ASSOCIATION ANALYSIS OF CHOLESTEROL 7- ALPHA HYDROXYLASE (CYP7A1) AND CHOLESTEROL 24-HYDROXLASE (CYP46A1) GENETIC POLYMORPHISMS AND MULTIPLE SCLEROSIS RISK IN TURKISH POPULATION

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ABSTRACT

ASSOCIATION ANALYSIS OF CHOLESTEROL 7- ALPHA HYDROXYLASE (CYP7A1) AND CHOLESTEROL 24-HYDROXLASE (CYP46A1) GENETIC POLYMORPHISMS AND MULTIPLE SCLEROSIS RISK IN TURKISH POPULATION

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Multiple Sclerosis (MS) is the most common demyelinating disorder of the central nervous system. Under the effects of certain environmental factors, MS develops in genetically susceptible individuals. People with MS have significantly lower vitamin D levels. UV-B radiation catalyzes the photo-conversion of 7-dehydrocholesterol, produced in cholesterol production pathway, to vitamin D in the skin. Cholesterol 7α-hydroxylase (CYP7A1) in the liver and Cholesterol 24S-hydroxylase (CYP46A1) in the brain are responsible for cholesterol removal in human body. A Single Nucleotide Polymorphism (SNP) conferring A→C transition, named rs3808607 in CYP7A1 and present at -204 location of form the transcriptional start site, might play a critical role in gene expression and its enzyme activity. T → C SNP named rs754203 in CYP46A1, which is located in intronic region of CYP46 gene, 151 bases 5′ to exon 3, is investigated in terms of human health or disease although it does not affect the amino acids sequences or the structure of the protein expressed by gene.

In this study, rs3808607 and rs754203 polymorphisms were investigated for their effect on the risk of MS. 138 MS patients and 100 healthy controls were tested by PCR-RFLP method to determine their genotypes. Analyses of them were conducted

by forming three genetic models of SNPs: Dominant, Recessive and Additive Model. For CYP7A1 rs3808607 and CYP46A1 rs754203, all genotypes and allele frequency analyses were performed with all subjects, male and female subgroups only. However, any significant difference was not found in them.

To understand whether there were any relations of blood lipids and vitamin D levels in MS patients and controls with rs3808607 and rs754203, SNPs were split according to mentioned genetic models. For rs3808607, only its recessive model, TC and vitamin D contents of patients, carrying the wild type allele were significantly lower than controls with same genotypes. The other comparisons didn't show any meaningful difference. As regard to CYP46A1 rs754203 SNP, of all the comparisons, only statistical significance was that vitamin D levels of patients with heterozygote mutant genotype was lower than controls with same genotype. CYP46A1, CYP7A1, rs3808607, rs754203, SNP, Turkish population

As a conclusion, in this study, there were no any significant associations between genotypes and allele frequencies of CYP7A1 rs3808607 and CYP46A1 rs754203 SNPs and MS risk. In terms of lipid parameters and vitamin D levels, these genes and lipid parameters were not found to be related in patients and controls, however, vitamin D levels were found to be lower in patients compared to controls in some subgroups.

Keywords: CYP46A1: rs754203, CYP7A1: rs3808607, MS, SNP, Turkish Population

ÖZ

TÜRK POPÜLASYONUNDA KOLESTEROL 7-ALFA HİDROKSİLAZ (CYP7A1) VE KOLESTEROL 24-HİDROKSİLAZ (CYP46A1) GEN POLİMORFİZİMLERİ İLE MULTİPLER SKLEROZ RİSKİ İLİŞKİSİNİN İNCELENMESİ

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Multipler skleroz (MS), merkezi sinir sisteminde en yaygın görülen demiyelinizasyon hastalığıdır. Belirli çevresel faktörlerin etkisi altında, genetik olarak yatkın kişilerde görülmektedir. MS hastaları düşük seviyede vitamin D'ye sahiptir. Kolesterol sentez ara ürünlerinden biri olan 7-dehidrokolesterolden, UVB-ışınlarının fotokimyasal dönüşümü sonucu deri de vitamin D üretimi gerçekleşir. Karaciğerde kolesterol 7α-hidroksilaz (CYP7A1) ve beyinde kolesterol 24S-hidroksilaz (CYP46A1) vücuttan kolesterolün uzaklaştırılmasından sorumludur. A→C dönüşümünü gösteren, CYP7A1 geninde transkripsiyonun başlama noktasından -204 konumunda bulunan, rs3808607 tek nükleotid polimorfizmi (SNP) gen ifadesinde ve enzim aktivitesinde önemli rol oynar. rs754203 isimli CYP46A1 geninin 5' noktasındaki ekzon 3'e 151 bazlık mesafede ki intronda bulunan T →C SNP'si de aminoasit sekansını ya da gen tarafından üretilen protein yapısını etkilememesine rağmen insan sağlığı ve hastalıkları yönünden çalışılmıştır.

Bu mevcut çalışmada, rs3808607 ve rs754203 polimorfizimleri MS riski üzerindeki etkileri yönünden araştırılmıştır. 138 MS hastası ve 100 sağlıklı kontrolle PCR-RFLP metodu kullanılmış olup; istatistiksel analizler SNP'lerin üç farklı genetik model-

Dominant, Resesif, Eklemeli model- oluşturularak yapılmıştır. CYP7A1 rs3808607 ve CYP46A1 rs754203 için bütün genotipleme ve allel frekans analizleri bütün bireylerle, yalnızca erkekler ve yalnızca kadın bireylerin olduğu gruplarla yapılmıştır fakat, onların hiçbirinde istatistiksel olarak anlamlı bir farklılık bulunamamıştır.

rs3808607 ve rs754203'lü MS hastalarının ve kontrollerinde, kan lipid ve vitamin D değerleri arasında ilişki olup olmadığını anlamak için, SNP'ler bahsedilen genetik modellere göre ayrılmıştır. rs3808607 için, sadece resesif modelinde, yabanıl tip allel taşıyan hastaların TC ve vitamin D içerikleri, aynı genotipli kontrollerden istatistiksel olarak anlamlı şekilde daha düşük bulunmuştur. Diğer karşılaştırmalarda ise herhangi bir farklılık bulunamamıştır. CYP46A1 rs7554203 için ise, karşılaştırmaların tamamına bakıldığında, bulunan tek istatistiki farklılık, heterozigot mutant genotipe sahip hastaların vitamin D değerleri, aynı genotipli kontrollerden daha düşük olmasıydı.

Sonuç olarak, bu çalışmada, CYP7A1 rs3808607 and CYP46A1 rs754203 SNP'lerin genotip ve allel frekansları ile MS riski arasında bir ilişki görülmedi. Lipit ve vitamin D parametreleri açısından değerlendirildiğinde ise, bu genler ve hasta ya da kontrollerin lipit içerikleri arasında bir ilişki bulunamazken, onların bazı alt guruplarında vitamin D değerlerinin hastalarda kontrollerden daha düşük olduğu görülmüştür.

Anahtar Kelimeler: CYP46A1: rs754203, CYP7A1: rs3808607, MS, SNP, Türk Popülasyonu

To my parents

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24OHC: 24S-hydroxycholesterol

25(OH)D: 25-hydroxyvitamin D

AD: Alzheimer Disease

ALS: Amyotrophic Lateral Sclerosis

ApoE: Apolipoprotein-E

BBB: Blood-Brain Barrier

CA: Cholic Acid

CDCA: Chenodeoxycholic Acid

CI: Confidence Interval

CIS: Clinically Isolated Syndrome

CNS: Central Nervous System

CSF: Cerebrospinal Fluid

CYP 450: Cytochrome P450

CYP46A1: Cholesterol 24S-hydroxylase

CYP7A1: Cholesterol 7α-hydroxylase

DHCR7: 7- Dehydrocholesterol reductase

DMT: Disease Modifying Therapies

EDTA: Ethylene Diamine Tetra Acetic Acid

EBV: Epstein-Barr virus

HDL: High-Density Lipoproteins

HMGCoAR: Hydroxy Methyl Glutaryl - Coenzyme A reductase

IDL: Inter- Mediate-Density Lipoproteins

LDL: Low-Density Lipoproteins

LOAD: Late-Onset AD

MHC: Major Histocompatibility Complex

MRI: Magnetic Resonance Imaging

MS: Multiple Sclerosis

OR: Odds Ratio

PPMS: Primary Progressive MS

RE: Restriction endonuclease

RFLP: Restriction Fragment Length Polymorphism

RIS: Radiologically Isolated Syndrome

RRMS: Relapsing-Remitting MS

RXR: Retinoid X Receptor

SNP: Single-Nucleotide Polymorphisms

SPSS: Statistical Package for Social Sciences

T2DM: Type 2 Diabetes Mellitus

TC: Total Cholesterol

TG: Triglycerides

VDR: Vitamin D Receptor

VDRE: Vitamin D Response Elements

VLDL: Very-Low-Density Lipoproteins

CHAPTER 1

INTRODUCTION

1.1. Multiple Sclerosis

After finding distinct postmortem scars in the brain of a woman who had tremor, unclear speech, and abnormal eye movement, Multiple sclerosis (MS) was described by French neurologist Jean-Martin Charcot almost 150 years ago (Charcot, 1868). MS is defined as both a chronic demyelinating disease which affects the brain, spinal cord, and optic nerves, and also an inflammatory disease of the central nervous system (CNS), causing loss of myelin sheaths and a degeneration of axons preferably in the white matter and cortex (Fox et al., 2016; Riedhammer & Weissert, 2015).

While several pathological processes including inflammation, demyelination, and axonal damage are contributing to the disease manifestations, the exact etiology of MS is still unknown (Fox et al., 2016). Chronic inflammation is one of the major hallmarks of multiple sclerosis pathology (Lassmann & Horssen, 2011). In addition to its contribution to demyelination and neurodegeneration, it starts at small veins and venules; and areas with high density of such vessels are preferential sites for initial MS lesions (Lassmann & Horssen, 2011; Silva et al., 2018).

Many different immune cells are considered to be involved in the disease process with leakage of the blood–brain barrier (BBB) in which the endothelial cells make a permanent cellular barrier between the blood and the interstitial fluid: Myelin-reactive CD4+T cells that carry the immune response to the nervous system, CD25+regulatory T cells which can control autoreactive CD4+cells, myelin-reactive B cells, CD8+killer cells, macrophages and brain microglia. In CNS, Th1, Th17, and B cells infiltrate discrete areas of tissue, where they interact with antigen-presenting cells (astrocytes,

microglia, macrophages, and dendritic cells), inducing the production of proinflammatory cytokines and oxidative stress. B lymphocytes function as specific antigen-presenting cells for T cells and produce specific antibodies for myelin antigens, making myelin the target of immune cells that mistake it for a foreign antigen. At the end of this process, they create damage to oligodendrocytes (e.g., demyelination) and neurons (e.g., axonal transection), resulting in the formation of a sclerotic plaque (Silva et al., 2018; Ziemssen, 2005; Gharagozloo et al., 2018) (**Figure 1.1**).

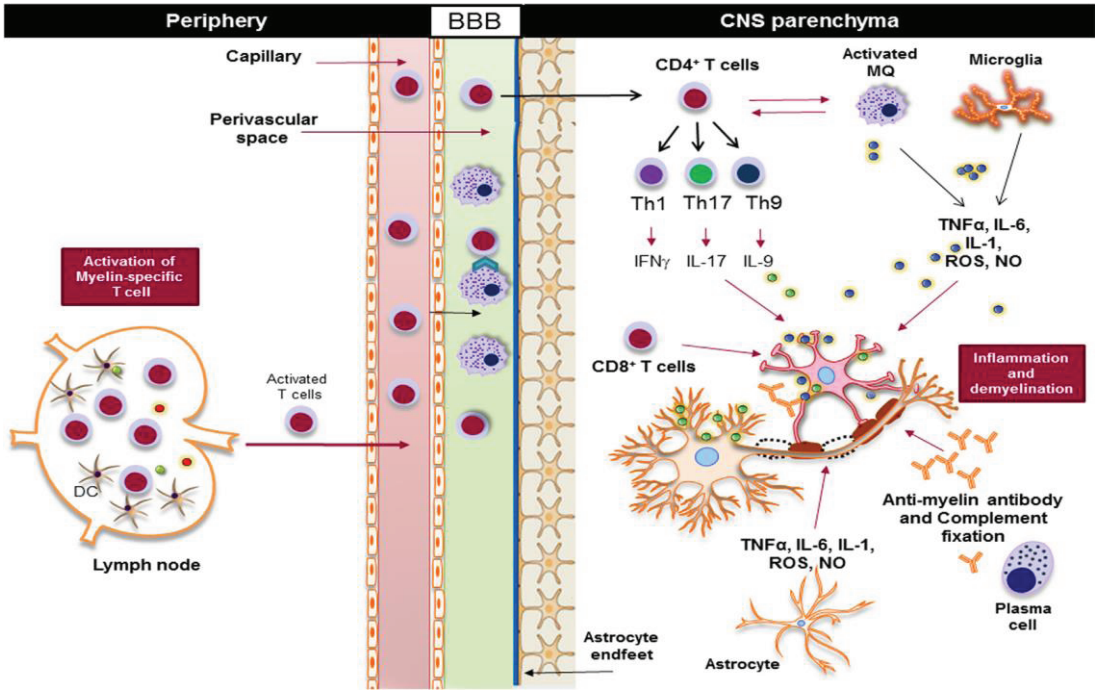


Figure 1. 1. Physiological orders of MS incidence.

(Gharagozloo et al., 2018).

When myelin, which is axon's insulator, and oligodendrocyte are destructed by inflammation, impulses through the brain and spinal cord can deform, which leads to the sensational and dynamic disorders such as sensory alterations, physical and/or

mental disability, fatigue, balance disorders, muscle weakness, spasticity, urinary incontinence, cognitive impairment, neuropathic pain, and visual deficiency in MS patients (Mohajeri et al., 2011; Silva et al., 2018). Furthermore, deterioration of body composition that is due to the changes in fat and bone mass can be seen in these patients, therefore risks for diseases such as coronary artery heart disease, non-insulin dependent diabetes mellitus, lipid metabolism abnormalities, and osteoporotic fractures increase in them (Dionyssiotis et al., 2014). The failure of remyelination, which is partly due to the to an insufficient capacity of resident oligodendrocyte precursor cells to proliferate, migrate, differentiate and initiate myelin membrane growth, also contributes to axonal damage in MS patients (Berghoff et al., 2017).Most common demyelinating disease, MS is seen in high-income countries and characterized by an unequal global distribution: While it is highest in North America (140/100,000 population) and Europe (108/100,000), it is lowest in East Asia (2.2/100,000 population) and sub-Saharan Africa (2.1/100,000) (Leray et al., 2016). This is usually clarified by ethnic differences in predisposition of a disease or exposure to one or more environmental factors (Sellner et al., 2011).

Although it mainly can be seen in young adults between 20 and 50 years of age, with a peak occurring at 30 years of age, childhood or older age cases can rarely occur (Milo & Kahana, 2010). Approximately 2.5 million people worldwide suffer from MS (Jelcic et al., 2018), and evidences suggest that its incidence is increasing like other autoimmune diseases. According to statistics from the Multiple Sclerosis International Federation, in Europe alone the disease costs more than €15 billion each year in terms of health-care costs and lost productivity (Sawcer et al., 2014).

MS patients have excess mortality (1.3/3) and reduced life expectancy (6-14 years) compared with the general population. Although MS is linked to increased frequency among women (in the female/male sex ratio from 2:1 to nearly 3:1), women with MS have better survival capacity than men (Leray et al., 2015).

1.1.1. Diagnosis of MS

Incidence of MS is rising over time, and this can be related with advances in recognition methods and longer life expectancy (Shao et al., 2018). If the diagnosis of multiple sclerosis is done incorrectly, this may lead to great worry to patients and may cause unneeded treatment with high-priced medicines. To minimalize this risk and maintain a high level of disease specificity, a set of recommended diagnostic criteria is used; and thanks to the clinical and paraclinical data of an algorithm within the criteria, diagnostic certainty can be divided three categories: MS, possible MS, and not MS (Freedman et al., 2005). In research and clinical practice, the 2017 McDonald criteria for its diagnosis is widely used (Thompson et al., 2018) and it emphasize a clinical diagnosis, referring any paraclinical measure [i.e., magnetic resonance imaging (MRI), evoked potentials, or cerebrospinal fluid (CSF)] (Freedman et al., 2005). All of them are not usually necessary, but can provide supportive evidence of multiple sclerosis (Brownlee et al., 2017).

Firstly, MRI is highly sensitive to detect characteristic CNS lesions; therefore, diagnosis of MS is based on the existence of clinical symptoms and signs and findings on MRI (Polman et al., 2011). Secondly, partially demyelinated axons conduct impulses at reduced velocity. For that reason, delays in conduction of evoked potentials can be seen in MS patients (Compston & Coles, 2002). Thirdly, a normal or mildly raised white cell count (<25 cells per cm^3; primarily lymphocytes) and protein (usually <1 g/L), a raised IgG index (in addition to IgM, IgA), which contribute directly to an immune response including neutralization of toxins and viruses, can be measured with CSF of cases (Brownlee et al., 2017; Schroeder & Cavacini, 2010; Henriksson et al., 1985). Finally, although routine testing for systemic autoimmune disorders has a very low yield in patients with presentations typical of MS, laboratory examinations are frequently demanded as part of the diagnostic work-up for MS (Brownlee et al., 2017). Still, the diagnostic approaches have many limitations and are often not specific enough for a diagnosis of MS, particularly in the early stages of the disease (Przybek et al., 2015).

1.1.2. Classification of MS

The US National Multiple Sclerosis Society (NMSS) Advisory Committee on Clinical Trials in Multiple Sclerosis defined the clinical subtypes of multiple sclerosis as benign MS and malignant MS in 1996 (Lublin & Reingold, 1996); however, the descriptions have been changed over time due to the confusion or lack of consensus (Lublin et al., 2014).

The clinical progression of MS can range from a sudden and severe inflammation disorder, which can be fatal within months, to an asymptomatic condition, which can only be realized at autopsy incidentally (Sadovnick et al., 1997). Based upon the disease course, MS is categorized in different forms: relapsing–remitting MS (RRMS), secondary progressive MS, primary progressive MS, being like "benign", "long-term relapsing-remitting", and "primary chronic progressive", respectively (Brownlee et al., 2017; Silva et al., 2018; Sadovnick et al., 1997).

RRMS is more common, affecting 85–90% of MS patients. It is described by relapses which are attacks of neurological dysfunction ongoing at least 24 hours in the absence of fever or infection, followed by stages of remission; and recovery from relapses is variable and could be partial (Brownlee et al., 2017). These series of relapses and remissions often become progressive (Sadovnick et al., 1997); and almost 70% of patients develop progression 10–15 years after an initial relapsing-remitting course, which is called secondary progressive MS (Ontaneda et al., 2017). About 15% of patients begin with a progressive disease course from initiation, which is called primary progressive MS; and it displays no relapses or periods of remission (Ontaneda et al., 2017; Silva et al., 2018) (**Figure 1.2**).

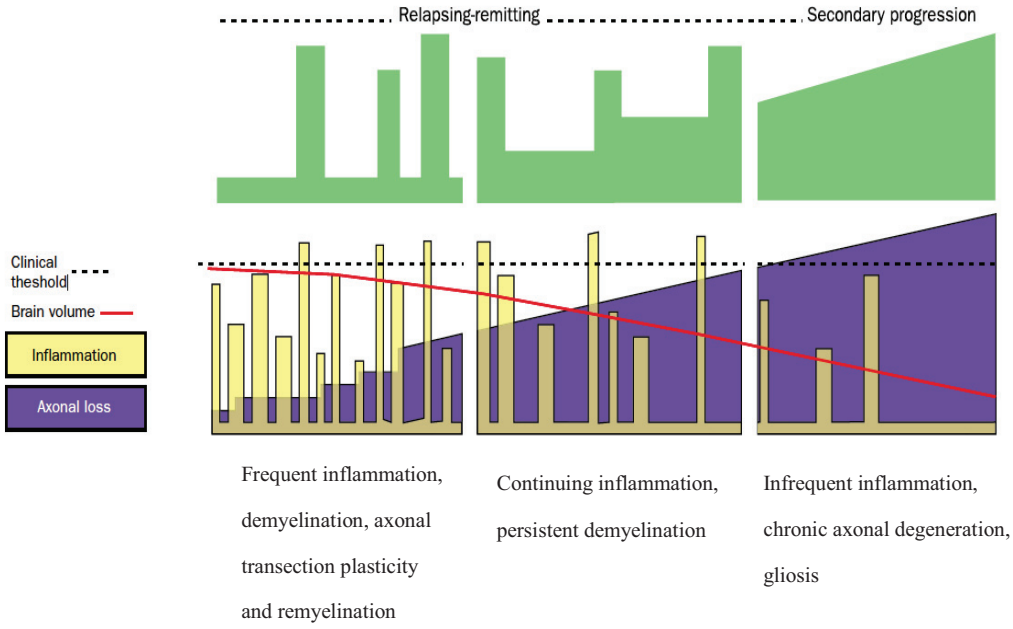


Figure 1. 2. Changed clinical phenotypes of multiple sclerosis and the underlying pathology. (Compston & Coles, 2002).

In addition to those, International MS panel in 2013 added another two types of MS definition: clinically isolated syndrome (CIS), and radiologically isolated syndrome (RIS) (Koriem, 2016). CIS is identified as the initial clinical presentation of a disease, indicating features of inflammatory demyelination, which could be seen in MS; however, it has to meet criteria of spreading in time (Lublin et al., 2014). On the other hand, the unanticipated MRI detection in the brain of symptomless subjects, which are the indicative of multiple sclerosis has been called as radiologically isolated syndrome (RIS) (La abiano-Fontcuberta et al., 2016).

1.1.3. Treatment of MS

Although multiple sclerosis is not usually a fatal disease, it affects the patients in terms of both decreasing the quality of life and increasing the economic expenditure related

to disease (Sadovnick et al., 1997). While there is not an available effective therapy for PPMS in the absence of obvious active inflammation, disease course of RRMS can be modulated with various disease modifying therapies (DMT), leading to less exacerbations and less rapid accumulation of disability in the early stages of the disorder. Treatment of PPMS is primarily confined to symptomatic and supportive care; therefore, this makes PPMS a significant unmet clinical need in neurologic care (Fox et al., 2016; Rolf et al., 2016).

Used for the treatment of MS, immunomodulatory therapies such as interferon-ß (IFN-ß), glatiramer acetate and mitoxantrone are not entirely effective (Mohajeri et al., 2011). For example, interferons and glatiramer acetate improved only the relapse rate in one-third of patients but did not alter the advanced progress of the disease (Dahbour et al., 2017). The reasons of this ineffectiveness are their insufficiency in controlling self-reactive lymphocytes and promoting remyelination and regeneration mechanisms, contributing to cumulative disability and irreversible axonal/neuronal impairment (Mohajeri et al., 2011). Other than immunomodulatory therapies, biological, monoclonal, and oral agents have been introduced with good outcome for treatment of MS (Dahbour et al., 2017). Complementary treatments such as vitamin D, Yoga, medicinal plants, oxygen therapy, acupuncture and reflexology (zone therapy) are used by almost half of MS patients. They are lack of any side effect but the efficiency of these treatments is still not enough (Koriem, 2016).

1.1.4. Risk Factors of MS

Being a chronic demyelinating central nervous system disease, MS includes oligodendrocyte loss and failure to remyelinate damaged brain areas, leading to a progressive neurological disability (Santoro et al., 2017). MS has a complex, multifactor etiology that is not entirely comprehended; however, it is assumed that in addition to autoimmune processes, genetic and environmental factors may be related with the formation of demyelinating lesions (Deckx et al., 2016).

