%)**	

*p<0,05

4.3. ALLELIC DISCRIMINATION OF APOE VARIANTS

Table 4.3 shows allelic frequency analysis of *ApoE* in cases and controls. When groups were compared with each other, any statistically significant result was not detected (p>0,05).

Table 4.3. Allele frequency of *ApoE* in study groups

Allele Variants	**Groups**		**p values**
	Controls (n=50)	**Cases (n=51)**	
APOE			
ε_1	0 (0,0%)	1 (2,0%)	0,320
ε_2	16 (32,0%)	11 (21,6%)	0,236
ε_3	31 (62,0%)	38 (74,5%)	0,177
ε_4	1 (2,0%)	3 (6,0%)	0,362

4.4. GENOTYPING

Table 4.4 illustrates genotype distribution of cases and controls. When groups were compared with each other, heterozygous genotype (CA) in the rs4588 region and GG genotype in rs2228570 region were found statistically significant in cases. (p=0,014 and p=0,042; respectively).

Table 4.4. Genotypes in cases and controls

Polymorphism and genotype variation	**Groups**		**p values**
	Controls (n=50)	**Cases (n=51)**	
VDBP			
rs4588			
CC	37 (74,0%)	28 (54,9%)	
CA	**7 (14,0%)**	**20 (39,2%)**	**0,014***
AA	6 (12,0%)	3 (5,9%)	
rs7041			
GG	13 (26,0%)	12 (23,5%)	
GT	18 (36,0%)	24 (47,1%)	0,507
TT	19 (38,0%)	15 (29,4%)	
VDR			
rs2228570			
AA	13 (26,0%)	5 (9,8%)	
AG	24 (48,0%)	23 (45,1%)	**0,042***
GG	**13 (26,0%)**	**23 (45,1%)**	

*p<0,05

4.5. RELATION BETWEEN RISK FACTORS AND POLYMORPHISMS

When the relation between risk factors and mutations were investigated, it has been found that there was a significant association between high triglyceride level (108,35±53,71) and rs4588 variant (heterozygous genotype) (n=27) (p=0,047). It was also observed that the presence of ε_2 variant of *ApoE* (n=27) was related to low LDL level (93,63±29,41) which was statistically significant (p=0,006). Additionally, high LDL level (115,27±36,16) was

detected within 69 individuals in the presence of ε_3 variant of *ApoE* which was also statistically significant (p=0,049).

5. DISCUSSION

MS is one of the neurodegenerative disease affecting over the two million people worldwide. Identification of MS-related markers either genetic or environmental factors is precisely important for the new therapeutic approaches. Here, the study aims to investigate associations between genetics and environmental factors which are possibly about vitamin D and cholesterol metabolism in MS patients. Epidemiologic study containing largest global distribution displayed that the universality of MS in Turkish population was detected as 43.2 in 100,000 people however, it was only covered Middle Black Sea Region [110]. Therefore, the purpose of the study was to investigate the possible association between MS and vitamin D as well as the relation between MS and cholesterol metabolism.

Vitamin D is a hormone taken part in calcium metabolism and bone maintenance as well as in immune-homeostasis [36, 65, 71, 72]. Epidemiological studies revealed that less than having 20-15 ng/mL 25(OH)D within body, considered as deficiency, is more frequent in patients with MS. It is also well known that MS is a complex disease which is related to both genetics and environmental factors. Here, the possible relation between MS and rs2228570 (*Fok I*) polymorphism which plays a specific role in 25(OH)D metabolism was investigated. As a result, it was found that mutation in rs2228570 was statistically significant in cases (p=0,042). Moreover, having G allele in rs2228570 region was significantly associated with MS disease risk (p=0,004). Similarly, large participants of USA nurses, it is observed a significant association between vitamin D and the *Fok I* polymorphism on MS risk (p=0,04) [111]. The F-allele (corresponded to nucleotide G) interacted with lower serum vitamin D levels in cases as well as in healthy controls [87]. Contrast to these findings, investigation of the allele and genotype frequency of rs2228570 in *VDR* within wide cohort have concluded that allelic and genotype frequency was not significantly different between MS patients and control [112]. In addition, the rs2228570 previously selected for positive MS susceptibility and serum vitamin D in patient with MS showed slight transmission where the patient carried HLA-DRB15* negative (p=0,03) [94]. In the present study, when group comparison regarding the genotypes in each region has been made, mutant genotype (GG) in rs2228570 region were also found relatively significant in cases (p=0,042).