1.1.4.1. Genetic Factors

Even though a lot of genetic changes are responsible for its symptoms, MS is not a genetic disease. Having roles of genetic factors in MS progression was first raised in the 1890s, due to the identification of familial aggregation (Dyment et al., 2004). MS incidence is high among the family of MS patient, and this increases to 30% in identical twins while the rate is 5% in nonidentical twins (Koriem, 2016). In addition to family history of disease, ethnic background of a person strongly affects the possibility of developing MS, suggesting that genetic susceptibility is a key factor of risk (Hollenbach & Oksenberg, 2015).

The exact cause of MS remains unknown; however, there are more than 100 confirmed genes or gene loci associated with MS (Harirchian et al., 2017; Kular et al., 2018). One locus exerting a moderate effect and many loci with modest effects are related inheritance of the disease (Kular et al., 2018). The strongest genetic associations with MS are located within the human leukocyte antigen (HLA) complex; especially the extended haplotype of the HLA class II region "DRB5*01:01-DRB1*15:01" confers the greatest risk for developing MS (Hedström et al., 2018; Kular et al., 2018). HLAs, cell surface proteins that play a vital role in regulation of immune responses through their ability to bind and present processed peptides to T cells, are encoded by the major histocompatibility complex (MHC). MHC is a principal susceptibility locus for many human autoimmune diseases, in which self-tissue antigens are bound and presented to pathogenic lymphocytes by the HLA molecules encoded by susceptibility alleles (Fridkis-Hareli, 2013).

Besides HLA, 23 important targets including interleukin-7 receptor α (IL7RA), interleukin-2 receptor α (IL2RA), C-type lectin domain family 16-member A (CLEC16A), CD58, tumor-necrosis-factor receptor superfamily member 1A (TNFRSF1A), interferon regulatory factor 8 (IRF8), and CD6 (CD6), have been found with modest effects in MS (Ramagopalan et al., 2010).

1.1.4.2. Environmental Factors

The genes are obligatory for developing MS; however, genetic epidemiological findings demonstrate a prominent role for the environment in determining MS risk (Ramagopalan et al., 2010). It is believed that environmental factors can trigger the autoimmune response in genetically susceptible individuals (Perez-Perez et al., 2018). Epstein-Barr virus (EBV), smoking, and latitude/vitamin D, in addition to geographical region are shown the strongest factors for involvement in MS; especially some factors can operate very early in life (Ramagopalan et al., 2010).

The role of EBV in the MS pathogenesis is that EBV-infected B cells and plasma cells accumulate in the brain of MS patients; and strong EBV specific cell-mediated immune responses are noticed in these patients (Agostini et al., 2018). As for smoke exposure, it was shown that smoke exposure displays a significantly higher association with MS, especially, amongst the people having a genetic predisposition to the disease (Hedström et al., 2018). Sunlight exposure and correlated vitamin D status were investigated to find relations between latitude and MS prevalence; and less sunlight exposure is inversely linked to MS susceptibility (Ramagopalan et al., 2010).

As another risk factor, it was stated that since nanoparticles can easily cross the BBB due to the lipid-soluble specification, stemming from their association with phospholipids, calcifying nanobacteria or nanoparticles may have a role in pathophysiology of MS, creating nano ruptures of the axonal membrane, and eventually causing neurodegeneration (Can Demirdöğen, 2019).

In addition to these factors, remarkable changes of lifestyle, especially for women, have been related with the recent alterations of MS epidemiology. Obesity, hormonal replacement therapy, and later childbirth have been added to risk factors' list of MS. Also, high incidence of adverse health attitude can be seen in MS patients (Sellner et al., 2011). For instance, the prevalence of obesity is rapidly growing world-wide because of bad lifestyle habits; and these people have lower vitamin D levels and less

sun exposure owing to their weights. Therefore, this triggers the rising of MS incidents (Luque-Córdoba & Luque de Castroa, 2017).

1.2. Vitamin D and MS

Although it is unclear whether vitamin D is related with the key functional disability hallmark in MS, it is among the strongest one of the identified environmental factors for MS risk and clinical course (Luque-Córdoba & Luque de Castroa, 2017). Vitamin D is a lipid-soluble vitamin, but acts like a hormone. Different from a vitamin, which is an essential organic compound that cannot be produced by the body and must be consumed, it can be synthesized in the body (Sintzel et al., 2018).

It has two different forms derived from distinct pathways: cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2) (**Figure 1.3**) (Iacopetta et al., 2018). The only structural difference between vitamin D2 and D3 is that there is a double bond between C-22 and C-23 and a C-24 methyl group in the side chain of vitamin D2 (Holick, 2003). Vitamin D3 is produced in the skin of humans and is taken from the diet containing the animal-based foods, especially fish oil while vitamin D2, which is not mainly human-made, is obtained from plant sources (Gil et al., 2018).

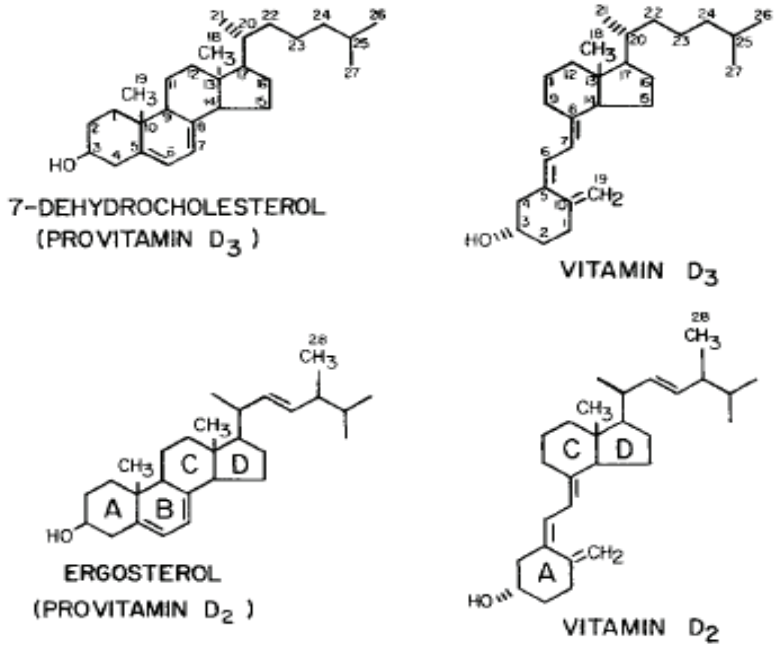


Figure 1. 3. Structure of vitamin D3 and D2 and their precursors 7-dehydrocholesterol and ergosterol, respectively.

(Holick, 2003).

Sun exposure, vitamin D supplementation and diet are the best recognized environmental factors linked to vitamin D levels (Browne, et al., 2014). UV-B radiation catalyzes the photo-conversion of 7-dehydrocholesterol to vitamin D (cholecalciferol) in the skin (Krementsov et al., 2018). On the other hand, dietary vitamin D (either vitamin D3 or D2) is adsorbed at the small intestine with other dietary fats, such as cholesterol; and exogenous vitamin D is packed into chylomicrons (which are the lipoprotein particles, consisting of triglycerides, phospholipids, cholesterol, and proteins mixture), and then, they are transported to the liver (Agnello et al., 2018). After that, Vitamin D (produced from the skin and absorbed through dietary intake) needs two hydroxylation to become biologically active. Therefore, it is converted to calcidiol [25-hydroxyvitamin D3 or 25(OH)D3] by three enzymes,

CYP2R1, CYP27A1, and CYP3A4 in the liver, and then to calcitriol [1,25-dihydroxyvitamin D3 or 1,25$(OH)_2$D3] by CYP27B1 in the kidney or in target tissue (**Figure 1.4**).

The plasma vitamin D-binding protein (VDBP) works for the transport of vitamin D3 from the skin to the liver, and transport of 25(OH)D from the liver to the kidney and other cells, and also transport of 1,25$(OH)_2$D from the kidney to the target cells. The inactivation of both 25(OH)D and 1,25$(OH)_2$D as excretion product is catalyzed with the help of the CYP24A1, converting 25(OH)D into 25,24$(OH)_2$D and 1,25$(OH)_2$D into calcitroic acid and 1,25$(OH)_2$D-26-23-lactone (**Figure 1.4**) (Agnello et al., 2018; Krementsov et al., 2018).

Although the hormonally active form of vitamin D is 1,25$(OH)_2$D, which can bind and activate the nuclear vitamin D receptor (VDR) in many different target tissues, including bone, kidneys, intestine and immune system, 25-hydroxyvitamin D [25(OH)D] is used to evaluate serological levels of this vitamin due to its longer half-life than 1,25$(OH)_2$D (Krementsov et al., 2018; Perez-Perez et al., 2018; Zahoor & Haq 2017).

After binding to the vitamin D receptor (VDR), 1,25$(OH)_2$D can exert its numerus biological functions. This ligated receptor makes a heterodimer complex with the retinoid X receptor (RXR)-alfa; and that complex migrates to the nucleus which binds vitamin D response elements (VDRE) in the DNA to induce or to repress transcription of target gene, by recruiting the related coregulators (Kägi et al., 2018; Agnello et al., 2018) (**Figure 1.4**). It has been reported that vitamin D regulates more than 900 genes (Wang et al., 2005).

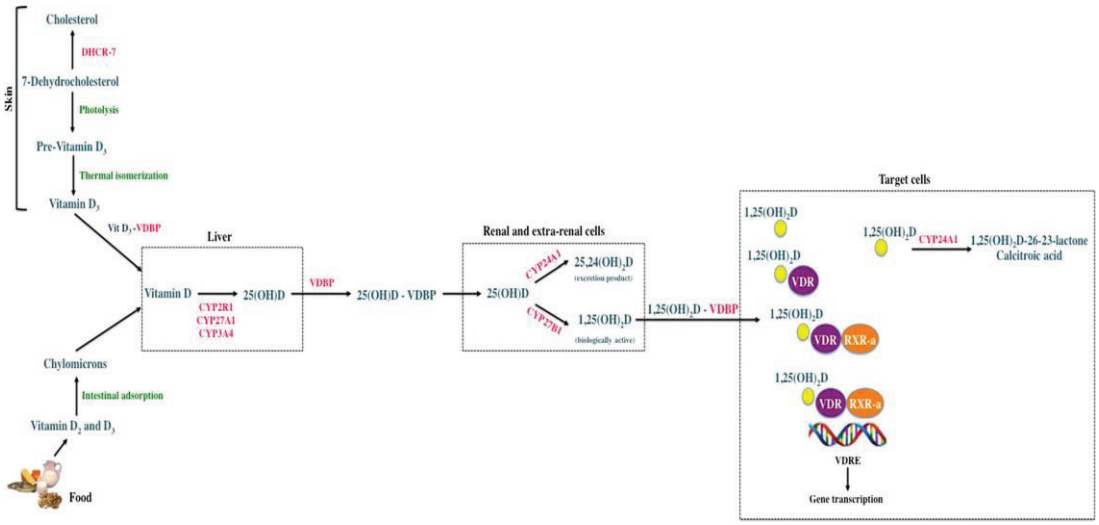


Figure 1. 4. Vitamin D metabolic pathway.

(Agnello et al., 2018).

The best-known function of vitamin D is to regulate calcium physiology in which it stimulates the absorption of calcium in the small intestine by binding to calcium transporting proteins. Furthermore, it induces osteoclastic maturation by contributing to an increase in bone resorption and the release of calcium into the blood and supports the bone development by assisting in the mineralization of the collagen matrix. Therefore, its deficiency causes rickets in children and osteomalacia in adults (Rosen et al., 2016; Ascherio et al., 2010).

In addition to functions of calcium homeostasis, vitamin D plays crucial roles on brain development and function, apoptosis and cell proliferation, regulation of blood pressure and insulin secretion. Also, it has effects on immune cells' differentiation and modulation of immune responses (Rosen et al., 2016; Ascherio et al., 2010). After the uncovering of the vitamin D receptor and key vitamin D metabolizing enzymes expressed by immune cells, importance of vitamin D on the regulation of these cells has gained more attention in last years (Kamen & Tangpricha, 2010). For instance, to increase the number of regulatory T-cells (T-Reg), which leads to decrease production of inflammatory cytokines, and to modulate the expression of MHC II-complexed

antigens on the surface of immune cells, which inhibits the production of Type 2 helper T cell cytokines, and to affect propagation and differentiation of B cells, which generates lower immunoglobulin secretion and affects T cell maturation, are some roles of vitamin D in the immune system (Kusumadewi et al., 2018; Tiwari et al., 2018).

Case-control studies have revealed that people with MS have significantly lower vitamin D levels. In one of those studies, insufficiency of it was seen in 61% of MS patients (Ozgocmen et al., 2005). However, there are many questions about whether low vitamin D levels increase the risk of MS or MS decreases vitamin D levels. In other words, whether exacerbated by heat MS symptoms may result in sun avoidance and contribute to lower vitamin D levels and whether vitamin D deficiency/ insufficiency triggers MS are still inconclusive and are needed to be clarified (Elkama & Karahalil, 2018). **Table 1.1** shows the recommended optimum levels of 25OHD by Vitamin D Council.

Table 1. 1. *The recommended optimum levels of 25OHD by Vitamin D Council.*

25OHD Levels			
Deficiency	**Insufficient**	**Sufficient**	**Toxic**
<0–30 ng/ml	31–39 ng/ml	40–80 ng/ml	>150 ng/ml

1.3. Lipid Metabolism and MS

Many reports indicate a connection between the lipoprotein profile of MS patients and MS disease processes like lesion formation, blood-brain-barrier dysfunction and disability; however, the current results about possible changes of the lipoprotein profile in MS patients are still inconclusive (Jorissen et al., 2018).

Brain cholesterol constitutes ~25% of the total body cholesterol, which involves in maintaining neural development, synaptic plasticity, and brain function (Zhang et al., 2018). Being main component of myelin and statins, cholesterol may prevent remyelination by inhibiting cholesterol synthesis in the brain (Weinstock-Guttman et al., 2011). However, Berghoff et al. showed that cholesterol intake from diet increased proliferation of oligodendrocyte precursor cell, which is important for remyelination, and promoted differentiation of oligodendrocyte in the cuprizone-treated mice (toxicant-induced MS models) (Berghoff et al., 2017).

Being fatty acid esters of glycerol, triglycerides represent fat stores of animals and lipid component of dietary fat (Cox & García-Palmieri, 1990). They have a small role in neuronal lipid metabolism, however, serve a function as the storage form of lipid precursors (Tracey et al., 2018). Lipids particularly lipoproteins, are connected with the neural functions' regulation in CNS (Weinstock-Guttman et al., 2011). Lipoproteins are important mediators of cholesterol and lipids transport and have critical roles in the regulation of inflammatory responses (Jorissen et al., 2018; Weinstock-Guttman et al., 2011[a]). Plasma lipoproteins are classified into five main classes based upon hydrated density; size; electrophoretic mobility; and their relative content of cholesterol, triglycerides, and protein: very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), inter- mediate-density lipoproteins (IDL), high-density lipoproteins (HDL) and chylomicrons (Cox & García-Palmieri, 1990). In here, we studied only HDL and LDL and their possible relations with MS.

Due to the its key role in reverse cholesterol transport, HDL has anti-atherogenic features (Jorissen et al., 2018) While it has anti-inflammatory effects on monocytes, it has been revealed that it prevents production of the pro-inflammatory cytokine interleukin-1beta and tumor necrosis factor (Jorissen, et al., 2018; Weinstock-Guttman et al., 2011[b]). Furthermore, HDL's main connected protein, ApoA-I, diminishes inflammation in CNS by preventing interaction between T cells and macrophages (Jorissen et al., 2018). Oxidative stress is involved in the MS pathogenesis, and it is known that HDL has anti-oxidant properties on endothelial cells (Jamroz-Wisniewska

et al., 2009; Jorissen et al., 2018). For those reasons, HDL can be associated with the MS.

An early participation of LDL in the formation of MS lesions has been shown in many investigations. Owing to blood–brain barrier damage, a great amount of the plasma LDL moves into the parenchyma of MS plaques and is altered oxidatively in the lesion. Lipid peroxidation and oxidized LDL intake from penetrating macrophages might have a significant role in initial demyelination stages of MS plaque (Giubilei et al., 2002).

There is limited information about effects of serum triglycerides and cholesterol levels on MS disease progression (Weinstock-Guttman et al., 2011[a]). After first clinical demyelinating event was seen in CIS patients, increased total cholesterol was related with increased brain MRI lesion, such as new T2 lesions and grey matter atrophy (Mukhopadhyay et al., 2016). Also, Jamroz-Wisniewska and coworkers found that MS patients had a higher incidence of hypercholesterolemia (Jamroz-Wisniewska et al., 2009).

As a conclusion, serum cholesterol and lipoprotein levels are associated with MS disease progression; however, the mechanisms responsible for these disease relations are not identified precisely (Narayanaswamy, et al., 2015). However, some researches have shown that increased levels of LDL, TC, TG, and reduced levels of HDL are significantly associated with worsening disability of MS patients (Jorissen et al., 2018; Mukhopadhyay et al., 2016).

1.3.1. Cholesterol Metabolism

Cholesterol is indispensable molecule for mammals, because of its structural functions such as regulation of fluidity, stability, integrity and permeability in the cell membranes (**Figure 1.5**). In addition to its critical role in the cell as a signaling

molecule, it is necessary for synthesis of other significant molecules such as bile acids, steroid hormones and vitamin D (Méndez-Acevedo et al., 2017). It is believed that systemic and cellular cholesterol participate in the regulation of immune cell activity (Andersen, 2018). For example, it is needed for sufficient development levels of the human lymphocytes to do their cytotoxic function (Qrafli et al., 2014).

All the cells have ability of synthesizing, releasing and using the cholesterol in the human body in order to maintain their cholesterol homeostasis. However, some of them specialize to produce it for other cells, which need exogenous cholesterol due to their limited production capacity. Under normal conditions, while almost 60% of it is synthesized (approximately 700 mg/day) in the body, the remaining is taken from the diet (Leoni & Caccia, 2015).

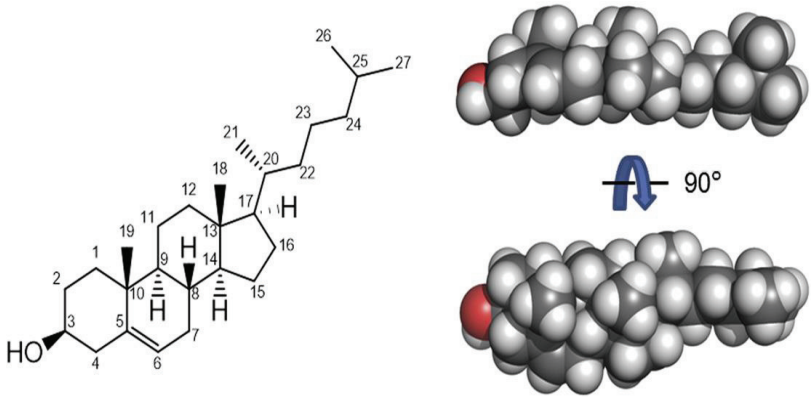


Figure 1. 5. The molecular structure of cholesterol.

(Grouleff et al., 2015).

Since excessive amount of free cholesterol might be toxic for the cells, either storing of it in the esterified form or exporting of it to other cells or releasing of it via oxidation into oxysterols are done to maintain the homeostasis (Leoni & Caccia, 2013; Leoni & Caccia, 2015). While it is synthesized in the ER membrane, it transported to other organelles via a combination of vesicular and non-vesicular transport processes

(Maxfield & Tabas, 2005; Yamauchi & Rogers, 2018). After absorbed by small intestine, cholesterol and other lipids such as phospholipids and triglycerides are loaded on chylomicrons and sent to the liver, which is the major organ of the cholesterol metabolism (Leoni & Caccia, 2015; Lorbek et al., 2012). The LDL cycle is required for the exogenous cell supply. On the other hand, HDL mechanism (reverse cholesterol transport) is important for removing of excess cholesterol from the cell (Leoni & Caccia, 2013). Besides, the ATP-binding cassette (ABC)-transporters which are highly conserved protein family involved in the membrane transport of a variety of substrates (such as ions, amino acids, peptides, sugars, vitamins, steroid hormones, phospholipids and cholesterol) have functions to regulate cholesterol homeostasis in the CNS and peripheral tissues (Kaminski et al., 2000; Aikawa et al., 2018; Leoni & Caccia, 2015).

Cholesterol biosynthesis from initial molecule, two-carbon acetyl-CoA, is highly complex; it involves a series of steps in which over 30 enzymes and 18 ATP molecules are required for production of only one cholesterol molecule (Yamauchi & Rogers, 2018; Valenza & Cattaneo, 2011). However, it can be categorized into five stages:

1) Synthesis of mevalonate from acetyl-coenzyme A (CoA) by hydroxymethylglutaryl-coenzyme A reductase (HMGCoAR) that is the enzyme of the rate-limiting step.

2) Production of isoprenoid units (5C) from mevalonate (6C) by loss of CO_2;

3) Formation of squalene (30C) via condensation of six isoprenoid units

4) Cyclization of squalene for making lanosterol (30C);

5) Formation of cholesterol (27C) by restructuring the lanosterol (30C) molecule (Leoni & Caccia, 2013; Valenza & Cattaneo, 2011) (**Figure 1.6**).

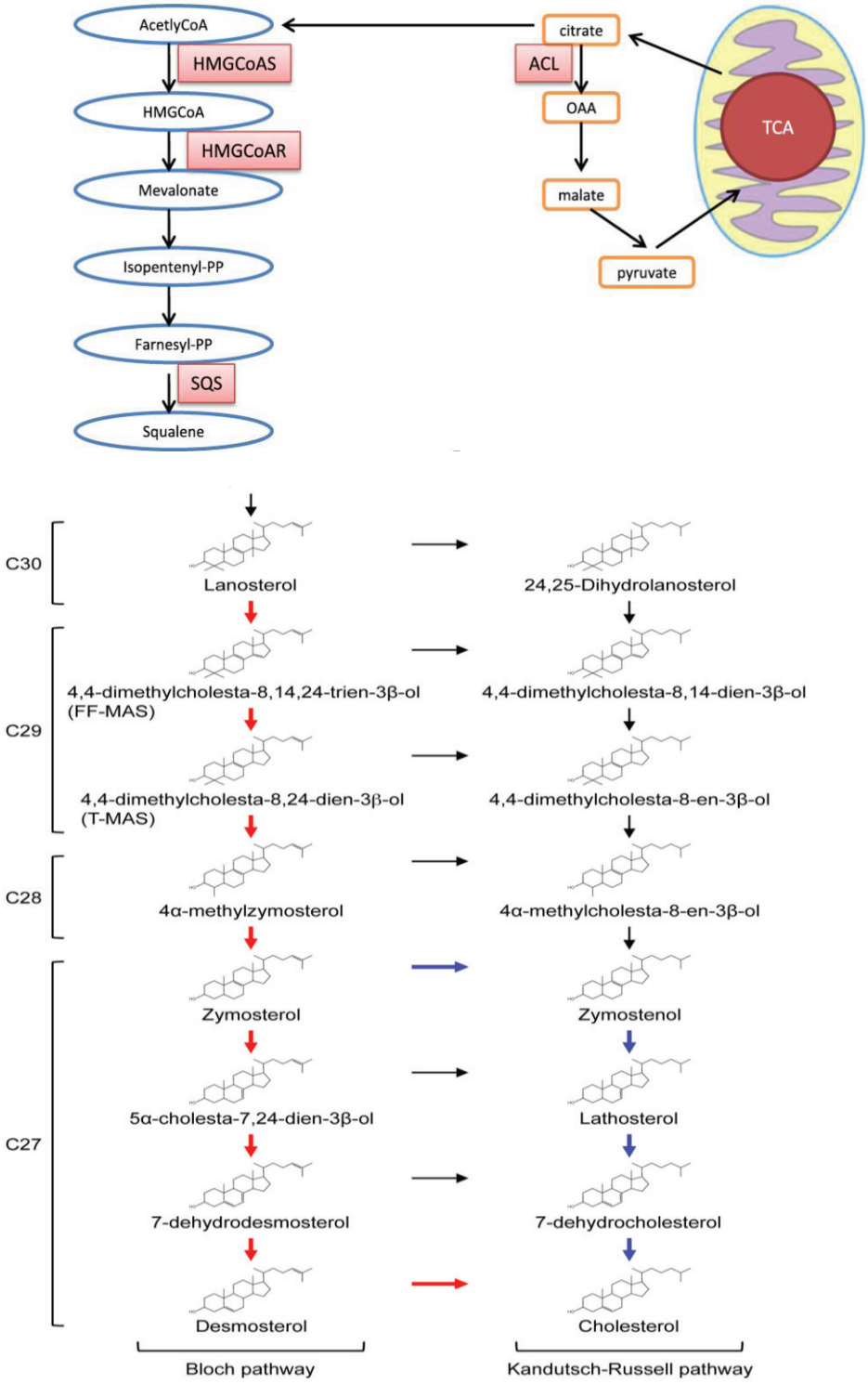


Figure 1. 6. Cholesterol biosynthetic pathway.

(Leoni & Caccia, 2015; Yamauchi & Rogers, 2018).

Cholesterol production process continues two ways after formation step of squalene: The Block pathway, in which desmosterol is the main intermediate, and the Kandutsch– Russell pathway in which lathosterol and 7-dehydrocholesterol are main intermediates (Kölsch et al., 2010). Lanosterol, lathosterol and desmosterol, which are the cholesterol precursors, are quantified as surrogate markers to assess tissue or whole-body cholesterol synthesis (Leoni & Caccia, 2015).

Several important molecules such as isoprenoids (farnesyl and geranylgeranyl diphosphates) which have significant role in many processes changing from intracellular signaling to inflammatory responses, are produced in cholesterol biosynthesis pathway. In addition to them, other different molecules such as heme-A and ubiquinone (coenzyme Q10), which are important for electron transport, and vitamin D can be obtained from this pathway (Valenza & Cattaneo, 2011).

If there are any defects in cholesterol biosynthesis, these may cause both lessening levels of newly manufactured cholesterol and decreasing number of intermediate products. Therefore, many key metabolic and cellular tasks, for instance, mitochondrial function, vesicular transport, cellular growth, immune responses etc. will be affected by these defects (Valenza & Cattaneo, 2011).

Cholesterol homeostasis is maintained via its elimination pathway and bile acids are the final products of this pathway, corresponding the major part of daily turnover of cholesterol (Lorbek et al., 2012; Chiang, 2013). In that process, there are ring structure's alterations of cholesterol, side chain's oxidation and shortening, and finally bile acids' conjugation with an amino acid (Ferdinandusse & Houten, 2006) (**Figure 1.7**).

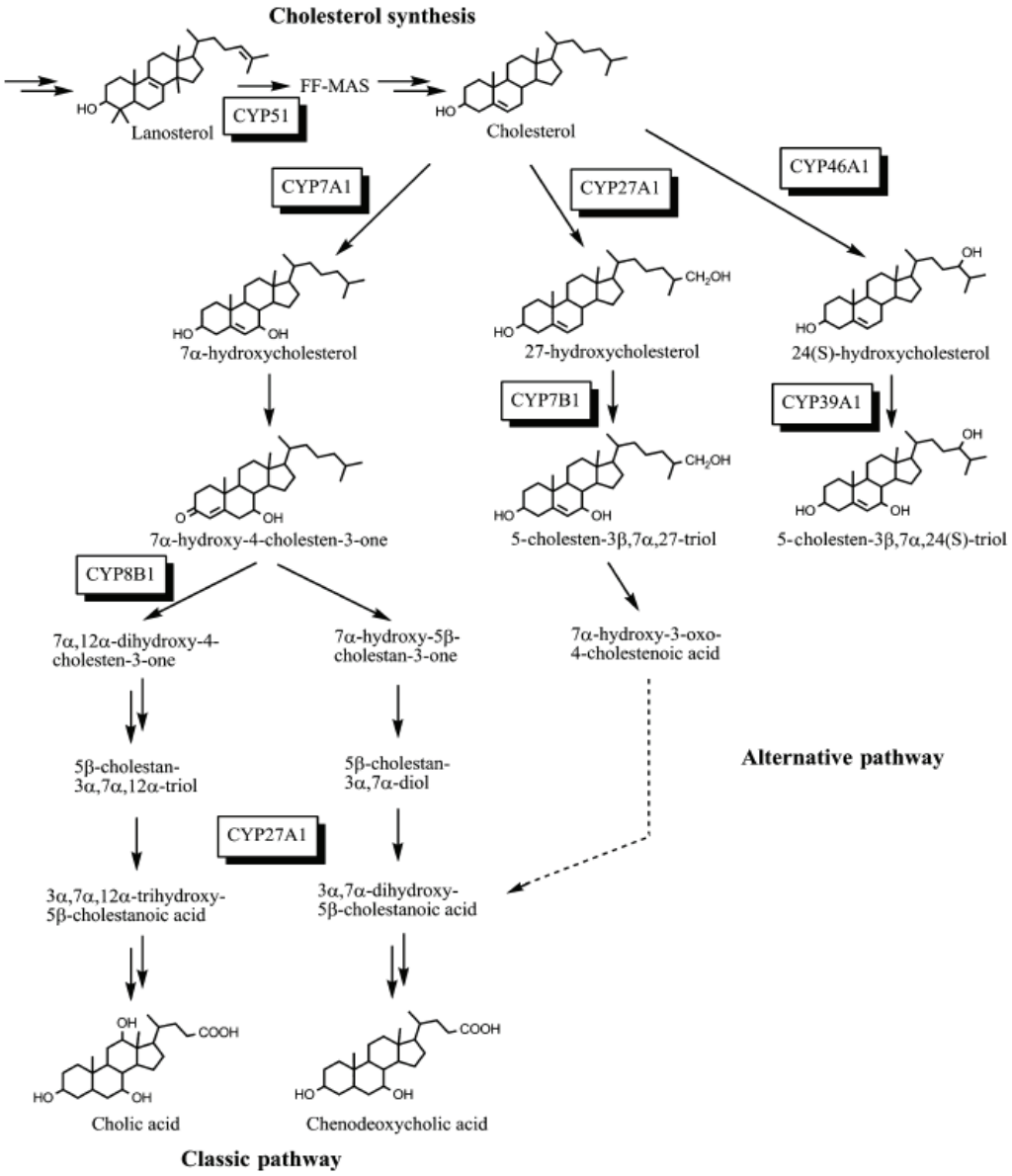


Figure 1. 7. Schematic demonstration of cholesterol and bile acids biosynthesis pathways. (Lorbek et al., 2012).

The classic (neutral) and the alternative (acidic) pathways are responsible for bile acid formation. The majority of bile acid synthesis is obtained through the classical pathways in the liver. Although Cholesterol 7-α-hydroxylase (CYP7A1) is the rate-limiting enzyme in this pathway, bile acid profiles are determined by CYP8B1. Cholic

Acid (CA) and Chenodeoxycholic Acid (CDCA) are the end up products of this pathway. On the other hand, cholesterol is oxidized by sterol-27-hydroxylase (CYP27A1), the rate-limiting enzyme in the alternative pathway. That step follows 7-α-hydroxylation of the oxysterol intermediates by oxysterol 7-α-hydroxylase (CYP7B1) (Zaborska & Cummings, 2018; Monte et al., 2009). This pathway causes mostly the formation of CDCA in humans (Lorbek et al.,2012). As the classic pathway account ~ 90% humans and ~ 75% in mice for the production of bile acids, the acidic pathway contributes the remaining ones. (Ferdinandusse & Houten, 2006). However, the acidic pathway can become the major pathway when CYP7A1 activity is deficient (Lorbek et al., 2012).

Bile acids are needed for intestinal lipids and fat-soluble vitamins absorption, and have highly significant functions as signal molecules. They activate nuclear receptors and G protein-coupled receptor (GPCR) signaling to regulate biological processes such as nutrient metabolism, inflammation, and liver regeneration (Chiang et al., 2013).

As oxidized metabolites of cholesterol or its precursors, oxysterols are critical metabolites regulating cholesterol levels and affecting the production of bile acids and have functions to modulate the immune system. Crick et. al., showed that demyelination led to activation of bile acid biosynthesis, especially acidic pathway in neurological disease such as MS, AD and ALS (Crick et al., 2017).

1.3.1.1. Brain Cholesterol Metabolism

Except for brain, cholesterol homeostasis in mammalian body keeps going with dietary uptake, de novo production in each organ and lipoprotein-mediated carriage through the blood circulation (Pfrieger & Ungerer, 2011). Because of the blood-brain barrier in which there is a permanent cellular barrier between the blood and the interstitial fluid made by endothelial cells by restricting the entrance of cholesterol-rich lipoproteins, brain meets its cholesterol's requirement via local synthesis (Laterra et al., 1999; Björkhem et al., 2013).

The cholesterol is synthesized by neuronal cells through the embryogenesis and the early life to obtain rapid brain growth; however, its production rate is reduced to very low degree in the mature brain owing to reaching a constant cholesterol level (Leoni & Caccia, 2011; Lu et al., 2018). While astrocytes have 2–3 times more production capacity of cholesterol than neuronal cells, oligodendrocytes can produce it at higher level than astrocytes for myelinization during development (Leoni & Caccia, 2011; Valenza & Cattaneo, 2011).

Brain cholesterol is approximately 25% of the total body cholesterol, which indicates its central role in the development and function of the brain; and as being a crucial constituent of the myelin membrane, it is located in myelin (70%), glial cells (20%) and neurons (10%), mostly with un-esterified form (>99.5%) (Leoni & Caccia, 2014; Ayciriex et al., 2017; Lavrnja et al., 2016). In addition to its significant functions like the structural component of membranes and lipid rafts, cholesterol is involved in formation of axons and dendrites during development, myelination during CNS maturation, neuro-steroid production, microtubule stability, neuronal repair and remodeling, learning, and memory (Lavrnja et al., 2016; Ayciriex et al., 2017; Valenza & Cattaneo, 2011). Also, the brain cholesterol has 250-300 times longer half-life than that in the circulation, which gives extra-importance as a structural component (Leoni & Caccia, 2013).

Cholesterol level is balanced by two different ways in human brain: the first and being less effective one is Apolipoprotein-E (ApoE, fat-binding protein)-dependent transportation through the cerebrospinal fluid, by which 1-2 mg/day is left from brain; second one is formation of a hydroxylated metabolite, 24S-hydroxycholesterol (24OHC), from cholesterol, in which almost 6–8 mg of 24OHC is daily released from the brain into the circulation (Leoni, 2009; Borroni et al., 2004). In addition to neuroprotective tasks, ApoE which is the key apolipoprotein constituent of HDL-like particles in the CNS, has many roles not only in brain cholesterol and lipid metabolism but also in regulating inflammatory responses (Dayger et al., 2013). However, its main

function in the CNS is the lipid transport among neurons and glial cells (Vitali et al., 2014).

On the other hand, 24S-hydroxycholesterol (24OHC), which is the main cholesterol elimination product of the brain cholesterol (**Figure 1.7**), is obtained from conversion of cholesterol to more water-soluble metabolite by the cytochrome P-450 family 46, subfamily A, polypeptide 1 (CYP46A1) or cholesterol 24S-hydroxylase, by inserting hydroxyl group to cholesterol (He et al., 2012; Li et al., 2013; Fernández del Pozo et al., 2006). In excess of 80% of 24OHC is transferred into circulation with esterified fatty acids on lipoproteins (LDL or HDL) for the liver clearance of 24OHC (almost 7 mg/24 h) that converts 24OHC into bile acids and other conjugated for final removal, in order to keep it at the constant level in plasma (Leoni & Caccia, 2013) (**Figure 1.8**).

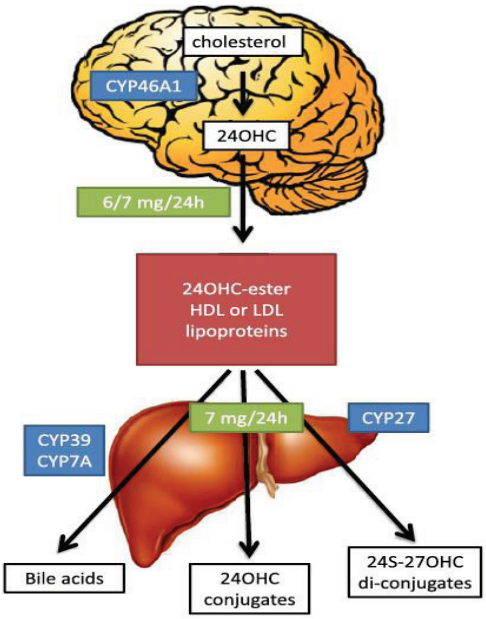


Figure 1. 8. Representation of major elimination pathway in brain cholesterol.

(Leoni & Caccia, 2013).

The epidemiological and molecular evidences have shown that there is a link between cholesterol and neurodegenerative diseases (Leoni & Caccia, 2011). Described by cyclic loss and repair of myelin sheaths correlated with chronic inflammation and neuronal loss, MS is a central nervous system demyelinating disorder (Houdebine et al., 2017). Extensive demyelinated regions of MS lesions have great amounts of macrophages filled with myelin, which is used by them to generate oxysterols. Both oxidative derivatives of cholesterol, oxysterols, which work as signal molecules to increase the autoimmunity, and cholesterol, which may promote inflammatory mediators' production through macrophages, have role in MS pathogenesis owing to their implication in inflammation, demyelination, oxidative stress, and neuro-degeneration (Houdebine et al., 2017; Lavrnja et al., 2016). There is not available enough information about the specific changes in the expression of key regulators of cholesterol homeostasis; however, as an example, it was found that demyelination which stems from the effects of the inflammation and neurodegeneration might increase the levels of 24OHC transiently in the circulation of acute stage-MS patients (Lavrnja et al., 2016).

1.3.1.2. Cholesterol and Vitamin D Interdependence

There are two cholesterol formation pathways: the Bloch pathway and Kandutsch–Russell pathway. In the first one, the major cholesterol precursor desmosterol is used for cholesterol production, and in the second one, lathosterol is needed for that purpose (**Figure 6**). Lathosterol and 7-dehydrocholesterol are central intermediates in Kandutsch–Russell pathway (Kölsch et al., 2010).

7-dehydrocholesterol is the key precursor for vitamin D3 production in the skin (Browne et al., 2014). 7-dehydrocholesterol is converted into cholesterol by 7-dehydrocholesterol reductase that is encoded by the *DHCR7* gene in humans (Genetics Home Reference, 2018) (**Figure 4**). This step is highly important and makes contact between the cholesterol and vitamin D pathways.

There are some evidences that activity of HMGCoA reductase, which is rate limiting enzyme of cholesterol synthesis, is inhibited via 25-hydroxy- vitamin D (Weinstock-Guttman et al., 2011). Therefore, it was suggested that higher vitamin D levels may be related with the lipid profile by lessening total cholesterol amounts (Karmon et al., 2013).

Cholesterol elimination products, bile acids regulate the digestion and absorption of cholesterol, triglycerides, and fat-soluble vitamins in the intestine (Ciaula et al., 2017). Therefore, this also forms another link between the cholesterol and vitamin D.

1.4. Cytochrome P450s

The name of the cytochrome P450 proteins are derived from their bonding to membranes within a cell (cyto) and containing a single heme pigment (chrome and P) that has a 450-nm absorption peak when reduced and form a complex with CO (Nebert & Russell, 2002; Pikuleva, 2006; Lynch & Price, 2007). Based upon their amino acid sequence similarity, phylogenetic and syntenic relationships, they are classified into families and subfamilies (Lee et al., 2018). If sharing identity is more than 40%, a number is given to mean a particular family, while it is more than 55%, a letter is assigned to refer a particular subfamily (Nebert & Russell, 2002).

The P450s have enzymatic functions as monooxygenases which bind with dioxygen to their ferrous (Fe^{2+}) heme iron and insert an atom of oxygen into the substrate, and the other oxygen atom is reduced to H_2O (Munro et al., 2018). After binding the substrate, cytochromes P450 generally takes the first electron via an electron transfer chain from NAD(P)H (Bernhardt, 2006). The most common reaction catalyzed by them as monooxygenase is like this:

$$RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+$$ (Bernhardt, 2006).

The CYP450 enzymes extensively distributed between eukaryotic and prokaryotic organisms. In human, they are represented by 57 genes, which comprise the fifty microsomal and seven mitochondrial P450s (Yamamoto et al., 2018; Lin et al., 2018). They participate in the several reactions of exogenous and endogenous products such as the drugs' biotransformation (about 75% of them), the metabolism of chemical carcinogens like the degradation of insecticides and herbicides, the biosynthesis of physiologically essential compounds such as steroids, eicosanoids, fatty acids, fat-soluble vitamins: A, D, E, K, cholesterol and bile acids, and also the alteration of alkanes, terpenes, and aromatic compounds (Bernhardt, 2006; Feng et al., 2018; Sobjanek et al., 2015). These reactions catalyzed by them are hydroxylation and epoxidation (e.g. of saturated and unsaturated fatty acids); N- and S-oxidation reactions; oxidative demethylation, deamination and dealkylation; oxidative and reductive dehalogenation; isomerization, aromatic hydroxylation and oxidation of alcohols and aldehydes, oxidative C–C bond cleavage (Munro et al., 2018).

1.4.1. CYP7A1

Cholesterol 7α-hydroxylase (CYP7A1) is the first and rate-limiting enzyme in classic pathway which is the main way of cholesterol removal from the body by converting it into bile acids (Iwanicki et al., 2015; Vera & Ribeiro, 2011). CYP7A1 enzyme is from the cytochrome P450 protein family, and its gene spans almost 10kb on chromosome 8q11-12 and consists of six exons, five introns, one 5′-UTR, and one 3′-UTR (Iwanicki et al., 2015; Qrafli et al., 2014). Besides consensus recognition sequences for several transcription factors have been identified in its 5' flanking area, a TATA-box and a modified CAAT-box have been recognized in its promoter region (Nakamoto et al., 2006).

Bile acids, cholesterol and hormones regulate its activity, which is highly variable among healthy individuals (Srivastava et al., 2008; Dias & Riberio, 2011). A complex nuclear receptor mediated network that controls expression of CYP7A1 gene and

preserves the balance between cholesterol and bile acid, has been described in several research (Nakamoto et al., 2006). Because of the function of the CYP7A1 as the central component of cholesterol conversion in the liver, the polymorphisms of CYP7A1 gene are studied for its possible effects on lipid metabolism (Iwanicki et al., 2015). For instance, CYP7A1 modulates plasma TG levels owing to the interaction between the regulation of bile acid and the production and secretion of TG or TG-rich lipoproteins. Therefore, genetic variations in the CYP7A1 gene, for example a loss-of-function mutation of this gene which leads to the defect of cholesterol 7α-hydroxylase activity in humans, have been connected with disorders of cholesterol and bile acid metabolism such as hypercholesterolemia, hypertriglyceridemia, or premature atherosclerosis (Nakamoto et al., 2006; Shen et al., 2012).

1.4.2. CYP46A1

Being independent from nutritional intake, brain cholesterol is locally produced due to its restricted transfer through BBB (Li et al., 2013). To maintain the brain cholesterol homeostasis, excess brain cholesterol which derives from its increased synthesis or its accumulation due to the neuronal cell death should be eliminated (Garcia et al., 2009). Therefore, brain cholesterol is converted into more water-soluble metabolite 24(S)-hydroxysterol (24OHC) by cytochrome P-450, family 46, subfamily A, polypeptide 1 (CYP46A1) or cholesterol 24S-hydroxylase (Li et al., 2013). 24OHC has ability to pass the blood-brain barrier and diffuse into the circulation for further bio-transformations in the liver (Mast et al., 2017[a]; Golanska et al., 2005).

As well as controlling the rate of cerebral cholesterol elimination, CYP46A1 regulates the level of cerebral cholesterol synthesis (Mast et al., 2017[b]). It stays on human chromosome 14q32.1 and contains 15 exons and 14 introns (Mousavidehmordi et al., 2016). There is no TATA or CAAT in its promoter region, however it was found that this region had a high GC content, which is a property frequently present in genes thought to have an essentially housekeeping function (Ohyama et al., 2006).

Encoding a neuron-specific enzyme, CYP46A1 predominantly expressed in neuronal cell bodies and dendrites of only a subset of neurons, such as neurons of the cerebral cortex, hippocampus, dentate gyrus, and thalamus and retina because cholesterol homeostasis is essential to do their specific functions of these cells (Lavrnja et al., 2016; Li et al., 2018). It stays in the endoplasmic reticulum and needs a source of reducing counterparts (NADPH) and the redox partner NADPH–cytochrome P450 oxidoreductase, which transfers electrons from NADPH to CYP46A1 (Mast et al., 2017[a]). It was shown that steroid hormones, insulin, growth hormone, thyroid hormone, cAMP, cholesterol, 24OHC, bile acids were not affective on the transcriptional regulation of CYP46A1 (Lu et al., 2018; Ohyama et al., 2006). However, oxidative stress and endogenous neurotransmitters can upregulate CYP46A1. As well as, its promoter can be induced by epigenetic modifications and the specificity protein (Sp) transcription factors at the basal expression level (Lu et al., 2018; Mast et al., 2017[b]).

As a surrogate marker of neuronal damage and brain atrophy, 24S-HC plasma level has been used in neurodegenerative disorders such as Alzheimer's (AD) and Parkinson's disease, and MS (Lu et al., 2018). It was shown by *in vitro* experiments that 24S-hydroxycholesterol gives a potent neurotoxin effect (Li et al., 2018). If there is an increase in the activity of CYP46A1, it causes an accumulation in the cerebrospinal fluid of 24S-hydroxycholesterol. On the other hand, if there is a decreasing in its activity, this leads to accumulation of cholesterol in the brain, which brings about amyloid beta plaque' formation in AD (Fernández del Pozo et al., 2006). Although in experimental autoimmune encephalomyelitis (EAE) which is the best characterized MS mouse model, CYP46A1 mRNA levels was decreased at the onset of disease, this event was returned to the controllable level during recovery period and protein content of CYP46A1 did not change through the course of the disease (Lavrnja et al., 2016).

1.5. Genetic Polymorphism

Alleles are defined as different forms of a gene in the genome. Normal or changed proteins with any, small or big alteration in their tasks, or sometimes with loss-of-function may be seen due to the allelic modifications. Genetic polymorphism happens if an allele exists at least in the 1% of the population, and it is thought as a normal variant of a gene. An alteration in protein function resulting from a polymorphism is generally minor; however, its effect may be increased by environmental factors, such as diet, smoking or infective factors, rising the risk of emerging a certain disease (Bevilacqua et al., 2018).

Genetic polymorphism in the DNA regulatory regions can affect the promoter activity, gene expression, immune responses by modulating the epigenetic alterations and transcription factor binding; therefore, it is also connected with disease development (Weng et al., 2018; Ramsuran et al., 2018). In order to identify genes involved in a disease process, large numbers of polymorphic markers have been used in genetic analyses, in which whether a genomic region is linked to a disease phenotype is searched (Engle et al., 2006). While nucleotide variations involve nucleotide substitutions, duplications, insertions and fusions, a nucleotide is switched with any other three kinds of nucleotides in a single-nucleotide substitution (Matsuda, 2017). The single nucleotide polymorphisms (SNPs) is the most studied types of genetic polymorphisms (Bevilacqua et al., 2018).