When cases and controls are compared regarding the genotypes in each region, it was also found that heterozygous genotype (CA) in rs4588 were statistically significant in case group (p=0,014). In a very recent study which was investigated the relation between vitamin D deficiency and MS risk in black people and Latin Americans due to the differences in rs7041 and rs4588 polymorphisms displayed that the G allele (corresponds to C allele in present study) predominated in rs4588 for all groups, but whites and Latins were less likely to have this allele and at least one copy of the allele T was found in individuals (corresponds to A allele in present study) more in rs4588 than in black people. Significantly lower 25(OH)D levels in all groups who have carried T allele at rs4588 [113]. However, another study having been investigated the association between *VDBP* gene polymorphism and MS risk in Italian case-control cohort showed no relation between those polymorphisms. It should be noted that we could not detect a significant relation between rs7041 and MS risk, similarly, a study indicated no differences for rs7041 either alleles, genotypes or haplotypes in the light of MS risk and disease progression [89]. But several studies suggested that rs7041 polymorphism is considerably related to MS risk [113, 114].

Identification of biomarkers at early stages of disease progression is accepted as an effective method to diagnose patients at risk for the neurodegenerative disorder. ε_4 variant of *ApoE* is best known as a risk factor associated with Alzheimer [107]. It is well known that *ApoE* is one of the protein construction takes part in cholesterol metabolism, and its variants of ε_1 and ε_2 can decrease the plasma level of the cholesterol whereas ε_4 form increase it [103]. And it is possible to say that ε_2 variant has a protective effect on individuals against neurodegenerative disease while ε_3 variant is more common in the community [115]. LDL has been known as the main carrier of plasma cholesterol and has an ability to enter the parenchyma of early MS lesions because of BBB damage [116]. Here, not surprisingly, it was detected that the presence of ε_2 variant of *ApoE* (n=27) was related to low LDL level. Additionally, high LDL level was detected within 69 individuals in the presence of ε_3 variant of *ApoE* which was also statistically significant.

Relation of *ApoE* ε_4 allele and MS has been investigated for 20 years and thus, detected by several studies [117-119]. However, several numbers of retrospective studies have not found any relationship [120, 121]. Here, current study could not detect a significant

relation between ε_4 and MS risk. This is probably due to the involvement of a low number of individuals in the study.

When the relation between risk factors and mutations were investigated, it has been found that there was a considerable association between high triglyceride and heterozygous genotype in rs4588 within 27 individuals (p=0,047). Therefore, it is possible to say that CA genotype in rs4588 may cause an increase in triglyceride level within the body. However, no similar supporting or contrast result has been obtained in the literature.

Lipid metabolism in the body may affect MS disability and disease progression directly or indirectly as they have critical importance considering it regulates immune responses and myelin formation process and repair in CNS. Deterioration of lipid metabolism may affect integration of myelin and cause neuron loss. There is new epidemiological evidence that lipid profile (lower HDL whereas higher LDL and triglyceride) and dyslipidaemia are associated with more serious disability and rapid disease progression in MS [122]. When the characteristics were compared between groups in this study, higher total cholesterol, triglyceride and LDL level as well as lower HDL were found statistically significant in cases.

Vitamin D deficiency has been considered as an influencer factor during MS for more than 30 years [36]. Epidemiological evidence supports the idea that both vitamin D and the susceptibility and seriousness of autoimmune disorders such as MS are related. Correlations between high disease prevalence, possible death risk from MS and high vitamin D result in hypothesis that high levels of vitamin D may have beneficial effects on disease progression [36, 123]. Moreover, in a prospective population-based cohort study, the occurrence of relapse was reduced by 12 per cent as each 10 nmol/L leds to rising in vitamin D [82]. Here, it was demonstrated that vitamin D deficiency is associated with MS which has found statistically significant (p<0,001). Due to these findings, it was considered that the presence of both vitamin D deficiency and gene mutations which are related to vitamin D metabolism might affect the course of MS.

6. CONCLUSION

In this present study, possible relation between genetic and environmental factors which play role vitamin D and cholesterol metabolism in MS patients was investigated. It was obtained that both genes in cholesterol metabolism and in vitamin D metabolism have an impact on disease progression.

Studies concluded that clinicians should recommend to MS patients, who have no chance to get benefits from sunlight or who have lower serum vitamin D level, to include extra vitamin D into their diets to balance 25(OH)D levels around 50 nmol/L. Also, patients with MS should take care of their daily intake of cholesterol since it has a crucial role not only in MS also in several autoimmune or metabolic disease such as Alzheimer and diabetes.

Basic research is required to explain the mechanisms governing the association of genotypes and response to the metabolism of cholesterol and 25(OH)D, whereas clinical studies with larger cohorts will probably answer the question of whether it is meaningful to incorporate polymorphisms in the individualisation of treatment in MS patients.

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