1.5.1. Single-Nucleotide Polymorphisms (SNPs)

Although there are two copies of each gene in every individual, copies of a certain gene present within a population may not have same nucleotide sequences, which can be seen throughout the genome of all species and this makes up the basis for human diversity (Alwi, 2005). Alterations in the DNA sequence at individual nucleotide bases are called single-nucleotide polymorphisms (SNPs- pronounced as “snips.”) (Dias et al., 2018; Huss, 2015). They are the most common genetic variants, occurring

naturally in one of every 1000 base pairs and presenting over 10 million in the human genome (Dias et al., 2018; Marqui, 2015). The individual genotype at an SNP site is AA, BB, or AB as a genomic locus in which two or more alternative bases occur more than 1% of the population (Huss, 2015). If the frequency is lower than this percentage, it is named as a mutation.

SNPs tend to assemble in regions, having a high recombination rate and microsatellite density and might arise in all genic and intergenic regions (Jackson et al., 2016). Found in non-coding sequences, such as introns, are highly effective on regulatory processes, like gene splicing, promoter activity, or enhancer binding (Jackson et al., 2016). In most cases, SNPs do not cause a difference in the amino acid sequence, therefore, they do not alter the function or expression of genes, classified as synonymous. On the other hand, some of them are biologically functional, and lead to a change in the amino acid eventually the altered protein function, which is called nonsynonymous/missense (Jackson et al., 2016; Dias et al., 2018). SNPs from coding and promoter regions could be expected to change gene function and, consequently, the formed protein (Marqui, 2015). Still, functions of most are unknown and need to be studied their influence on human health (Dias et al., 2018).

Since inherited variances in DNA sequence play a role phenotypic variation, they affect individual's anthropometric features, disease risk and response to the environment (Group, 2001). Therefore, an assessment of SNP frequencies between different populations with distinct ethnic background will entirely be beneficial to determine the alterations in drug pharmacokinetics and estimate interindividual differences and predisposition to possible adverse results (Dias & Riberio, 2011). Future researches of scientists will be to find SNPs associated with complex diseases like heart disease, diabetes, metabolic disorder and cancer, so that such genetic diagnosis can contribute to personalized therapy for choosing drugs and treatment regimens (Matsuda, 2017).

1.5.2. Polymorphism of Cytochromes P450 (CYP450)

Having highly important functions such as in drug responses and in the endogenous compounds' metabolism (Section 1.4), Cytochromes P450 has been studied in many researches in terms of genetic polymorphism. For these enzymes, several polymorphisms have been defined and their functional effects could be a moderate reduction, a defect or an increase of enzymatic activity (Dias & Riberio, 2011). Hence, it is important to study them and their relations between disorders.

1.5.2.1. CYP7A1: rs3808607 A → C

In mediating cholesterol metabolism, functions of CYP7A1 could be isoform specific (Wang, et al., 2016). Many polymorphisms have been shown in the regulatory regions of the CYP7A1 gene (Vlachová et al., 2016). A single nucleotide polymorphism conferring A→C transition, named rs3808607, which is present at -204 location of form the transcriptional start site or -278 from the translation initiation codon, might play a critical role in gene expression and its enzyme activity (Srivastava et al., 2008; Cai et al., 2014). Associations between this polymorphic region and various metabolic disorders have been investigated in several studies due to the critical roles of the CYP7A1 in cholesterol and bile acid homeostasis (Qrafli et al., 2014; Xiang et al., 2012). Some of these studies reported the relations between the rs3808607 and diseases such as hypercholesterolemia, hypertriglyceridemia, gallbladder stone, gallbladder cancer, proximal colon cancer, neuromyelitis optica, hypertension and coronary artery disease in adults and children in Caucasian and Asian populations with conflicting results, due to the different study design, experimental procedures, ethnic background, exposed environmental factors and so on (Cai et al., 2014; Iwanicki et al., 2015; Qrafli et al., 2014; Nakamoto et al., 2006). Genetic variations in CYP7A1, especially A-204C promoter variant, have influenced the serum levels of total cholesterol, LDL and triacylglycerol, which were shown in several studies (Wertheim et al., 2012; Iwanicki et al., 2015).

This polymorphic site founds in a highly important region (**Figure 1.9**) for several reasons. Firstly, it was shown that there are several cell-specific enhancer elements between −432 and −220 in CYP7A1 promoter, such as the functional binding sites for hepatocyte nuclear factor-3 (HNF-3), HNF-4, and a ubiquitous transcription factor, which emphasizes its regulatory importance (Molowa et al., 1992). Secondly, deletion of the segment from -213 to- 91 leads to a 40% reduction in promoter activity, which suggests that a positive element was removed (Srivastava et al., 2008). Thirdly, as being responsive to several effectors such as insulin, glucocorticoids, and thyroid hormone, several regulatory elements present within -764 to +46 of promoter region of CYP7A1 in human (Cooper et al., 1997). Therefore, staying in this crucial location, rs3808607 can interact with regulatory elements and effectors to modulate the transcriptional activity of CYP7A1, which affects eventually its functions (Fu et al., 2011).

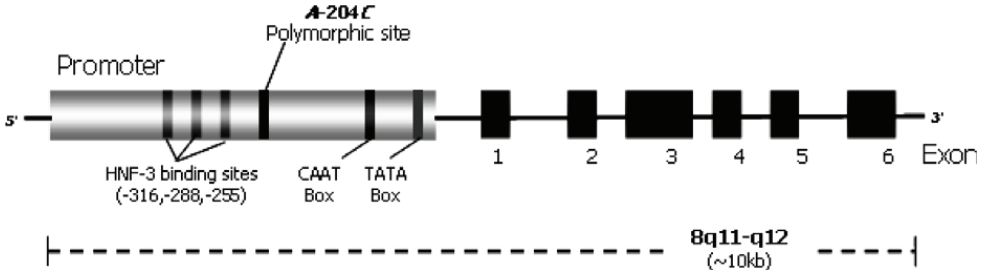


Figure 1. 9. Schematic demonstration of position of A-204C polymorphism (rs3808607) in cholesterol 7a-hydroxylase (CYP7A1) gene promoter.

HNF-3: Hepatocyte Nuclear Factor 3. (Srivastava et al., 2008).

The A-to-C substitution in the CYP7A1 promoter have been associated with increased plasma total and LDL cholesterol concentrations, which was postulated that there was reduced CYP7A1 activity with carriers of the C allele (Wertheim et al., 2012; Xiang

et al., 2012). However, -204CC genotype was associated with reduced risk of colorectal cancer, suggesting a protective gene effect due to the reduced bile acid synthesis in CC carriers (Wertheim et al., 2012). In another study the CC genotype may affect transcriptional regulation of the gene, causing CYP7A1 deficiency, which triggers several alterations in hepatic fatty acid metabolism and gallbladder bile composition, leading to more susceptibility to gallbladder cancer pathogenesis. In addition, while the CC genotype of rs3808607 was making a significant risk for gallstone disease in Indian people, its AA genotype was discovered to be related with this disease in Chinese population (Srivastava et al., 2008; Jiang et al., 2004). Ethnic difference of the study population can exert a major effect on the disease predisposition.

1.5.2.2. CYP46A1: rs754203 T → C

Cholesterol- 24S-hydroxylase (CYP46A1) which is a brain specific cholesterol-metabolizing enzyme has role in the removal of cholesterol from neuronal structures (Fourgeux et al., 2009) (Section 1.4.2). Intronic variations could be seen important in terms of having roles on the rate of transcription of gene products by affecting splice sites, or RNA stability and modulating nuclear transcriptional factors, which means that they regulate functionality or synthesis rate of a genes product (Borroni et al., 2004; Mossböck et al., 2011). There are many reports about investigations between these types of SNPs and human health or disease. CYP46A1 T → C single nucleotide polymorphism named rs754203 is one of them (Papassotiropoulos et al., 2003). It locates in intronic region of CYP46 gene, 151 bases 5′ to exon 3, which does not affect the amino acids sequences or the structure of the protein expressed by the gene (Papassotiropoulos et al., 2003; Jin et al., 2013).

In many studies, this polymorphic region has been associated with several diseases in different populations with controversial results like in the rs3808607 of CYP7A1. For example, while intron 2 T to C substitution in CYP46 was related with increased risk

of Alzheimer's disease (AD) in a Chinese population, there was not enough association about this polymorphism and AD in Hungarian and Italian populations (Rizvi et al., 2017; Fu et al., 2009). Besides, Mousavidehmordi et al., reported that TT genotype or T allele frequency of CYP46A1 may be a risk factor by increasing the susceptibility to AD in the Iranian population, whereas Borroni et al. described that CC genotype might act as a risk factor for LOAD (Late-Onset AD) in Italian people (Mousavidehmordi et al., 2016; Borroni et al., 2004). In addition to these studies, Juhász et al., could not find any relation between this SNPs of CYP46A1 and increasing susceptibility to LOAD in the Hungarian population (Juhász et al., 2005). All these results suggest that predisposition of a disease related with a polymorphism may be distinct in different ethnic groups (Garcia et al., 2009).

1.6. Aim of the Study

Multiple Sclerosis is the most common autoimmune, inflammatory, demyelinating, neurodegenerative disorder of the central nervous system. Under the effects of certain environmental factors, it develops in genetically susceptible individuals. While HLA-DRB1*15:01 allele exerts the greatest genetic effect on MS risk with other loci having very modest effects; obesity, smoking, Epstein-Barr virus and lower vitamin D levels have been associated with the development of MS. As a central step, the invasion of autoreactive CD4+ T-cells (Th-1) into the CNS has been assumed, and other CD4+ cells, such as Th2 and Th17, CD8+ T cells, and B cells are also involved in MS pathogenesis. 85%–90% of MS patients are referred to as relapsing- remitting MS, in which episodes of acute neurological deterioration is followed by partial or complete recovery. On the other hand, primary progressive multiple sclerosis is seen almost 10–15% of MS patients which is described by slowly progressive increase in neurological disability from onset, usually without relapses. It generally starts in the most productive years of life, between 25-40 years, and decreases the life quality of those people.

It was shown in many case-control studies that people with MS have significantly lower vitamin D levels. Vitamin D has several important functions in human body such as in calcium homeostasis, apoptosis, cell proliferation, brain development and function, differentiation of immune cells and modulation of immune responses. Its level is increased in the human body by sun exposure, vitamin D supplementation and diet. UV-B radiation catalyzes the photo-conversion of 7-dehydrocholesterol, produced in Kandutsch–Russell pathway that is one of the cholesterol production pathways, to vitamin D (cholecalciferol) in the skin. This is a linker between Vitamin D and cholesterol pathways.

Cholesterol has major roles in many biological processes. It plays a structural role because of being an important component of cellular membranes. The precursors of steroid hormones and bile acids are produced from cholesterol. In addition to these, it is involved in formation of axons and dendrites during development, myelination during CNS maturation, neuro-steroid production, microtubule stability, neuronal repair and remodeling, learning, and memory. To maintain the cholesterol homeostasis, excess cholesterol in many tissues should be removed by different cytochrome P450 enzymes that hydroxylate cholesterol at specific positions, and bile acids are produced. Cholesterol 7α-hydroxylase (CYP7A1) in the liver and Cholesterol 24S-hydroxylase (CYP46A1) in the brain are responsible for cholesterol removal in human body. CYP7A1 is the first and rate-limiting enzyme in the bile acids production pathway and it converts to cholesterol into 7 α-hydroxycholesterol. CYP46A1 converts the brain cholesterol into more water-soluble metabolite 24(S)-hydroxycholesterol (24OHC), levels of which has been thought being important in neurodegenerative disorders such as AD, PD and MS. Also, CYP7A1 modulates plasma TC, TG HDL, LDL levels, therefore genetic variations in the CYP7A1 gene have been connected with disorders of cholesterol and bile acid metabolism.

Lipoproteins (VLDL, LDL, IDL, HDL, chylomicrons) are important mediators of cholesterol and lipids transport and have critical roles in the regulation of inflammatory responses. Although many reports indicate a correlation between the

lipoprotein profile of MS patients and MS progressions, the outcomes about possible changes of the lipoprotein profile in MS patients in literature are still conflicting due to the study design, experimental procedures, ethnic background, exposed environmental factors etc. However, some researchers have shown that increased levels of LDL-C, TC, TG, and reduced levels of HDL are significantly associated with worsening disability of MS patients.

The rs3808607 has been studied with several diseases such as hypercholesterolemia, hypertriglyceridemia, gallstone disease, gallbladder cancer, proximal colon cancer, neuromyelitis optica with different population. In addition, it was shown that A-204C promoter variant influenced serum levels of total cholesterol, LDL and triacylglycerols. However, obtained results may be different in terms of effective allele on disease progression. For example, Srivasta et al., showed that the CC genotype gave a significant risk for gallstone disease in Indian people, whereas Jiang et al., revealed that AA genotype is related with this disease in Chinese population. Also, the rs754203: T → C region in CYP46A1 has been studied with several diseases such as AD, primary open-angle glaucoma, diabetes mellitus, age-related macular degeneration in different population with controversial results. For instance, Mousavidehmordi et al., reported that TT genotype or T allele frequency of it may be a risk factor by increasing the susceptibility to AD in the Iranian population, whereas Borroni et al. described that CC genotype might act as a risk factor for AD in Italian people.

In present study, rs3808607 in CYP7A1 gene, and rs754203 in CYP46A1 polymorphisms were investigated for the risk of multiple sclerosis. Because of their important roles in cholesterol metabolism, the associations between both cholesterol and vitamin D pathways and their effects in multiple sclerosis progression, these regions were selected for that study.

The aim of the study is to investigate associations rs3808607 (A → C) in CYP7A1 and rs754203 (T → C) CYP46A1 single nucleotide polymorphic regions with multiple

sclerosis in Turkish population and also their relations with lipid profiles (TC, TG, HDL, LDL) and vitamin D levels in MS cases and healthy controls. To determine whether there are these relations, the steps given below have been performed:

*Collecting total blood samples from multiple sclerosis patients and healthy controls in a Turkish population,

*Isolation of genomic DNA from blood samples,

*Amplification of interested regions in CYP7A1 gene and CYP46A1 gene by PCR,

*Restriction endonuclease digestion with special enzyme of amplified regions to determine the genotype of every individual,

*Identification of genotype and allele frequencies for CYP7A1 and CYP46A1 genes for MS-case and healthy control groups,

* Evaluation of genotype and allele frequencies between patients and controls and their relations with blood-lipid profiles and vitamin D levels.

CHAPTER 2

MATERIAL AND METHODS

2.1. Materials

2.1.1. Population and Blood Sampling

Blood samples were collected from 138 multiple sclerosis (MS) patients and 100 symptom-free controls thanks to the collaboration with the Department of Neurology, Gülhane Education and Research Hospital, Ankara, Turkey from September 2016 to July 2018. All of the participants in patient and control groups were Caucasian people who were from same geographic region, Central Anatolia, Turkey. After learned the medical histories of patients, a variety of tests were performed to the patients by neurologists. MS patients were diagnosed according to the 2010 McDonald Criteria, based upon the neurological examination, brain MRI scans, Lumbar puncture and blood tests. The patients who were included in our study, were preferably without any other major disease such as carcinoma, any failure in clotting, hepatic and renal failure and no familial background in terms obesity, diabetes mellitus (DM), heart related diseases and had no having any other neurological diseases. Control group subjects were chosen randomly and these people were not diagnosed with MS or any other neurological disease. Familial background of them were checked whether there is any individual having obesity, diabetes and heart related disease. Based upon the results, 100 of 140 control volunteers have been included in this study.

All participants were subjected to routine laboratory tests including complete blood counting, leucocyte differential, erythrocyte sedimentation rate, routine biochemical tests such as fasting glucose and lipid profile and liver function. Also, creatine, sodium, potassium, bilirubin and 25-OH vitamin D levels were measured. The values of Total Cholesterol, Triglycerides, LDL-cholesterol, HDL- cholesterol and 25-OH

vitamin D were used in our study. All laboratory measurements were conducted blinded to medical conditions of the subjects.

Both participants of patients and control groups were informed about our study and consent forms were signed before procedures were applied (see **Appendix A**). Besides, this study was approved by the Ethical Committee of Gülhane Education and Research Hospital (approval is available in the **Appendix B**) and was performed according to the principles of the Declaration of Helsinki.

2.1.2. Chemicals and Enzymes

To perform the experimental procedures, some chemicals were needed such as agarose (A9539), ethidium bromide (E-7637), ethylene diamine tetra acetic acid disodium salt (EDTA; E-5134), 2-amino-2 (hydroxymethyl)-1,3-propandiol (Tris; T-1503), which were all purchased from Sigma Chemical Company, Saint Louis, Missouri, USA. Boric acid (110607) and absolute ethanol (3221) were used products as of Riedel de Haen, Seelze, Germany.

GenJET whole blood genomic DNA purification mini kit (#K0782), *Taq* DNA polymerase, which is supplied with amplification buffer and $MgCl_2$ (#EP0402), dNTP set (#R0181), GeneRuler 50 bp DNA Ladder (#SM0371), GeneRuler ultra low range DNA ladder (#SM1211), GeneRuler low range DNA ladder (#SM1191) were bought from Thermo Fisher Scientific Inc., California. As restriction enzymes, Bsa I (R0535S) and Msp I (RS106S) (supplied with their buffers), were ordered from New England Biolabs Inc., USA. All chemicals used in this study were of molecular grade and were gotten from commercial sources having the highest level of purity.

2.1.3. Primers

Two primer pairs, which were selected according the literature search, were purchased from Oligomer (Oligomer Biotechnology, Ankara, Turkey) to amplify our interest

regions. The commercial primer stocks were at 100 µM/µl concentration and were stored at -20°C. In order to use in the polymerase chain reaction (PCR), the primers were prepared as aliquots of 10 pmole/µl.

The sequences of forward and reverse primers used for analysis of rs3808607: A→C polymorphism of CYP7A1 gene and rs754203: T→C polymorphism of CYP46A1 gene were shown in **Table 2.1**

Table 2. 1. *The information of forward and reverse primers used for analysis of rs3808607 polymorphism of CYP7A1 gene and rs754203 polymorphism of CYP46A1.*

Gene & SNP Name	**Forward and Reverse Primer Information**				**References**
		Sequence (5'-3')	**Length (bp)**	**Tm (°C)**	
CYP7A1, rs3808607	**Forward Primer**	AATGTTTTTCCCAGTTCTCTTTC	23	55	(Wang, et al., 1998)
	Reverse Primer	AATTAGCCATTTGTTCATTCTATTAG	26	55	
CYP46A1, rs754203	**Forward Primer**	AATGCATGCTACCAAAAGAG	20	53	(Papassotiropoulos, et al., 2003)
	Reverse Primer	AATCATTTGATTCCCAGGAC	20	53	

2.2. Methods

Genomic DNA was extracted from blood samples of patient and control groups' subjects by using GenJET whole blood genomic DNA purification mini kit according to the manufacturer's instructions. Patients and controls were genotyped for the rs3808607 polymorphism of CYP7A1 and rs754203 polymorphism of CYP46A1 variants by PCR-RFLP methods. Preparation of reagents used for PCR, restriction endonuclease digestion and agarose gel electrophoresis were described in the **Appendix C**.

2.2.1. Preparation of Human Genomic DNA Sample for PCR

2.2.1.1. Isolation of Genomic DNA from Whole Blood Samples

The whole blood samples were collected by Gülhane Education and Research Hospital into EDTA containing tubes to prevent the blood clotting. Isolations of genomic DNA from case and control groups were performed with GenJET whole blood genomic DNA purification mini kit (#K0782) according to the instructions of manufacturer.

In that process, 200 µl of whole blood sample was transferred into an Eppendorf tube and 20 µl of proteinase K (supplied with kit) was added on it. In order to obtain a uniform solution, they were mixed by vortexing or pipetting, and then, the mixture was incubated at 56 °C for 10 minutes. After incubation, 200 µl of absolute ethanol was supplemented to the mixture and was mixed by pipetting. The prepared mixture was transferred to the spin column (which is product of kit) and centrifugation for 1 min at 6 000xg (~8.000 rpm) was performed to column by using Sigma 1-15 benchtop microfuge (Sigma Postfach 1713-D-37507, Osterode). Then, the collection tube containing the flow-through solution was discarded; and the column was placed into a new 2 ml collection tube (included in kit). In next step, the 500 µl of wash buffer I (which was used after Ethanol was added into it according to manufacturer instructions) was put to the column, and the tube was centrifuged for 1 min at 8 000xg (~10 000 rpm). Then, the flow-through was discarded and the column was placed back into the collection tube. Later, 500 µl of wash buffer II (ethanol was added into it according to manufacturer instructions before used) was put to the column; and it was centrifuged for 3 min at maximum speed (≥ 20 000xg or ≥ 14 000 rpm). After the centrifugation, the collection tube containing the flow-through solution was discarded and the column was transferred into a sterile 1.5 ml microcentrifuge tube (not included with kit). In order to elute the genomic DNA from the column, 200 µl of elution buffer was added to the center of the membrane. Afterwards, it was incubated for 2 min at room temperature and centrifuged for 1 min at 8 000xg (~10 000 rpm). In final step,

the purification column was discarded and the isolated DNA was used immediately or stored at -20°C.

2.2.1.2. Quantification and Qualification of Genomic DNA Samples by Spectrophotometry

In order to determine the concentration and purity of the isolated DNA, Nanodrop^{TM}2000 (Thermo Scientific) was used. This machine gives to us the concentration (µg/ml) of DNA sample as well as absorbance ratios of sample in 260 nm to 280 nm (A_{260}/A_{280}) and 260 nm to 230 nm (A_{260}/A_{230}) wavelengths. Because the nucleic acids and proteins give the maximum absorbance at 260 nm and 280 nm, respectively, A_{260}/A_{280} ratio is used to evaluate the purity of the nucleic acids. A ratio of ~1.8 is accepted as "pure" for DNA samples. Also, EDTA, carbohydrates and phenolic reagents like TRIzol have absorbance near 230 nm. Therefore, A_{260}/A_{230} ratio is used as a secondary measure of nucleic acid purity. This ratio is generally expected in the range of 2.0-2.2. If it is lower than expected, it may show the presence of contaminants which absorb the light at 230 nm.

2.2.1.3. Qualification of Genomic DNA Samples by Agarose Gel Electrophoresis

Biogen horizontal gel electrophoresis system was used with 1% agarose gel to assess the intactness of DNA samples. There is a tray in 8 cm x 9 cm dimensions in this system. Reagents used in agarose gel electrophoresis were given in **Appendix C**.

0.6 g agarose to 60 ml 0.5 X TBE buffer in pH 8.3 containing 45 mM Tris, 45 mM Boric Acid and 1 mM EDTA, was used to prepare 1% agarose gel. The mixture was heated in microwave oven until all of the agarose particles dissolved. The solution was cooled to approximately 60°C while stirring, to maintain homogenous cooling. When the solution cooled down enough, ethidium bromide was put from stock solution of 10 mg/ml in water with final concentration of 0.5 µg/ml and the solution was

thoroughly mixed. While the 1 % agarose was being heated, the mold, plastic tray and comb were cleaned with 70 % ethanol. After plastic tray was settled in the mold, prepared warm agarose solution with ethidium bromide was poured into the mold. If there were any air bubbles, especially between or under the teeth of comb, they were removed with the help of the pipette tip, and the comb was properly placed at 0.5-1.0 mm above the plate. Approximately 20-40 minutes at room temperature was enough to obtain completely solid gel.

After the agarose gel was solidified, it was placed to the electrophoresis tank filled with 0.5 X TBE buffer such that way the slots of the gel were faced towards the negative pole-cathode. The comb was removed from the gel, carefully. Before loading the DNA samples into the well, 10 µl (0.25 - 0.5 mg) of DNA sample was mixed with 1 µl of 6X gel loading dye (supplied with DNA ladder) by pipetting. Besides, a DNA ladder was loaded into one well of the gel to identify the approximate size of DNA bands. It was prepared according to manufacturer instructions: 3 µl of distilled water, 1 µl of loading dye and 2 µl of stock ladder (6X) were mixed, and 5 µl of this mixture (1X) was loaded into one slot of the gel.

After the loading was finished, lid of the tank was closed and a power supply was connected to the electrical leads. The constant voltage of 100 volts was set in the power supply, so that no more than a voltage of 5 V/cm (which is measured as the distance between electrodes) was applied. Until the loading dye reached to the bottom of the gel, the gel was run -approximately 90 minutes-. Under the UV light, gel imaging was done and then its photography was taken by Vilber Lourmat Gel Imaging System (Marne La Vallee, Cedex, France) and InfinityCapt (version 12.9) computer software. In pure DNA preparations, a single band in agarose gel electrophoresis was obtained while RNA contaminated preparations showed two bands. If there was a smear formation, it meant that DNA was degraded.

2.2.2. Genotyping of rs3808607 Polymorphism in CYP7A1 Gene and rs754203 Polymorphism in CYP46A1

In this study, genetic polymorphism of CYP7A1 gene (rs3808607) and polymorphism of CYP46A1 gene (rs754203) were investigated in multiple sclerosis patients and healthy control groups of Turkish population. Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) analysis was performed in order to determine the genotyping of interested regions (**Table 2.2**). For that technique, firstly, the interest regions of genes were propagated by using specific primers through the PCR, and followed by restriction enzyme digestion. While Techne Progene (Cambridge, UK) thermocycler was used for PCR, DB-120 Heat Block (Biosan Ltd, Latvia) was used to incubate the samples during digestion. The procedure details of PCR and digestion were mentioned in following sections.

Table 2. 2. *Information about interested genes and their polymorphic regions, PCR product size, restriction endonucleases and fragment sizes for CYP7A1 and CYP46A1 genes.*

Gene	Polymorphism	Regions of Amplification	PCR Product Size	Restriction Endo-nuclease	Fragment Sizes (bp)
CYP7A1	(A→C)	Promotor Region	393 bp	BsaI	AA:300 -93 AC:300-261-93-39 CC:261-93-39
CYP46A1	(T→C)	Intron Region	182 bp	MspI	TT:182 CC:137-45 TC:182-137-45

2.2.2.1. Genotyping of rs3808607 Single Nucleotide Polymorphism on CYP7A1 Gene

2.2.2.1.1. Polymerase Chain Reaction for CYP7A1 rs3808607 SNP

A polymorphism (rs3808607) in the promoter region of CYP7A1 named -278 A→C from translation codon or -204 A → C from the transcriptional start site was amplified by PCR. The used reagents (details were given in **Appendix C**) were

*_Taq_ DNA polymerase

*PCR amplification buffer with $(NH_4)_2SO_4$

*dNTP mixture

*$MgCl_2$ solution

*Forward and reverse primers

*Pure H_20 (section 2.1.3)

In order to obtain single specific product for interested SNP regions, conditions and components of PCR were optimized. Three different concentrations of primer (30 pmole, 40 pmole, 50 pmole) and three concentrations of $MgCl_2$ (1M, 1.5M, 2M) with two different *Taq* buffers [*Taq* Buffer with KCl and *Taq* Buffer with $(NH_4)_2SO_4$] were tested under three different annealing temperatures of primers which were Tm, recommended by the manufacturer (Tm:55 °C), Tm+1 (56 °C), Tm-1 (54 °C). The best concentration of solutions used for optimized PCR mixture were given in **Table 2.3**.

PCR conditions except the annealing temperature were set according to study of Wang and coworkers (Wang, et al., 1998). The conditions for best PCR product output were shown in **Table 2.4**.

The amplified PCR products were checked with 1% agarose gel electrophoresis, which was described in section 2.2.1.3. After 5µl of PCR product and 1 µl of loading dye (6X) were mixed by pipetting, the mixture was filled into the well. Also, 5 µl of GeneRuler 50 bp DNA ladder was loaded to one of the well to determine the size of PCR products. The gel was run 90 min at 90 V, until the dye reached to the bottom of the gel. Then, the gel was analyzed under the UV light and its photography was taken. 393 bp of PCR product was expected to be seen in the gel.

Table 2. 3. *The component of PCR mixture for CYP7A1 rs3808607 polymorphism.*

Constituent	**Stock Concentration**	**Volume Added**	**Final Concentration in 50 µl Reaction Mixture**
Amplification (*Taq*) Buffer [with $(NH_4)_2SO_4$]	10X	5 µl	1X
$MgCl_2$	25 mM	4 µl	2 mM
dNTP Mixture	10 mM	1 µl	0.2 mM
Forward Primer	10 pmole/µl	5 µl	50 pmole
Reverse Primer	10pmole/µl	5 µl	50 pmole
Taq DNA Polymerase	5U/µl	0.5 µl	2.5 U
Template DNA	Varies	Varies	~200 ng
Sterile Apyrogen H_2O		Up to 50 µl	

Table 2. 4. *The thermal cycling program to amplify CYP7A1 rs3808607 SNP.*

	Temperature (°C)	Time	
Initial Denaturation	94	4 min	
Denaturation	94	30 sec	30X
Annealing	55	30 sec	
Extension	72	30 sec	
Final Extension	72	7 min	

2.2.2.1.2. Restriction Endonuclease Digestion of CYP7A1 rs3808607 Polymorphism

Restriction fragment length polymorphism (RFLP) was used determination of genotypes of rs3808607 in CYP7A1 gene. Bsa I (Eco31I) restriction enzyme was used to digest PCR products of CYP7A1 with the help of the (5' ..GGTCTC.. 3') recognition sequence. In individuals carrying the wild type A allele, the enzyme cut the DNA from only one site by forming 300 bp and 93 bp fragments. However, if the persons have the mutant allele C, it cuts the DNA from additional one site by forming the bands of 261 bp, 93 bp and 39 bp, and in heterogeneous individuals in terms of this SNP, it forms fragments of 300 bp, 261 bp, 93 bp and 39 bp (**Figure 2.1**).

In order to obtain the clearest digestion products in the agarose gel electrophoresis, optimizations for restriction enzyme digestion mixture and incubation conditions were carried out. Three different amounts of PCR product (2µl, 5µl, 10µl) were tested with three different enzyme concentrations (2U, 5U, 10U from 1U/µl stock enzyme solution) at different incubation time as 1 hours (h), 1.5 h, 2 h, 2.5 h, 3 h, 17 h at two different incubation temperature (37°C and 50°C) which were selected from literature

(Barcelos et al., 2019; Qrafli et al., 2014; Srivastava et al., 2008). The optimized condition used for restriction endonuclease digestion mix was summarized in **Table 2.5**.

Table 2. 5. *The components of restriction endonuclease digestion mixture for CYP7A1 rs3808607 polymorphism.*

Constituent	**Stock Concentration**	**Amount / Volume to be added**	**Final Concentration in 50 µl Reaction Mixture**
PCR Product		10 µL	
NEBuffer	10X	5 µL	1X
Bsa I	1U/ µl	10 µL	10U
Sterile Apyrogen H_2O		25 µL	
Total		50 µl	

10 µl PCR product and 10 U Bsa I restriction enzyme were incubated within the digestion mixture at 50°C for 17 hours. After incubation, the digested products were examined in 3% agarose gel and 5 µl of GeneRuler ultra low range DNA ladder was also loaded to one of the wells of the gel to define the length of fragments. The gel was run for 90 min at 90 V.

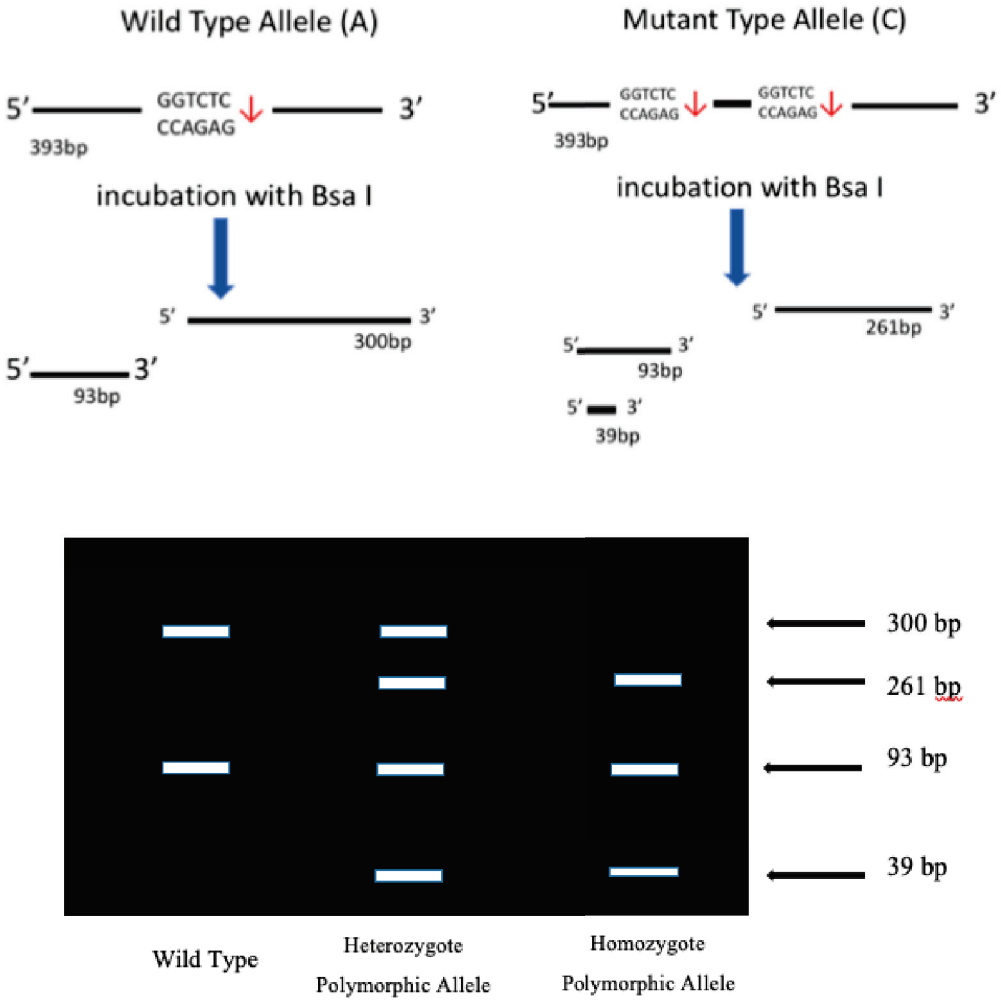


Figure 2. 1. Schematic representation for determination of genotypes of CYP7A1 rs3808607 A → C single nucleotide polymorphism.

In the left panel, the wild type allele AA can be seen. BsaI cuts the 393 bp PCR product by forming two bands of 300 bp and 93 bp. In the right panel, the mutant allele (CC) can be seen. BsaI also cuts from another one site and forms three fragments of 261 bp, 92 bp and 39 bp. A representative agarose gel electrophoresis photo of wild type, homozygote and heterozygote mutant genotypes are given on the bottom site. Used reagents for determination of genotypes were mentioned in Appendix C.

Amplified region of CYP7A1 gene including rs3808607 single nucleotide polymorphism was illustrated in **Figure 2.2** with highlighted forward and reverse primers, recognition site of restriction enzyme and single nucleotide substitution.

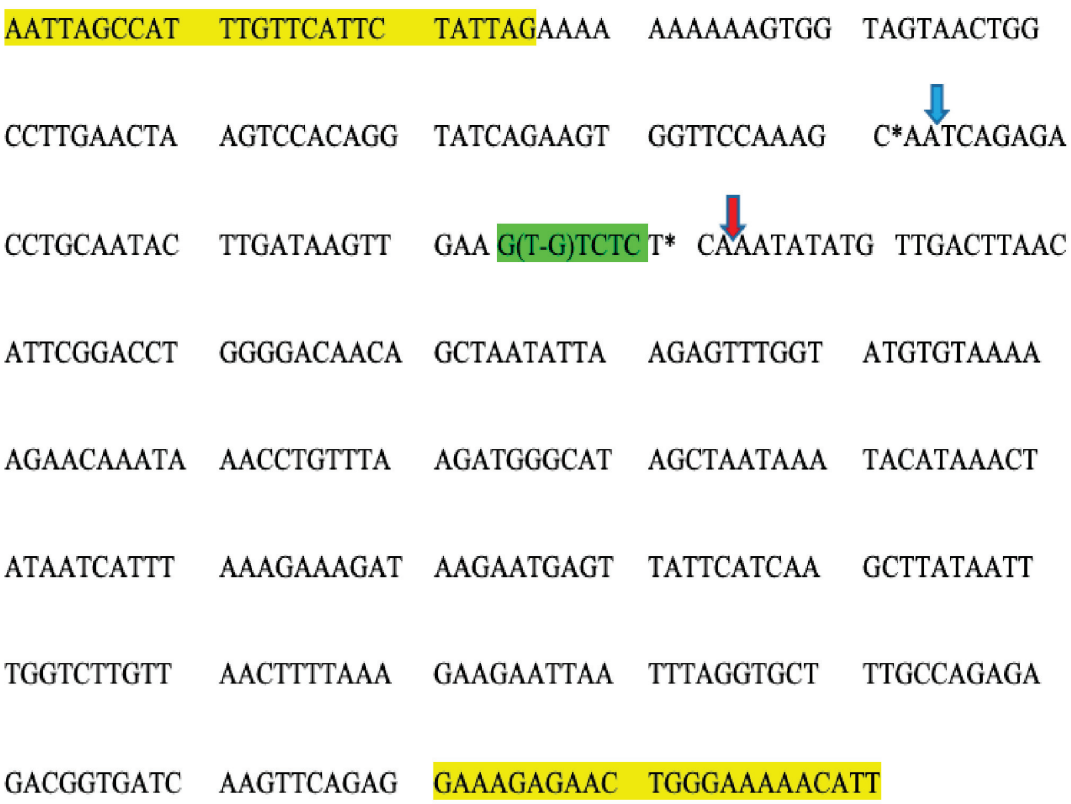


Figure 2. 2. Schematic representation of the Cholesterol 7-alpha hydroxylase gene (CYP7A1) nucleotide sequence that includes rs3808607 single nucleotide polymorphism.

The yellow highlighted sequences are the forward and reverse primers used for PCR procedure. The green region is enzyme recognition site. In wild type, the enzyme is cut from blue arrow site and form fragments of 300 bp and 93 bp. In mutant type, it forms three bands of 261 bp, 93 bp and 39 bp by cutting from blue and red arrows sites. The nucleotide sequence was taken from https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=3808607#fasta. Restriction points were determined with the help of the http://www.restrictionmapper.org/.

2.2.2.2. Genotyping of CYP46A1 rs754203 Single Nucleotide Polymorphism on CYP46A1 Gene

2.2.2.2.1. Polymerase Chain Reaction for CYP46A1 rs754203 SNP

T → C polymorphism (rs754203) in the intron 2 region of CYP46A1 gene was amplified by PCR. The used reagents (details were given in **Appendix C**) were

**Taq* DNA polymerase

*PCR amplification buffer with $(NH_4)_2SO_4$

*dNTP mixture

*$MgCl_2$ solution

*Forward and reverse primers

*Pure H_2O (section 2.1.3).

In order to obtain single and specific product for interested SNP region, amounts of PCR components were optimized. Three different concentrations of primer (30 pmole, 40 pmole, 50 pmole) and three different concentrations of $MgCl_2$ (1M, 1.5M, 2M) with two different *Taq* buffers [with KCl or $(NH_4)_2SO_4$] were tested under the PCR conditions, which were determined according to study of Papassotiropoulos and colleagues (Papassotiropoulos et al., 2003). The amounts of compounds used for optimized PCR mixture were given in **Table 2.6** and PCR conditions for this interested region were shown in **Table 2.7.**

The amplified PCR products were checked with 3% agarose gel (its method was described in section 2.2.1.3). After 5µl of PCR product and 1 µl of loading dye (6X) were mixed by pipetting, the mixture was filled into the well. Also, 5 µl of Gene-Ruler 50 bp DNA ladder mix was loaded to one of the wells to determine the size of PCR products. The gel was run at 90 V, until the dye reached to the bottom of the gel -

approximately 90 min-. Then, the gel was analyzed under the UV light and its photography was taken. PCR product was expected to be seen at 182 bp in the gel.

Table 2. 6. *The component of PCR mixture for CYP46A1 rs754203 polymorphism.*

Constituent	**Stock Concentration**	**Volume Added**	**Final Concentration in 50 µl Reaction Mixture**
Amplification (*Taq*) Buffer with $(NH_4)_2SO_4$	10X	5 µl	1X
$MgCl_2$	25 mM	2 µl	1 mM
dNTP Mixture	10 mM	1 µl	0.2 mM
Forward Primer	10 pmole/µl	4 µl	40 pmole
Reverse Primer	10 pmole/µl	4 µl	40 pmole
Taq DNA Polymerase	5U/µl	0.5 µl	2.5 U
Template DNA	Varies	Varies	~200 ng
Sterile Apyrogen H_2O		Up to 50 µl	

Table 2. 7. *The thermal cycling program to amplify CYP46A1 rs754203 SNP.*

	Temperature (°C)	Time	
Initial Denaturation	94	5 min	
Denaturation	94	30 sec	8 X
Annealing	59	30 sec	
Extension	72	30 sec	
Denaturation	94	30 sec	27 X
Annealing	55	30 sec	
Extension	72	30 sec	
Final Extension	72	7 min	

2.2.2.2.2. Restriction Endonuclease Digestion of CYP46A1 rs754203 Polymorphism

Restriction fragment length polymorphism (RFLP) was used determination of genotypes of rs754203: T → C in CYP46A1 gene for this study. MspI (HpaII) restriction enzyme was used to digest PCR products of CYP46A1. In individuals carrying the wild type T allele, the enzyme cannot cut the DNA. However, if the person has the mutant allele C, it cuts the DNA with the help of the forming CCGG recognition site through this transition, creating bands of 137 bp and 45 bp. Furthermore, it forms fragments of 182 bp 137 bp and 45 bp in heterogeneous individuals in terms of this SNP (**Figure 2.3**).

In order to obtain the clearest digestion products in the agarose gel electrophoresis, optimizations for restriction enzyme digestion mixture and incubation conditions were

carried out. Three different amounts of PCR product (2µl, 5µl, 10µl) were tested with different incubations time like 1hours (h), 1.5 h, 2 h, 2.5 h, 3 h, and 17 h at 37°C with three different enzyme concentration (2U, 5U, 10U from 1U/µl stock enzyme solution), separately. The optimized conditions used for this digestion mix were summarized in **Table 2.8**.

Ten µl PCR product was incubated with 10 U MspI restriction enzyme at 37°C for 17 hours. After incubation, the digested products were examined in 3% agarose gel and 5 µl of GeneRuler low range DNA ladder mix, which was prepared according to manufacturer's instructions, was also loaded to one of the wells to define the length of the fragments. The gel was run at 90 V for 90 min.

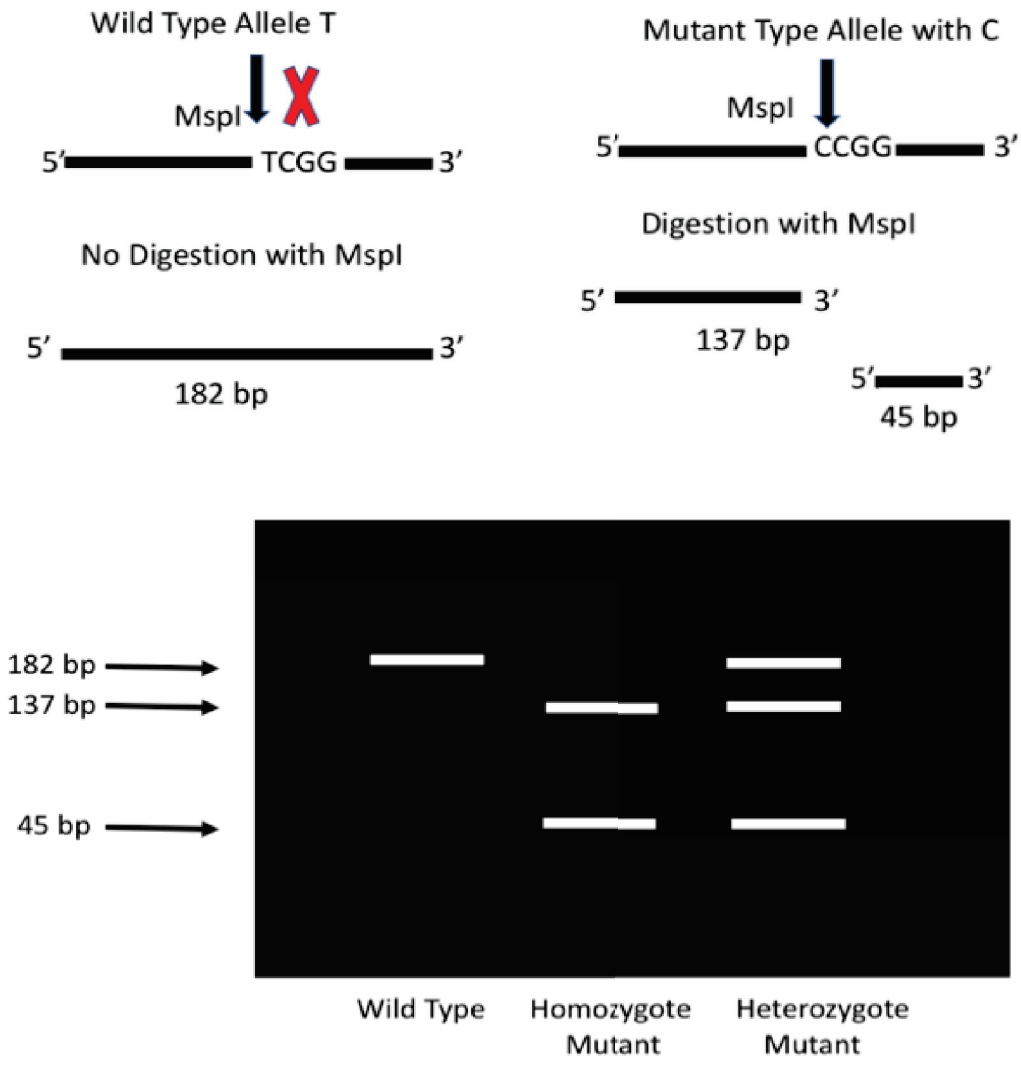


Figure 2. 3. Schematic presentation of genotypes for CYP46A1 rs754203 single nucleotide polymorphism.

In the left panel, the wild type allele "T" allele can be seen. MspI does not find its cutting site on having this type genotype in individuals and therefore, unfragmented 182 bp band represents the wild type allele "T". In the left panel, the mutant allele (CC) can be seen. MspI cuts the 182 bp PCR product and cause two fragments of 137 bp and 45 bp. A representative agarose gel electrophoresis photo of wild type, homozygote and heterozygote mutant genotypes is given in the bottom site. Used reagent for determination of genotypes were mentioned in Appendix C.

Table 2. 8. *The components of restriction endonuclease digestion mixture for CYP46A1 rs754203 polymorphism.*

Constituent	**Stock Concentration**	**Amount / Volume to be added**	**Final Concentration in 50 µl Reaction Mixture**
PCR Product		10 µL	
NEBuffer	10X	5 µL	1X
MspI	1U/ µl	10 µL	10U
Sterile Apyrogen H_2O		25 µL	
Total		50 µl	

Amplified region of CYP46A1 gene including rs754203 single nucleotide polymorphism was demonstrated in **Figure 2.4** with highlighted forward and reverse primers, recognition site of restriction enzyme and single nucleotide substitution.

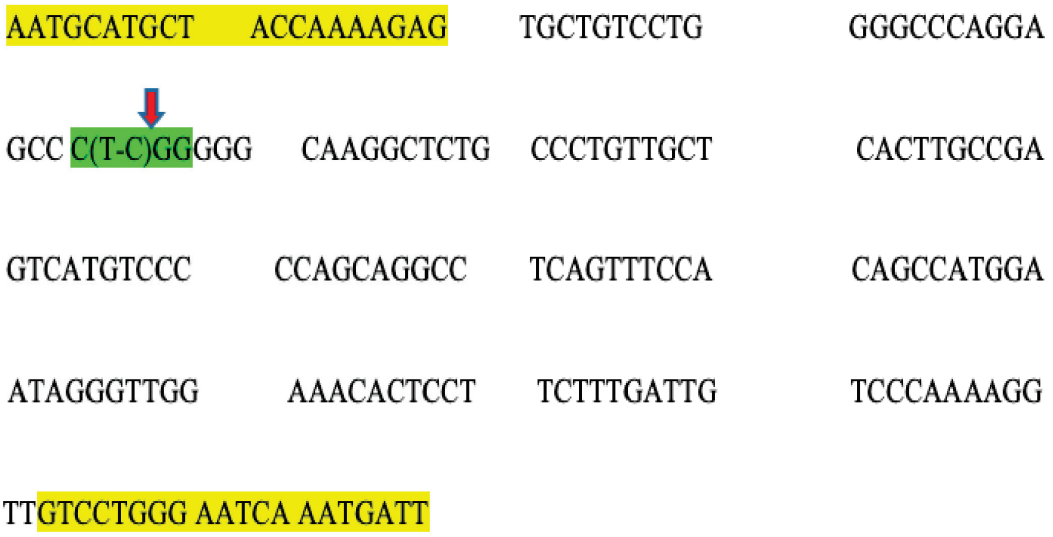


Figure 2. 4. Schematic representation of the cholesterol 24- hydroxylase (CYP46A1) gene nucleotide sequence that includes rs754203: T → C single nucleotide polymorphism.

The yellow highlighted sequences are the forward and reverse primers used for PCR procedure. The green region is enzyme recognition site. In wild type allele (TT), the enzyme does not find its recognition site (CCGG); and unfragmented 182bp PCR product is seen in the gel. In mutant type allele (CC), it cuts the DNA from red arrow point; and forms two bands of 137 bp and 45 bp. The nucleotide sequence was taken from https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=754203#fasta. Restriction point was determined with the help of the http://www.restrictionmapper.org/.

2.2.3. Sanger Sequencing

After finishing the PCR allelic discriminations of MS case and healthy control groups in terms of rs3808607 in CYP7A1 and rs754203 in CYP46A1 SNPs, six of the samples of them were selected to check up the accuracy of genotyping. These samples were picked up from amongst possible wild type, homozygote mutant and heterozygote mutant types alleles for each polymorphic region; and Sanger sequencing was performed for them by Sentegen (Sentegen Biotechnology, Ankara, Turkey).

2.2.4. Statistical Analysis

Statistical analyses were performed by using SPSS (Statistical Package for Social Sciences, IBM Corp. Released 2016. IBM SPSS Statistics for Macintosh, Version 24.0. Armonk, NY: IBM Corp.).

Normality of the sample distribution of each continuous variable was tested with Kolmogorov- Smirnov test. In analyses of lipid parameters and vitamin D values, if they didn't show normal distribution, logarithmic transformation (log10) was applied to bring the data closer to normal distribution. When the new log-transformed variable showed normal distribution, parametric tests were used in the analyses. In the chapter of results, this event was indicated with "*" sign in the tables, once transformed data was used in the analysis. On the other hand, if the data didn't show the normal distribution after log 10 transformation, nonparametric tests were used with untransformed data. Depending on the shape of the distribution curves, Independent Sample t-Test and Mann Whitney U-Test were used to evaluate the differences of continuous variables. These continuous variables were expressed as mean ± standard deviation (SD) and median ± interquartile range (IQR) value at the end of the analysis.

Categorical variables were stated as proportions and their comparisons were done using Chi-square test. Allele frequencies were determined by the gene counting method. Chi-square was used to assess the departure from the Hardy-Weinberg equilibrium and also in order to compare genotype distributions and allele frequencies.

Evaluation for significance of the results was carried out with *p*-value, in which if the *p*-value was less than 0.05, this result was accepted as statistically significant, in contrast ones higher than 0.05 was evaluated as insignificant. As well as this, Bonferroni correction for multiple testing was applied.

CHAPTER 3

RESULTS

3.1. Study Population

The study population comprised of 138 Multiple Sclerosis patient and 100 healthy control subjects. Clinical parameters of both groups, like serum lipid (total cholesterol, total triglyceride, HDL-cholesterol, LDL-cholesterol) and 25-hydroxy-vitamin D values were measured by Gülhane Education and Research Hospital, Biochemistry Laboratory. These parameters and demographic information for patients and controls were demonstrated in **Table 3.1**.

Multiple Sclerosis is one of the most common causes of neurological disability in young and middle-aged adults. Therefore, age is an important factor in MS. In this study, the mean age was 33.54 ± 5.88 for patients and 32.79 ± 8.93 for controls; and any statistically significant difference was not found between them (p = 0.053). The women are more prone to be MS than men with 3:2 ratio. While our patients group included 95 females and 43 males, the controls group comprised of 61 female and 39 male subjects. Number of females in patients (68.8 %) were higher than controls (61.0 %) but this was at an insignificant level (p = 0.208).

According to clinical laboratory tests, Total Cholesterol (TC) level was significantly lower in MS patients (4.89 ± 0.97 mmol/L) than healthy controls (4.95 ± 1.11 mmol/L; p = 0.010). In addition, LDL-cholesterol level was significantly higher in the controls (3.07 ± 0.93 mmol/L;) than in patients (2.71 ± 0.82 mmol/L; p = 0.010). On the other hand, the triglycerides level and HDL-cholesterol levels were insignificantly higher in controls (1.49 ± 0.92 mmol/L and 1.31 ± 0.30 mmol/L, respectively) than in patients (1.16 ± 0.56 mmol/L and 1.28 ± 0.30 mmol/L, respectively), (p = 0.054 for TG and p = 0.725 for HDL-C). Since level of serum vitamin D affects the MS development and modifies the disease activity in MS patients, Vitamin D is evaluated as a risk factor for MS. In our study, vitamin D level was found significantly lower in MS patients (53.83 ± 38.57 nmol/L) than healthy controls (63.63 ± 29.89 nmol/L; p = 0.003).

Table 3. 1. Clinical laboratory data and general characteristics of MS patients (**P**) and healthy controls (**C**).

Parameters	**n**	**Patients (P: 138)**	**Controls (C: 100)**	***P***	**OR (95% CI)**
Age (years)[a]	P: 114 C: 100	mean ± SD: 33.54 ± 5.88 median ± IQR: 35.00 ± 8	mean ± SD: 32.79 ± 8.93 median ± IQR: 32.00 ± 11	0.053	-
Female, n (%) [b]	P: 138 C: 100	95 (68.8)	61 (61.0)	0.208	1.414 (0.823 – 2.423)
Total Cholesterol (mmol/L) [a]	P: 92 C: 100	mean ± SD: 4.89 ± 0.97 median ± IQR: 4.51 ± 0.95	mean ± SD: 4.95 ± 1.11 median ± IQR: 4.87 ± 1.21	**0.005**	-
Triglycerides (mmol/L) [a]	P: 93 C: 100	mean ± SD: 1.16 ± 0.56 median ± IQR: 0.99 ± 0.82	mean ± SD: 1.49 ± 0.92 median ± IQR: 1.20 ± 1.16	0.054	-
HDL - C (mmol/L) [a]	P: 84 C: 100	mean ± SD: 1.28 ± 0.30 median ± IQR: 1.24 ± 0.38	mean ± SD: 1.31 ± 0.30 median ± IQR:1.26 ± 0.48	0.725	-
LDL - C (mmol/L) [a]	P: 84 C: 100	mean ± SD: 2.71 ± 0.82 median ± IQR: 2.64 ± 0.87	mean ± SD: 3.07 ± 0.93 median ± IQR: 2.98 ± 1.05	**0.010**	-
Vitamin D (nmol/L) [a]	P: 78 C: 100	mean ± SD: 53.83 ± 38.57 median ± IQR: 43.78 ± 53.28	mean ± SD: 63.63 ± 29.89 median ± IQR: 61.73 ± 35.39	**0.003**	-

([a] Mann Whitney U Test is applied; [b] Chi-square test is applied; critical p value: 0.05)

3.2. Genotyping for Single Nucleotide Polymorphisms in CYP7A1 and CYP46A1

The PCR-RFLP method was used in order to determine genotypes of CYP7A1 rs3808607 A→ C SNP and CYP46A1 rs754203 T→ C SNP in all subjects, in which interested regions were amplified by PCR, then these products were digested by specific enzymes, and finally the restriction products were visualized by agarose gel electrophoresis.

3.2.1. Genotyping for rs3808607 Single Nucleotide Polymorphism of CYP7A1 Gene

Determination of rs3808607 A→ C SNP of CYP7A1 gene was conducted by PCR-RFLP method. After the polymerase chain reaction, the restriction enzyme digestion was performed and the specific enzyme for this procedure was BsaI.

3.2.1.1. Polymerase Chain Reaction Results for rs3808607 SNP of CYP7A1

rs3808607 is the most widely studied SNP in the promoter region of the CYP7A1 gene. It is an A/C transversion polymorphism at -278 from the translational codon, or -204 from transcriptional start site. This region was amplified by polymerase chain reaction, which was optimized to obtain the desired band without non-specific bands. The optimized PCR mixture and conditions were shown in Section 2.2.2.1.1. In briefly, almost 200 ng of DNA, 1X Amplification (*Taq*) Buffer with $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2 mM dNTPs, 50 pmole of each primer and 2.5 unit of *Taq* DNA polymerase were included in PCR reaction.

The amplified PCR products were analyzed on 1% agarose prepared by adding 0.6 g agarose to 60 ml of 0.5X TBE buffer. 10 µl of each PCR product was mixed with 1 µl of 6X gel loading dye and they were put into the well of the gel, which was repeated for each sample. 5 µl of 50 bp DNA ladder mix (prepared according to manufacturer instructions) was applied to the one well, and then the gel was run for 90 min at 90 V. Because of selected primers, the product size of the amplified region should be at 393

bp. **Figure 3.1** shows the agarose gel electrophoresis image of the CYP7A1 rs3808607 SNP from patients and controls after PCR reaction.

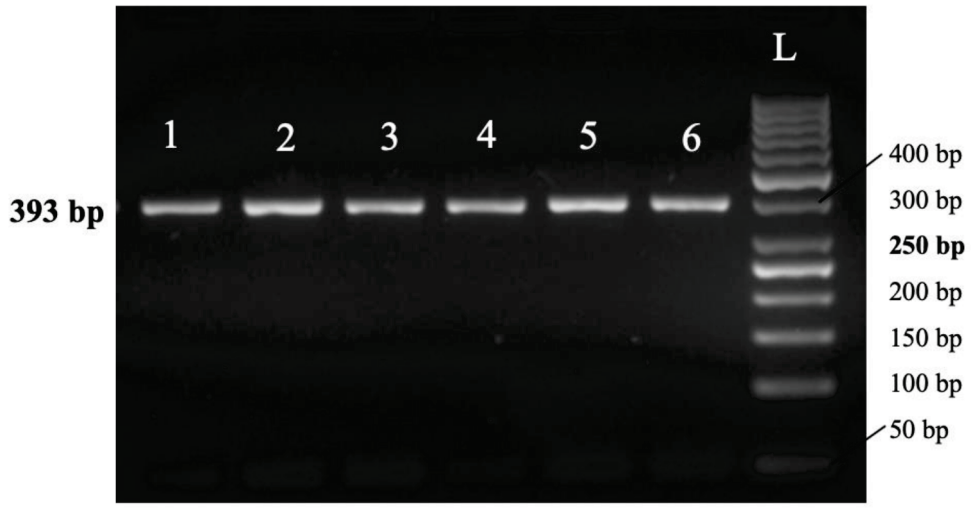


Figure 3. 1. Agarose gel (1 %) electrophoresis image for PCR products of rs3808607 SNP of CYP7A1 gene.

Ladder (50 -1000 bp) was shown as "L" on the picture. The other lanes represented the 393 bp-PCR products (Lane 1, 2, 3 were PCR products of patients, the others were from controls).

3.2.1.2. Restriction Endonuclease Digestion Results for rs3808607 SNP of CYP7A1

The allelic discriminations of patients and controls were done by restriction fragment length polymorphism method. A specific endonuclease enzyme, BsaI was used for CYP7A1 rs3808607 A → C SNP. If the individual carries the wild type allele "A", the PCR product contains one recognition site (5'...-GGTCTC↓-...3') for enzyme, independently from the presence or absence of SNP, and the enzyme cuts the DNA by forming two fragments: 300 bp and 93 bp. However, if the individual carries the polymorphic allele "C", an extra-suitable site forms and the enzyme cuts the DNA from two points by producing three fragments: 261 bp, 93bp and 39 bp. If the individual has the heterozygous genotype, it yields four different DNA fragments with 300 bp, 261 bp, 93 bp, and 39 bp. The digestion procedure details and optimized

conditions for it were mentioned in Section 2.2.2.1.2. The results for restriction fragments of CYP7A1 rs3808607 SNP were demonstrated in **Figure 3.2.**

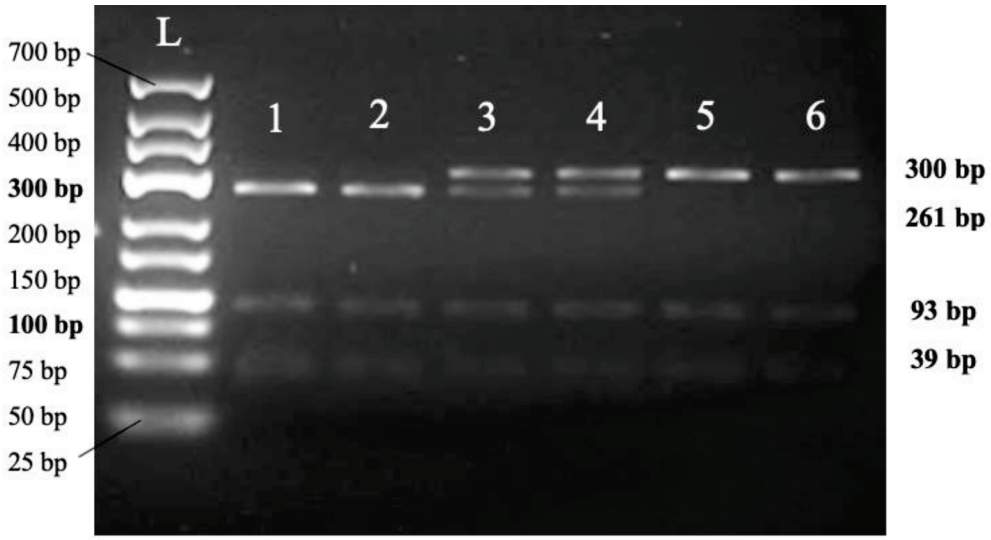


Figure 3. 2. Agarose gel (3 %) electrophoresis image for restriction endonuclease digestion with BsaI of amplified rs3808607 SNP of CYP7A1 gene.

Agarose gel (3 %) electrophoresis image for restriction endonuclease digestion with BsaI of amplified rs3808607 SNP of CYP7A1 gene. Ladder (25 -700 bp) was shown as "L" on the picture. The other lanes were the digestion products from patients (odd numbers) and controls (even numbers). Lane 1 and 2 represented the homozygote mutant individuals (CC), who formed three bands at 261 bp, 93 bp and 39 bp and lane 5 and 6 represented the homozygote wild type (AA) individuals, who made two bands at 300 bp and 93 bp after digestion. Lane 3 and 4 showed the heterozygote mutant (AC) individuals with four bands at 300 bp, 261 bp, 93 bp and 39 bp.

3.2.2. Genotyping for rs754203 Single Nucleotide Polymorphism of CYP46A1 Gene

Determination of genotypes for rs754203 T → C single nucleotide polymorphism of CYP46A1 gene was conducted by PCR-RFLP method. After the polymerase chain reaction, the restriction enzyme digestion was done in this process. MspI was selected as the specific enzyme.

3.2.2.1. Polymerase Chain Reaction Results for rs754203 SNP of CYP46A1

rs754203 single nucleotide polymorphism, which changes T to C, is located intron 2 region of the CYP7A1 gene. This region was amplified by polymerase chain reaction. In order to obtain desired PCR products, like only interested band on the gel by avoiding from forming of non-specific bands, PCR ingredients and conditions were optimized, which were mentioned in the Section 2.2.2.2.1. In short, almost 200 ng of DNA sample, 1X amplification (*Taq*) buffer with $(NH_4)_2SO_4$, 1 mM $MgCl_2$, 0.2 mM dNTPs, 40 pmole of each primer, 2.5 U of *Taq* DNA polymerase were included in the PCR reaction tube. After amplification, the products were analyzed through 3% agarose gel electrophoresis, in which 10 µl of each PCR product was mixed with 1 µl of 6X loading dye and they put into the well of the gel, and 5 µl of 25 -700 bp DNA ladder mix (after prepared according to the manufacturer instructions) was applied into one well of the gel, and then the gel was run for 90 min at 90 V.

The expected DNA fragments are the 182 bp length for rs754203 SNP of the CYP46A1 gene. **Figure 3.3** represents the gel photo of the amplified region of this gene. The bands were located approximately at 182 bp.

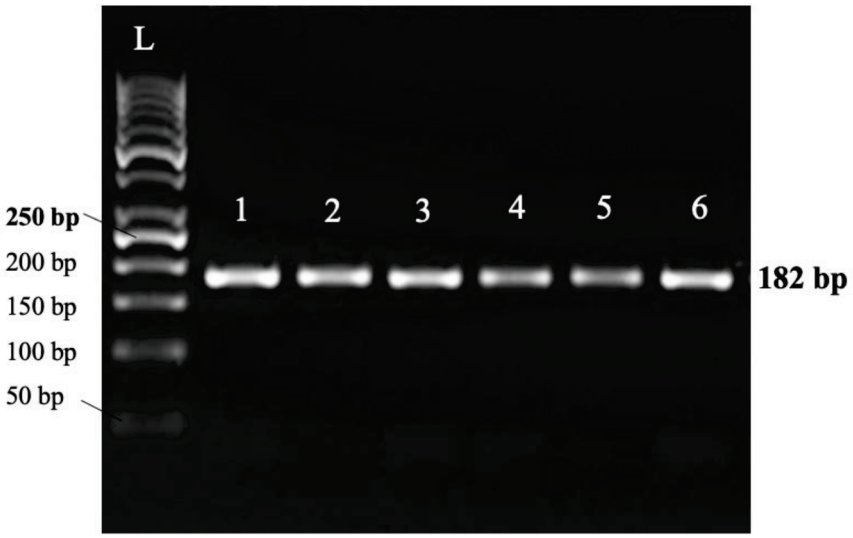


Figure 3. 3. Agarose gel (3 %) electrophoresis image for PCR products of rs754203 SNP of CYP46A1 gene.

Ladder (50 -1000 bp) was displayed as "L" on the picture. The other lanes demonstrated the 182 bp length PCR products (Lane 1, 2, 3 were selected from patients, the others were from controls).

3.2.2.2. Restriction Endonuclease Digestion Results for rs754203 SNP of CYP46A1

The allelic discriminations of patient and control subjects were done by restriction length fragment polymorphism method. As a specific enzyme, MspI was selected for CYP46A1 rs754203 SNP. In the wild type individuals, enzyme cannot digest the DNA due to the lack of its recognition site (5'...C ↓ CGG…3'), and a single band with 182 bp long is seen on the gel. On the other hand, in the mutated allele that bears the "C" allele at this position, the enzyme can find the cutting site on the DNA and forms two bands as 137 bp and 45 bp long. The digestion procedure and details for optimized conditions were given in Section 2.2.2.2.2. **Figure 3.4** illustrates the agarose gel photography of restriction endonuclease digestion products for rs754203 SNP of CYP46A1 gene.

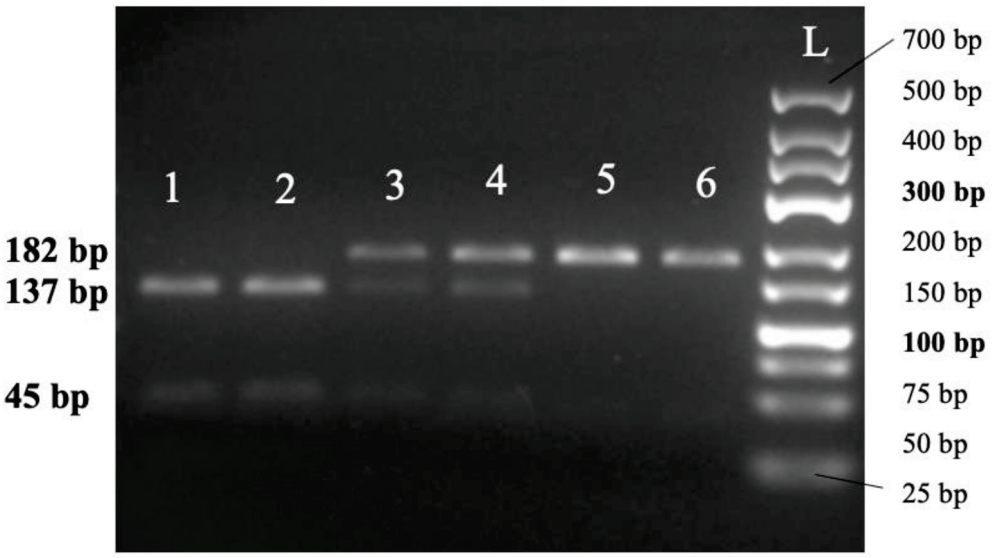


Figure 3. 4. Agarose gel (3 %) electrophoresis image for restriction endonuclease digestion with MspI of amplified rs754203 SNP of CYP46A1 gene.

Ladder (25 -700 bp) was shown as "L" on the picture. The other lanes were the digestion products from patients (odd numbers) and controls (even numbers). Lane 1 and 2 were from the homozygote mutant individuals (CC), who made two bands at 137 bp and 45 bp after digestion; Lane 5 and 6 were from the homozygote wild type (TT) individuals, who had only one band at 182 bp due to the absence of suitable recognition site; Lane 3 and 4 showed the heterozygote mutant (AC) individuals with three bands at 182 bp, 137 bp and 45 bp.

After finished the all allelic discriminations for patients and controls in terms of rs3808607 and rs754203 SNPs, six samples of them were sequenced by Sentegen (Sentegen Biotechnology, Ankara, Turkey) in order to test their genotyping accuracy. One sample from each wild type homozygote, mutant homozygote and heterozygote genotypes for both SNPs was sequenced and their results for our interested points matched with our genotyping outcomes.

3.3. Analysis of Genotypes and Allele Frequencies of rs3808607 SNP CYP7A1 and rs754203 SNP of CYP46A1

In this study, rs3808607 SNP of CYP7A1 gene and rs754203 SNP of CYP46A1 gene were analyzed at 138 MS patients and 100 healthy controls with respect to genotype and allele frequencies. The aim of this was to learn how their distributions were changing in MS patient and control groups in the Turkish population and also was to determine whether these SNPs have any effects on risk of the MS. For that purposes, the analyses were conducted by forming three genetic models of SNPs: Dominant, Recessive and Additive Model. In dominant model, the polymorphic allele was accepted as having dominant character compared to wild type allele. Therefore, the carries of mutant allele were pooled in a single group. On the other hand, in recessive model, it was assumed that for the polymorphic allele had recessive characteristic compared to wild type allele and therefore, wild type allele carriers were collected in a single group. In addition to these, additive model was formed to be based upon the fact that both wild type allele and mutant allele have equal genetic effects on heterozygote individuals. Hence, three different groups were formed within this model. All comparisons were done between these created groups.

Odds ratio was calculated the following formula in order to determine the risk assessment between groups:

$$OR = \frac{\text{\# of patients with risky allele / of patients without risky allele}}{\text{\# of controls with risky allele / of controls without risky allele}}$$

When calculating the odds ratio in dominant model, the individuals carrying polymorphic allele were assessed within the risky group and homozygous wild type individuals were included into without risky group. In contrast, only homozygous mutant allele individuals were counted as risky groups in recessive model and wild type allele carriers were considered as without risky groups. As for additive model, both homozygous mutant individuals and heterozygous mutant individuals were evaluated separately as having risky allele groups, and homozygous wild type subjects are counted in non-risky group. Two separate analyses, which were in individuals with heterozygote mutant versus homozygote wild type and homozygote mutant versus homozygote wild type, were conducted within this model. In multiple comparisons, Bonferroni corrected *p* value was calculated with this formula: new critical alpha = (general alpha rate/ per comparison). The general alpha rate is accepted as 0.05 and therefore, new critical alpha was determined as 0.05/5 = 0.01 in them.

3.3.1. Analysis of Genotypes and Allele Frequencies for rs3808607 SNP of CYP7A1 Gene

The distribution of genotypes and allele frequencies for rs3808607 single nucleotide polymorphism of CYP7A1 gene within the multiple sclerosis patients and healthy controls are shown in **Table 3.2**. In this study, a total of 138 MS patient and 100 control individuals were analyzed in terms of CYP7A1 rs3808607 A → C polymorphism. Among them, 46 (33.3%) patients and 44 (44.0%) controls had homozygous wild type (AA); 82 (59.4%) patients and 51 (51.0%) controls had heterozygous mutant type (AC); and 10 (7.2%) patients and 5 (5.0%) controls had homozygous mutant genotype (CC). In additive model, the comparisons were performed with the heterozygous mutant individuals (AC) versus homozygous wild type individuals (AA), and also with homozygous mutant individuals (CC) versus homozygous wild type individuals (AA). There was no significant difference among the patients and controls with respect to genotype distribution in this model of rs3808607 SNP (OR: 1.538, 95% CI: 0.895 – 2.642 and *p:* 0.118 for AC vs AA), (OR: 1.913, 95% CI: 0.606 – 6.044 and *p:* 0.403 for CC vs AA).

In dominant model, the comparison was done between the carriers of mutant allele "C" and the carriers of homozygous wild type genotype. When the OR was calculated, 92 patients and 56 controls were evaluated within the risky group (AC+CC), and 46 patients and 44 controls were included as non-risky groups (AA). However, no statistically significant difference was found between patients and controls (OR: 1.571, 95% CI: 0.925-2.670, and *p:* 0.094). In recessive model, the homozygous mutant individuals (CC) were compared with wild type allele carriers (AC+AA). While 128 patient and 95 controls were assumed as without risk group (AC+AA), 10 patients and 5 controls were put into the risky group category (CC). At the end of the analysis, statistically significant difference among the patients and controls was not seen (OR: 1.484, 95% CI: 0.491 – 4.485 and *p:*0.594).

The wild type allele (A) frequencies were 0.870 and 0.695 and the mutant allele (C) ones were 0.130 and 0.305 for patients and controls, respectively. But, statistically significant difference was not found between them (OR: 1.336, 95% CI: 0.506–1.968 and *p:* 0.143).

Table 3. 2. *Genotypes and alleles frequency distribution of patients and controls for CYP7A1rs3808607 A → C single nucleotide polymorphism.*

Genotype/Allele	Patients (n: 138) (n, %)	Controls (n: 100) (n, %)	Model	OR (95%CI)	*p* [e]
AA	46 (33.3)	44 (44.0)		Reference	Reference
AC	82 (59.4)	51 (51.0)	**Additive** [a]	1.538 (0.895 – 2.642)	0.118 *
CC	10 (7.2)	5 (5.0)		1.913 (0.606 – 6.044)	0.403 +
AA	46 (33.3)	44 (44.0)	**Dominant** [b]	1.571	
AC+CC	92 (66.7)	56 (56.0)		(0.925 – 2.670)	0.094 *
AA+AC	128 (92.8)	95 (95.0)	**Recessive** [c]	1.484	
CC	10 (7.2)	5 (5.0)		(0.491 – 4.485)	0.594 +
A	0.870	0.695	**Allele frequency** [d]	1.336	
C	0.130	0.305		(0.506 - 1.968)	0.143 *

[a]: AC vs AA and CC vs AA; [b]: AC + CC vs AA; [c]: CC vs AC + AA; [d]: C vs A (: Pearson's chi-squared test and +: Fisher's exact test were applied.) (Bonferroni corrected p value threshold: 0.01)*

3.3.1.1. Genotypes and Allele Frequencies of rs3808607 SNP of CYP7A1 Gene in Male Subgroups

The distribution of genotypes and frequencies of alleles for rs3808607 single nucleotide polymorphism of CYP7A1 gene in male subgroups of MS patients and healthy controls are shown in **Table 3.3.** 43 male MS subjects and 39 male healthy controls were studied regarding this A → C polymorphism. Among them, 14 (32.6 %) patients and 21 (53.8 %) controls were homozygote wild type (AA); 28 (65.1 %) patients and 16 (41.0 %) controls were heterozygote mutant (AC); and 1 (2.3 %) patients and 2 (5.1 %) controls were homozygote mutant genotype.

Like in the previous section, the analysis was conducted according to three genetic models. In additive model, although there was statistically significant difference between the heterozygote male individuals versus ones with wild type allele, after Bonferroni correction for multiple testing, it was not remained this significance (OR: 2.625, 95% CI: 1.053 – 6.545 and *p*: 0.036). Similarly, there was no statistically important difference in comparison of male-homozygote mutant genotype (CC) individuals and wild type genotype (AA) ones within this model (OR: 0.750, 95% CI: 0.062 -9.081 and *p:* 0.999). In dominant model, the polymorphic C allele carriers, 29 for patients and 18 for controls, were compared with homozygote wild type carriers, 14 for patients and 21 for controls. However, no statistically significant link was found in male patients and controls in terms of this SNP in that model (OR: 2.417, 95% CI: 0.986 – 5.922 and *p:* 0.052). In recessive model, the comparison was performed between homozygote mutant individuals and having wild type allele individuals. Likewise, there was no significant difference between groups in terms of genotype distributions of this SNP (OR: 0.440, 95% CI: 0.038 – 5.058 and *p:*0.602).

It was observed that A and C allele frequencies were 0.651 and 0.349 for patients and 0.744 and 0.256 for controls, respectively. No significant association for the allele frequencies was also found between the male subgroups (OR: 1.554, 95% CI: 0.791 – 3.050 and *p:* 0.199).

Table 3. 3. Distribution of genotypes and alleles of CYP7A1 rs3808607 A → C single nucleotide polymorphism in the male subgroups of MSpatients and healthy controls.

Genotype/ Allele	Patients (n=43) (n, %)	Controls (n=39) (n, %)	Model	OR (95%CI)	*P*
AA	14 (32.6)	21 (53.8)		Reference	Reference
AC	28 (65.1)	16 (41.0)	**Additive** [a]	2.625 (1.053 – 6.545)	0.036 *
CC	1 (2.3)	2 (5.1)		0.750 (0.062 – 9.081)	0.999 +
AA	14 (32.6)	21 (53.8)	**Dominant** [b]	2.417 (0.986 – 5.922)	0.052 *
AC+CC	29 (67.4)	18 (46.2)			
AA+AC	42 (97.7)	37 (94.9)	**Recessive** [c]	0.440 (0.038 – 5.058)	0.602 +
CC	1 (2.3)	2 (5.1)			
A	0.651	0.744	**Allele frequency** [d]	1.554 (0.791 – 3.050)	0.199 *
C	0.349	0.256			

[a]: AC vs AA and CC vs AA; [b]: AC + CC vs AA; [c]: CC vs AC + AA; [d]: C vs A. (: Pearson's chi-squared test and +: Fisher's exact test were applied). (Bonferroni corrected p value threshold: 0.01).*

3.3.1.2.Genotypes and Allele Frequencies of rs3808607 SNP of CYP7A1 Gene in Female Subgroups

Table 3.4 illustrates the distributions of genotypes and frequencies of alleles for rs3808607 single nucleotide polymorphism of CYP7A1 gene in female subgroups of MS patients and healthy controls.

95 female MS patients and 61 female healthy controls were studied in terms of rs3808607 A → C SNP of CYP7A1 gene. Among them, 32 (33.7 %), 54 (56.8 %) and 9 (9.5 %) individuals from patients, and 23 (37.7 %), 35 (57.4 %) and 3 (4.9 %) individuals from controls had homozygote wild type (AA), heterozygote mutant homozygote (AC), and homozygote mutant type genotype (CC), respectively.

In additive model, the comparisons were done with "AC vs AA" and "CC vs AA" genotypes in female subjects. Nonetheless, no significant differences were found for both comparisons (OR: 1.109, 95% CI: 0.56 – 2.198, *p:* 0.767 for AC vs AA; OR: 2.156, 95% CI: 0.525 – 8.852, *p:* 0.343 for "CC vs AA").

The comparison was conducted between individuals with mutant allele and individuals with homozygote wild type allele in dominant model; however, no significant association was seen between them (OR: 1.192, 95% CI: 0.610 – 2.329, *p:* 0.608). Similarly, no significant difference for the genotype distribution of rs3808607 SNP according to recessive model was seen in the female subgroups of patients and controls (OR: 2.023; 95% CI: 0.525 – 7.792, and *p:* 0.369).

Allele frequencies of this SNP was found to be nearly same in female patients and controls, which were 0.621 and 0.664 for A allele and 0.379 and 0.336 for C allele, respectively (OR: 1.205, 95% CI: 0.749 – 1.941 and *p:* 0.442).

Table 3. 4. Distribution of genotypes and alleles of CYP7A1 rs3808607 A → C single nucleotide polymorphism in the female MS patients and healthy controls.

Genotype/ Allele	Patients (n=95) (n, %)	Controls (n=61) (n, %)	Model	OR (95%CI)	*P*
AA	32 (33.7)	23 (37.7)		Reference	Reference
AC	54 (56.8)	35 (57.4)	**Additive** [a]	1.109 (0.56 – 2.198)	0.767 *
CC	9 (9.5)	3 (4.9)		2.156 (0.525 – 8.852)	0.343 +
AA	32 (33.7)	23 (37.7)	**Dominant** [b]	1.192	
AC+CC	63 (66.3)	38 (62.3)		(0.610 – 2.329)	0.608 *
AA+AC	86 (90.5)	58 (95.1)	**Recessive** [c]	2.023	
CC	9 (9.5)	3 (4.9)		(0.525 – 7.792)	0.369 +
A	0.621	0.664	**Allele frequency** [d]	1.205	
C	0.379	0.336		(0.749 – 1.941)	0.442 *

[a]: AC vs AA and CC vs AA; [b]: AC + CC vs AA; [c]: CC vs AC + AA; [d]: C vs A. (: Pearson's chi-squared test and +: Fisher's exact test were applied). (Bonferroni corrected p value threshold: 0.01)*

3.3.2. Analysis of Genotypes and Allele Frequencies for rs754203 SNP of CYP46A1 Gene

The 138 MS patients and 100 healthy controls were investigated in terms of conversion from "T" allele to "C" allele in the CYP46A1 gene and results of the genotype distributions and allele frequencies for this SNP were summarized in **Table 3.5**. The genotypes frequency distribution of the patient groups agreed with Hardy-Weinberg equilibrium (*p:* 0.302).

There were 69 (50.0%) and 58 (58%) homozygote wild type (TT), 59 (42.8%) and 34 (34.0%) heterozygote mutant (TC), 10 (7.2%) and 8 (8.0%) homozygote mutant genotype individuals from patients and controls, respectively. Like in previous section, three different genetic models were formed based upon whether having additive, dominant and recessive characteristics of polymorphic allele compared to wild type allele.

In additive model, both heterozygote individuals and homozygote mutant individuals were compared with wild type-homozygous (TT) carriers, separately. However, any statistically significant association was not found between patients and controls (OR: 1.459, 95% CI: 0.843 – 2.522, *p:* 0.176 for TC vs TT and OR: 1.051, 95% CI: 0.389 – 2.837 *p*: 0.999 for CC vs TT).

It was assumed that polymorphic "C" allele had dominant characteristic compared to wild type allele in the dominant model. Thus, "C" allele carriers were pooled in a single group. In contrast, "C" allele was considered the recessive one compared to wild type allele in the recessive model. Therefore, "T" allele carriers were pooled in a single group. However, there were no any significant relations between patients and controls with respect to genotype distributions according to both models (OR: 1.381, 95% CI: 0.822 – 2.320, *p:* 0.222 for TC+CC vs TT in dominant model; and OR: 0.898, 95% CI: 0.341 – 2.364, *p:* 0.828 for CC vs TC+TT in recessive model).

The distributions of both T and C allele in patients and controls were similar, which were 0.714 and 0.286 for patients and 0.750 and 0.250 for controls, that's why no significant difference was found between them (OR: 1.203, 95% CI: 0.796 – 1.818, *p:* 0.380).

Table 3. 5. *Genotypes and allele frequency distribution of patients and controls for CYP46A1rs754203 T → C single nucleotide polymorphism.*

Genotype/Allele	Patients (n=138) (n, %)	Control (n=100) (n, %)	Model	OR (95%CI)	*P*
TT	69 (50.0)	58 (58.0)		Reference	Reference
TC	59 (42.8)	34 (34.0)	**Additive** [a]	1.459 (0.843 – 2.522)	0.176 *
CC	10 (7.2)	8 (8.0)		1.051 (0.389 – 2.837)	0.999 +
TT	69 (50.0)	58 (58.0)	**Dominant** [b]	1.381	
TC+CC	69 (50.0)	42 (42.0)		(0.822 – 2.320)	0.222 *
TT+TC	128 (92.8)	92 (92.0)	**Recessive** [c]	0.898	
CC	10 (7.2)	8 (8.0)		(0.341 – 2.364)	0.828 +
T	0.714	0.750	**Allele frequency** [d]	1.203	
C	0.286	0.250		(0.796 – 1.818)	0.380 *

[a]: TC vs TT and CC vs TT; [b]: TC + CC vs TT; [c]: CC vs TC + TT; [d]: C vs T. (: Pearson's chi-squared test and +: Fisher's exact test were applied). (Bonferroni corrected p value threshold: 0.01)*

3.3.2.1.Genotypes and Allele Frequencies of rs754203 SNP of CYP46A1 Gene in Male Subgroups

The genotype distributions and allele frequencies of rs754203 SNP of CYP46A1 gene in male subgroups of patients and controls were given in **Table 3.6.**

43 male patients and 39 male controls were analyzed in term of this polymorphism. There were 21 (48.8%) homozygous wild type (TT), 17 (39.5%) heterozygous mutant type (TC) and 5 (11.6%) homozygous mutant type individuals in the patient group. These number were 23 (59.0%), 12 (30.8%) and 4 (10.3%), respectively for the control group.

The comparison analyses were performed to additive, dominant and recessive genetic models. However, no significant association was found between male subgroups of the patients and controls with respect to genotype distributions according to each formed model (OR: 1.552 95% CI: 0.602 – 3.998, *p:* 0.362 for TC vs TT and OR: 1.369, 95% CI: 0.324 – 5.788, *p:* 0.728 for CC vs TT in additive model), (OR: 1506, 95% CI: 0.628 – 3.611, *p:* 0.358 for TC+CC vs TT in dominant model), (OR: 1.151, 95% CI: 0.286 – 4.635, *p:* 0.999 for CC vs TC+TT in recessive model).

Likewise, distributions of T and C allele frequencies were not statistically significant between them (OR: 1.327, 95% CI: 0.671 – 2.626, *p:* 0.416).

Table 3. 6. Distribution of genotypes and alleles of CYP46A1 rs754203 T → C single nucleotide polymorphism in the male subgroups of MS patients and healthy controls.

Genotype/Allele	Patients (n=43) (n, %)	Control (n=39) (n, %)	Model	OR (95%CI)	*P*
TT	21 (48.8)	23 (59.0)		Reference	Reference
TC	17 (39.5)	12 (30.8)	**Additive** [a]	1.552 (0.602 – 3.998)	0.362 *
CC	5 (11.6)	4 (10.3)		1.369 (0.324 – 5.788)	0.728 +
TT	21 (48.8)	23 (59.0)	**Dominant** [b]	1.506	
TC+CC	22 (51.2)	16 (41.0)		(0.628 – 3.611)	0.358 *
TT+TC	38 (88.4)	35 (89.7)	**Recessive** [c]	1.151	
CC	5 (11.6)	4 (10.3)		(0.286 – 4.635)	0.999 +
T	0.686	0.744	**Allele frequency** [d]	1.327	
C	0.314	0.256		(0.671 – 2.626)	0.416 *

[a]: TC vs TT and CC vs TT; [b]: TC + CC vs TT; [c]: CC vs TC + TT; [d]: C vs T. (: Pearson's chi-squared test; and +: Fisher's exact test were applied). (Bonferroni corrected p value threshold: 0.01)*

3.3.2.2. Genotypes and Allele Frequencies of rs754203 SNP of CYP46A1 Gene in Female Subgroups

Within the female subgroups of patients and controls, the distributions of genotypes and allele frequencies for rs754203 T → C single nucleotide polymorphism of CYP46A1 gene are given in **Table 3.7.**

There were 50.5% homozygote wild type (TT), 42.2% heterozygote mutant type (TC) and 5.3% homozygote mutant type genotype (CC) individuals in the patient group with 95 females. On the other hand, there were 57.4% homozygote wild type, 36.1% heterozygote mutant type and 6.6% homozygote mutant type genotype individuals in the control group with 61 females.

The comparisons according to additive model were not found any statistical association between patients and controls in terms of genotype distribution (OR: 1.392, 95% CI: 0.709 – 2.735, *p:*0.336 for TC vs TT, and OR: 0.911, 95% CI: 0.228 – 3.641, *p:* 0.999 for CC vs TT). In dominant model, C allele carriers were compared to homozygous wild type genotype carriers, but no significant difference was seen between them (OR: 1.318, 95% CI: 0.690 – 2.519, *p:* 0.403). In addition, the assessment of recessive model did not show any statistically important difference between female subgroups of patients and controls in terms of this SNP genotype distribution (OR: 0.792, 95% CI: 0.204 – 3.072, *p:* 0.738).

Also, allele frequencies of wild type T and mutant type C were similar like 0.726 and 0.274 for patients and 0.754 and 0.246 for controls, respectively. Therefore, there were no significant difference among them (OR: 1.156, 95% CI: 0.686 – 1.946, *p:* 0.586).

Table 3. 7. Distribution of genotypes and alleles of CYP46A1 rs754203 T → C single nucleotide polymorphism in the female subgroups of MS patients and healthy controls.

Genotype/Allele	Patients (n=95) (n, %)	Control (n=61) (n, %)	Model	OR (95%CI)	*p*
TT	48 (50.5)	35 (57.4)		Reference	Reference
TC	42 (44.2)	22 (36.1)	**Additive** [a]	1.392 (0.709 – 2.735)	0.336 *
CC	5 (5.3)	4 (6.6)		0.911 (0.228 – 3.641)	0.999 +
TT	48 (50.5)	35 (57.4)	**Dominant** [b]	1.318	
TC+CC	47 (49.5)	26 (42.6)		(0.690 – 2.519)	0.403 *
TT+TC	90 (94.7)	57 (93.4)	**Recessive** [c]	0.792	
CC	5 (5.3)	4 (6.6)		(0.204 – 3.072)	0.738 +
T	0.726	0.754	**Allele frequency** [d]	1.156	
C	0.274	0.246		(0.686 – 1.946)	0.586 *

[a]: TC vs TT and CC vs TT; [b]: TC + CC vs TT; [c]: CC vs TC + TT; [d]: C vs T. (: Pearson's chi-squared test; and +: Fisher's exact test were applied). (Bonferroni corrected p value threshold: 0.01)*

3.3.3. Effects of Gender as a Risk Factor in Genotypes of CYP7A1 rs3808607 A → C SNP and Genotypes of CYP46A1 rs754203 T → C for Multiple Sclerosis Patient and Control Groups

Multiple Sclerosis affects over 2.5 million people in the world and is seen more often women than man with 3:1 ratio. In addition, its incidence differs according to ethnicity and geographical location. Therefore, in this section, being a female was analyzed as a risk factor with genotypes of CYP7A1 rs3808607 A → C and of CYP46A1 rs754203 T → C SNPs among the Turkish patients and controls groups. The analyses were performed according to assumed additive, dominant, recessive models of SNPs (mentioned in section 3.3). **Table 3.8** shows the summary of the relations between gender and genotypes of both rs3808607 and rs754203 with respect to additive model.

For CYP7A1 rs3808607 SNP, there are 32 and 23 females, 14 and 21 males with AA genotypes; 54 and 35 females, 28 and 16 males with AC genotypes; and 9 and 3 females, 1 and 2 males with CC genotypes from patients and controls, respectively. Based upon these, homozygote wild type genotype (AA) was seen with 2-fold higher risk in females compared to man among the patients and controls. However, this was not at the statistically significant level (OR: 2.087, 95% CI: 0.881 – 4.946, *p:* 0.093). AC genotypes of rs3808607 showed protective effects on females in terms of the risk of having MS compared to male, but still at statistically insignificant level (OR: 0.882, 95% CI: 0.418 – 1.861, *p:* 0.741). In addition, no significant association was found between being a female and the risk of having MS in homozygote mutant type (CC) individuals (OR: 6; 95% CI: 0.390 – 92.278, *p:* 0.242).

For CYP46A1 rs754203 SNP, 48 and 35 females and 21 and 23 males with TT genotypes; 42 and 22 females and 17 and 12 males with TC genotypes; and 5 and 4 females and 5 and 4 males with CC genotypes from patients and controls, respectively, were investigated. There were no significant associations between genotypes of rs754203 and gender in MS patients and controls. In other words, for individuals with homozygote wild type (TT), heterozygote mutant (TC) and homozygote mutant type

Table 3. 8. Stratification analysis of MS patients and healthy controls according to additive model of CYP7A1 rs3808607 and CYP46A1 rs754203.

CYP7A1		Patient (n)	Control (n)	OR (95% CI)	*p* #	CYP46A1		Patient (n)	Control (n)	OR (95% CI)	*p* #
AA	Female	32	23	2.087 (0.881 – 4.946)	0.093 *	TT	Female	48	35	1.502 (0.720 – 3.131)	0.257 *
	Male	14	21				Male	21	23		
AC	Female	54	35	0.882 (0.418 – 1.861)	0.741 *	TC	Female	42	22	1.348 (0.547 – 3.319)	0.516 *
	Male	28	16				Male	17	12		
CC	Female	9	3	6 (0.390 –92.278)	0.242 +	CC	Female	5	4	1 (0.156 – 6.420)	0.999 +
	Male	1	2				Male	5	4		
	Total	138	100				Total	138	100		

*(#: Female vs Male; *: Pearson's chi-squared test; and +: Fisher's exact test were applied) (Bonferroni corrected p value threshold: 0.01)*

(CC) genotypes, to be female was not evaluated as a risk factor for having MS (OR: 1.502, 95% CI: 0.720 – 3.131, *p:* 0.257 for TT carriers; OR: 1.348, 95% CI: 0.547 – 3.319, *p:* 0.516 for TC carriers; OR: 1, 95% CI: 0.156 – 6.420, *p:* 0.999 for CC carriers).

Same analyses were performed with dominant and recessive models of rs3808607 and rs754203, results of which were summarized in **Table 3.9**. In dominant models of them, polymorphic allele was assumed as having the dominant character compared to the wild type allele, and vice versa for recessive models.

The comparison for CYP7A1 rs3808607 according to dominant model could not find any statistically important difference both homozygous AA carriers and C allele carriers in terms of being female and having the risk of MS (OR: 2.087, 95% CI: 0.881 – 4.946, *p:* 0.093 for AA carriers and OR: 1.029, 95% CI: 0.505 – 2.099, *p:* 0.999 for AC/CC carriers). In addition, no significant associations were found in assumed recessive model of this SNP (OR: 1.306, 95% CI: 0.751 – 2.272, *p:* 0.344 for wild type A allele carriers and OR: 6; 95% CI: 0.390 – 92.278, *p:* 0.242 for CC carriers).

If the CYP46A1 rs754203 genotypes were inherited according to dominant model, TC and CC genotypes individuals were pooled into a single group; conversely, if the CYP46A1 rs754203 genotypes were inherited according to recessive model, TC and TT genotypes individuals were pooled together into single group. When being female as a risk factor for MS was analyzed among the patients and the controls with these types of genotypes, any statistically significant association was not found (OR: 1.502, 95% CI: 0.720 – 3.131, *p:* 0.277 for wild type TT carriers and OR: 1.315, 95% CI: 0.589 – 2.934, *p:* 0.504 for polymorphic C allele carriers in dominant model); (OR: 1.454, 95% CI: 0.825 – 2.563, *p:* 0.195 for wild type T allele carriers and OR:1, 95% CI: 0.156 – 6.420; *p:* 0.999 for mutant homozygote CC carriers in recessive model).

Table 3. 9. Stratification analysis of MS patients and healthy controls according to dominant and recessive models CYP7A1 rs3808607 and CYP46A1 rs754203.

CYP7A1		Patient (n:138)	Control (n:100)	OR (95% CI)	$p^{\#}$	CYP46A1		Patient (n:138)	Control (n:100)	OR (95% CI)	$p^{\#}$
Dominant Model	Female	32	23	2.087	0.093 *	Dominant Model	Female	48	35	1.502	0.277 *
AA				(0.881 – 4.946)		TT				(0.720 – 3.131)	
	Male	14	21				Male	21	23		
AC+CC	Female	63	38	1.029	0.999 +	TC+CC	Female	47	26	1.315	0.504 *
				(0.505 – 2.099)						(0.589 – 2.934)	
	Male	29	18				Male	22	16		
Recessive Model	Female	86	58	1.306	0.344 *	Recessive Model	Female	90	57	1.454	0.195 *
AA+AC				(0.751 – 2.272)		TT+TC				(0.825 – 2.563)	
	Male	42	37				Male	38	35		
CC	Female	9	3	6	0.242 +	CC	Female	5	4	1	0.999 +
				(0.390 – 92.278)						(0.156 – 6.420)	
	Male	1	2				Male	5	4		

*(#: Female vs Male; *: Pearson's chi-squared test; and +: Fisher's exact test were applied.) (Bonferroni corrected p value threshold: 0.01).*

3.3.4. The Combination Analysis of CYP7A1 rs3808607 A → C SNP and CYP46A1 rs754203 T → C SNP in Multiple Sclerosis Patients and Healthy Controls

138 MS patients and 100 healthy controls were studied for double combinations of CYP7A1 rs3808607 A → C SNP and CYP46A1 rs754203 T → C SNP. In total, nine different double-combinations were created like AATT, AATC, AACC, ACTT, ACTC, ACCC, CCTT, CCTC, CCCC. They were compared one by one with the general populations (for example, the comparison of AATT individuals versus without AACC individuals, so on) in order to determine whether having this type of double form of genotypes affects the risk of being MS. The results for all situations were summarized in **Table 3.10**.

Based upon this analysis, individuals having homozygote wild type genotypes for both rs3808607 and rs754203 SNPs (AA+TT), numbers of which were 21 for patients and 26 for controls, had approximately 2 times lower risk of having MS (OR: 0.511, 95% CI: 0.268 – 0.973, *p:* 0.039). However, no statistically significant associations were found in comparisons of all the other double combinations (all p values of them > 0.05). In addition, any individual with dual form of homozygous mutant genotypes of these SNPs (CC+CC) was not seen in our study population. Hence, the analysis could not be done for that.

Table 3. 10. *The double combination analysis of CYP7A1 rs3808607 A:C and CYP46A1 rs754203 T:C according to genotypes.*

Genotypes (CYP7A1+CYP46A1)	Patient (n: 138)	Control (n: 100)	OR (95 % CI)	*p*
AA+TT	21	26	0.511 (0.268 – 0.973)	**0.039** *
AA+TC	24	14	1.293 (0.632 – 2.647)	0.481 *
AA+CC	4	4	0.716 (0.715 – 2.936)	0.723 +
AC+TT	41	29	1.035 (0.588 – 1.822)	0.999 *
AC+TC	35	18	1.548 (0.818 – 2.931)	0.178 *
AC+CC	6	4	1.091 (0.300 – 3.972)	0.999 +
CC+TT	7	3	1.728 (0.436 – 6.852)	0.526 +
CC+TC	3	2	1.089 (0.179 – 6.640)	0.999 +
CC+CC	0	0	NA	NA

(: Pearson's chi-squared test; and +: Fisher's exact test were applied; NA: Not applicable;*

***AA**: Wild type homozygote for CYP7A1;*

***AC**: Heterozygote mutant for CYP7A1;*

***CC**: Homozygote mutant for CYP7A1;*

***TT**: Wild type homozygote for CYP46A1;*

***TC**: Heterozygote mutant for CYP46A1;*

***CC**: Homozygote mutant for CYP46A1.)*

(Critical alpha value: 0.05).

3.4. Analysis of Serum Lipids and Vitamin D Values According to Genotypes for CYP7A1 rs3808607 A → C SNP and CYP46A1 rs754203 T → C SNP

In this section, we wanted to analyze possible relations of blood lipids and vitamin D with different genotype groups of CYP7A1 rs3808607 SNP and CYP46A1 rs754203 SNP, groups of which were split according to additive, dominant and recessive model of polymorphic alleles, in MS patients and healthy controls. Although all information of 100 controls was full regarding lipids and vitamin D values, information of some people from 138 patients about TC, TG, HDL-C, LDL-C and Vitamin D was absent due to the incomplete testing in hospital.

3.4.1. Analysis of Serum Lipids and Vitamin D Values with respect to Genotypes for CYP7A1 rs3808607 A → C SNP

The potential effects of CYP7A1 -203 A > C SNP on plasma lipids and vitamin D concentrations in MS patients and healthy controls were investigated in this section. This analysis was conducted according to dominant, recessive and additive model of this SNP, respectively.

3.4.1.1. Analysis of Serum Lipids and Vitamin D Values According to Dominant Model of CYP7A1 rs3808607 A → C SNP

In dominant model of CYP7A1 rs3808607 SNP, the polymorphic "C" allele was assumed having dominant character compared to wild type allele "A". Therefore, TC, TG, HDL-C, LDL-C and Vitamin D levels were compared between AA genotype individuals and all of the AC and CC genotypes individuals. The first comparisons were done among patients and controls, which had same genotype of this SNP. Then, the comparisons were conducted between homozygote wild type AA carriers and C allele carriers from patient group. Also, control groups were compared in a similar way. All results were summarized in **Table 3.11**.

Table 3. 11. *Serum lipid levels and Vitamin D values respect to genotypes of CYP7A1 rs3808607 polymorphism with dominant model in MS patients and healthy controls.*

Genotype in Dominant Model		AA			AC+CC				Comparison between PATIENTS of AA and AC+CC	Comparison between CONTROLS of AA and AC+CC
	n	Patients	Controls	*p*	n	Patients	Controls	*p*	*p*	*p*
TC [a] **(mmol/L)**	P: 31 C: 44	mean ± SD: 4.50 ±0.82 median ± IQR: 4.40 ± 1.04	mean ± SD: 4.95 ± 0.99 median ± IQR: 4.86 ± 0.99	0.022	P: 61 C: 56	mean ± SD: 4.63 ± 1.04 median ± IQR: 4.53 ± 0.98	mean ± SD: 4.95 ± 1.21 median ± IQR: 4.87 ± 1.55	0.077	0.460	0.975
TG (mmol/L) *[b]	P: 31 C: 44	mean ± SD: 1.26 ± 0.65 median ± IQR: 1.10 ± 0.85	mean ± SD: 1.66 ± 1.02 median ± IQR: 1.41 ± 1.52	0.096	P: 62 C: 56	mean ± SD: 1.11 ± 0.50 median ± IQR: 0.99 ± 0.82	mean ± SD: 1.35 ± 0.82 median ± IQR: 1.19 ± 0.95	0.134	0.233	0.096
HDL-C (mmol/L) [a]	P: 29 C: 44	mean ± SD: 1.23 ± 0.31 median ± IQR: 1.14 ± 0.49	mean ± SD: 1.23 ± 0.32 median ± IQR: 1.13 ± 0.36	0.857	P: 55 C: 56	mean ± SD: 1.35 ± 0.29 median ± IQR: 1.27 ± 0.52	mean ± SD: 1.33 ± 0.28 median ± IQR: 1.27 ± 0.36	0.737	0.035	0.060
LDL-C (mmol/L) [b]	P: 29 C: 44	mean ± SD: 2.70 ± 0.65 median ± IQR: 2.67 ± 0.67	mean ± SD: 3.00 ± 0.82 median ± IQR: 2.80 ± 0.82	0.100	P: 55 C: 56	mean ± SD: 2.71 ± 0.90 median ± IQR: 2.56 ± 1.04	mean ± SD: 3.12 ± 1.02 median ± IQR: 3.02 ± 1.48	0.029	0.939	0.536
25-OH Vit D (nmol/L) [a]	P: 25 C: 44	mean ± SD: 49.41 ± 33.79 median ± IQR: 43.13. ± 53.83	mean ± SD: 64.31 ± 32.51 median ± IQR: 62.50 ± 45.30	0.036	P: 53 C: 56	mean ± SD: 55.92 ± 40.77 median ± IQR: 44.43 ± 53.28	mean ± SD: 63.11 ± 27.94 median ± IQR: 60.05 ± 31.64	0.027	0.619	0.923

*([a] Mann Whitney U Test is applied; [b] Independent Samples T-test; *Logarithmic transformation (lg10) into data is performed; Bonferroni corrected p value threshold: 0.01).*

Since multiple comparisons were done between five different groups (for patients or controls comparisons with themselves) and by using five parameters (for patients and controls comparisons with same genotypes), new critical Bonferroni corrected *p* value was calculated with this formula: 0.05/5= 0.01.

When the AA genotypes individuals were compared in terms of these parameters, it was found that the patients had significantly lower cholesterol concentrations (4.50 ± 0.82 mmol/L) (*p:* 0.022) than controls (4.95 ± 0.99 mmol/L). In addition, the mean value of patient groups for vitamin D was 49.41 ± 33.79 nmol/L, and it was 64.31 ± 32.51 nmol/L for controls, and this difference was at statistically meaningful level (*p:* 0.036). However, after Bonferroni correction, neither TC nor vitamin D remained statistically significant. Also, levels of other lipid parameters (TG, HDL-C, LDL-C) were not statistically different between them (*p:* 0.096, *p:* 0.857, *p:* 0.100, respectively).

In individuals with AC or CC genotype, which were pooled into a single group as C allele carriers, only LDL-C level from lipid parameters was significantly different between patients (2.71 ± 0.90 mmol/L) and controls (3.12 ± 1.02 mmol/L; *p:* 0.029). Also, vitamin D level significantly lower in patients (55.92 ± 40.77 nmol/L, *p:*0.027) than controls (63.11 ± 27.94 nmol/L) like AA genotypes individuals. However, after Bonferroni correction, neither of them were not statistically meaningful.

When the homozygous AA carriers and the C allele carriers in patients were compared with respect to lipid parameters and vitamin D, only statistically important difference was found at HDL cholesterol level (*p:* 0.035). The C allele carrier group had higher level of HDL-C (1.35 ± 0.29 mmol/L) than the other group (1.23 ± 0.31 mmol/L). But, this difference didn't remain after Bonferroni correction. Besides, the comparisons of these parameters within control subjects with AA genotype and AC or CC genotype were not found statistically significant (all *p* values > 0.01).

3.4.1.2. Analysis of Serum Lipids and Vitamin D Values According to Recessive Model of CYP7A1 rs3808607 A → C SNP

In recessive model of CYP7A1 rs3808607 SNP, the polymorphic "C" allele was assumed having a recessive character compared to the wild type "A" allele. Therefore, the comparisons were done between homozygote CC carriers and A allele carriers. Like in the previous section, the controls and patients with same genotype, both patients and controls among themselves were compared as regards to serum lipids and vitamin D values. All results from these analyses were shown in **Table 3.12**.

The patients had lower levels of the TC, TG, LDL-C from lipid parameters (4.55 ± 0.97 mmol/L, 1.15 ± 0.55 mmol/L, 2.70 ± 0.84 mmol/L, respectively) than controls (4.96 ± 1.12 mmol/L, 1.52 ± 0.93 mmol/L, 3.08 ±0.95 mmol/L, respectively) in the comparisons of the wild type A allele carriers. According to Bonferroni corrected *p* value threshold (0.01), only TC was found statistically different (*p:* 0.002) among them. Although *p* value of LDL-C (0.010) was so close the critical *p* value, it was not accepted statistically meaningful and likewise, there was no significant difference between them in terms of HDL-C levels (*p:* 0.690). Furthermore, vitamin D levels of patients were significantly lower (52.53 ± 37.35 nmol/L, *p:* 0.002) than those of controls (64.18 ± 30.13 nmol/L) within the A allele carriers even after Bonferroni correction. Whereas, no statistically significant results were found in the analysis of lipid parameters and vitamin D among the patients and controls with CC genotype (all *p* values > 0.01).

Neither in the comparisons of patients with AC/AA and CC genotypes among themselves, nor in the comparisons of controls with AC/AA and CC genotypes among themselves, no statistically significant differences was found regarding the blood lipid parameters and vitamin D concentrations (all *p* values > 0.01).

Table 3. 12. Serum lipid levels and Vitamin D values respect to genotypes of CYP7A1 rs3808607 polymorphism with recessive model in MS patients and healthy controls.

Genotype in Recessive Model		AA+AC				CC			Comparison between **PATIENTS** of AA + AC and CC	Comparison between **CONTROLS** of AA + AC and CC
	n	**Patients**	**Controls**	***p***	**n**	**Patients**	**Controls**	***p***	***p***	***p***
TC (mmol/L) [a]	P: 85	mean ± SD: 4.55 ± 0.97	mean ± SD: 4.96 ± 1.12	**0.002**	P: 7	mean ± SD: 5.01 ± 0.93	mean ± SD: 4.75 ± 1.09	0.223	0.151	0.543
	C: 95	median ± IQR: 4.48 ± 0.97	median ± IQR: 4.87 ±1.19		C: 5	median ± IQR: 4.90 ± 0.83	median ± IQR: 4.82 ± 1.85			
TG (mmol/L) [a]	P: 86	mean ± SD: 1.15 ± 0.55	mean ± SD: 1.52 ± 0.93	0.021	P: 7	mean ± SD: 1.39 ± 0.65	mean ± SD: 4.86 ± 0.34	0.223	0.329	0.100
	C: 95	median ± IQR: 0.99 ± 0.80	median ± IQR: 1.28 ± 1.25		C: 5	median ± IQR: 1.36 ± 1.37	median ± IQR: 0.85 ± 0.55			
HDL-C (mmol/L) [a]	P: 78	mean ± SD: 1.31 ± 0.31	mean ± SD: 1.27 ± 0.29	0.690	P: 6	mean ± SD: 1.36 ± 0.28	mean ± SD: 1.50 ± 0.50	0.709	0.627	0.296
	C: 95	median ± IQR: 1.25 ± 0.47	median ± IQR: 1.24 ± 0.36		C: 5	median ± IQR: 1.27 ± 0.54	median ± IQR: 1.27 ± 0.73			
LDL-C (mmol/L) [b]	P: 78	mean ± SD: 2.70 ± 0.84	mean ± SD: 3.08 ± 0.95	0.010	P: 6	mean ± SD: 2.78 ± 0.51	mean ± SD: 2.85 ± 0.65	0.464	0.834	0.606
	C: 95	median ± IQR: 2.60 ± 0.90	median ± IQR: 2.98 ± 1.17		C: 5	median ± IQR: 2.99 ± 0.83	median ± IQR: 3.16 ± 1.23			
25-OH Vit D (nmol/L) *[b]	P: 74	mean ± SD: 52.53 ± 37.35	mean ± SD: 64.18 ± 30.13	**0.002**	P: 4	mean ± SD: 77.86 ± 58.53	mean ± SD: 53.38 ± 25.11	0.624	0.203	0.434
	C: 95	median ± IQR: 42.78 ± 49.73	median ± IQR: 61.60 ± 34.79		C: 5	median ± IQR: 85.08 ± 107.56	median ± IQR: 65.57 ± 47.70			

*([a] Mann Whitney U Test is applied; [b] Independent Samples T-test; * Logarithmic transformation (lg10) into data is performed; Bonferroni corrected p value threshold: 0.01).*

3.4.1.3. Analysis of Serum Lipids and Vitamin D Values According to Additive Model of CYP7A1 rs3808607 A → C SNP

In additive model of CYP7A1 rs3808607 A → C SNP, both wild type allele "A" and polymorphic allele "C" were assumed as having equal effects on heterozygous individuals. Therefore, how relations of three different genotypes on blood lipids and vitamin D concentrations had changed among the patients and the controls were investigated. Besides, the patient groups and control groups among themselves were compared with all possible double combinations. All results of these comparisons were illustrated in **Table 3.13**.

In comparison of homozygous AA subjects, the patients had significantly lower levels of TC and vitamin D (4.50 ± 0.82 mmol/L, 49.41 ± 33.79 nmol/L, respectively) than controls (4.95 ± 0.99 mmol/L, 64.31 ± 32.51 nmol/L, respectively), but *p* values of them (0.039 for TC and 0.036 for vitamin D) did not continue their significance after Bonferroni correction.

Similarly, the other lipid parameters were not found statistically different between patients and controls with AA genotypes (*p* values for TG, HDL-C and LDL-C > 0.01).

When heterozygous mutant -AC- genotypes individuals were analyzed in terms of these parameters, the patients had significantly lower TG (*p:* 0.043), LDL-C (*p:* 0.030) and Vitamin D level (*p:* 0.014) than the controls (1.08 ± 0.48 mmol/L, 2.71 ± 0.94 mmol/L, 54.13 ± 39.29 nmol/L for patients; 1.40 ± 0.84 mmol/L, 3.14 ± 1.05 mmol/L, 64.07 ± 28.25 nmol/L for controls, respectively). However, according to Bonferroni *p* value threshold, none of them remained statistically significant. Also, TC and HDL-C levels were not significantly different within the patients and controls with AC genotypes (*p:* 0.083 for TC and *p:* 0.659 for HDL-C).

No statistically significant differences was found with respect to lipids and vitamin D values within the patients and controls with CC homozygous mutant type (all *p* values > 0.01).

Table 3. 13. Serum lipid levels and Vitamin D values respect to genotypes of CYP7A1 rs3808607 polymorphism with additive model in MS patients and healthy controls.

Genotype in Additive Model	AA				AC				CC				*AA vs AC [1]*		*AA vs CC [2]*		*AC vs CC [3]*	
													Patients	Controls	Patients	Controls	Patients	Controls
	n	Patients	Controls	*p*	n	Patients	Controls	*p*	n	Patients	Control	*p*	*p*	*p*	*p*	*p*	*p*	*p*
TC (mmol/L) [b]	P: 31 C: 44	mean ± SD: 4.50 ± 0.82 median ± IQR: 4.40 ± 1.04	mean ± SD: 4.95 ±0.99 median ± IQR: 4.86 ± 0.99	0.022	P: 54 C: 51	mean ± SD: 4.58 ± 1.05 median ± IQR: 4.52 ± 0.97	mean ± SD: 4.97 ± 1.22 median ± IQR: 4.87 ± 1.48	0.083	P: 7 C: 5	mean ± SD: 5.01 ± 0.93 median ± IQR: 4.90 ±0.83	mean ± SD: 4.75 ± 1.09 median ± IQR: 4.82 ± 1.85	0.223	0.707	0.940	0.154	0.667	0.310	0.698
TG (mmol/L) [*b]	P: 31 C: 44	mean ± SD: 1.26 ± 0.65 median ± IQR: 1.10 ± 0.85	mean ± SD: 1.66 ± 1.02 median ± IQR: 1.41 ± 1.52	0.096	P: 55 C: 51	mean ± SD: 1.08 ± 0.48 median ± IQR: 0.97 ± 0.79	mean ± SD: 1.40 ± 0.84 median ± IQR: 1.20 ± 0.98	0.043	P: 7 C: 5	mean ± SD: 1.39 ± 0.65 median ± IQR: 1.36 ± 1.37	mean ± SD: 0.86 ± 0.34 median ± IQR: 0.85 ± 0.55	0.223	0.143	0.175	0.654	0.092	0.131	0.162
HDL-C (mmol/L) [a]	P: 29 C: 44	mean ± SD: 1.23 ± 0.31 median ± IQR: 1.14 ± 0.49	mean ± SD: 1.23 ± 0.32 median ± IQR: 1.13 ± 0.36	0.857	P: 49 C: 51	mean ± SD: 1.35 ± 0.30 median ± IQR: 1.27 ± 0.51	mean ± SD: 1.31 ± 0.25 median ± IQR: 1.27 ± 0.36	0.659	P: 6 C: 5	mean ± SD: 1.36 ± 0.28 median ± IQR: 1.27 ± 0.54	mean ± SD: 1.50 ± 0.50 median ± IQR: 1.27 ± 0.73	0.709	0.043	0.085	0.220	0.186	0.999	0.454
LDL-C (mmol/L) [b]	P: 29 C: 44	mean ± SD: 2.70 ± 0.65 median ± IQR: 2.67 ± 0.67	mean ± SD: 3.00 ± 0.82 median ± IQR: 2.80 ± 0.82	0.100	P: 49 C: 51	mean ± SD: 2.71 ± 0.94 median ± IQR: 2.54 ± 1.11	mean ± SD: 3.14 ± 1.05 median ± IQR: 3.00 ± 1.53	0.030	P: 6 C: 5	mean ± SD: 2.78 ± 0.51 median ± IQR: 2.99 ± 0.83	mean ± SD: 2.85 ± 0.65 median ± IQR: 3.16 ± 1.23	0.464	0.972	0.466	0.785	0.704	0.858	0.549
25-OH Vit D (nmol/L) [a]	P: 25 C: 44	mean ± SD: 49.41 ± 33.79 median ± IQR: 43.13 ± 53.83	mean ± SD: 64.31 ± 32.51 median ± IQR: 62.50 ± 45.30	0.036	P: 49 C: 51	mean ± SD: 54.13 ± 39.29 median ± IQR: 42.43 ± 49.08	mean ± SD: 64.07 ± 28.25 median ± IQR: 58.51 ± 30.68	0.014	P: 4 C: 5	mean ± SD: 77.86 ± 58.53 median ± IQR: 85.08 ± 107.56	mean ± SD: 53.38 ± 25.11 median ± IQR: 65.57 ± 47.70	0.624	0.698	0.988	0.429	0.597	0.479	0.595

*([a] Mann Whitney U Test is applied; [b] Independent Samples T-test; * Logarithmic transformation (lg10) into data is performed Bonferroni corrected p value threshold: 0.01).*

([1]: The analysis was performed among patients themselves and among controls themselves with AA and AC genotypes individuals;

[2]: The analysis was performed among patients themselves and among controls themselves with AA and AC genotypes individuals;

[3]: The analysis was performed among patients themselves and among controls themselves with AC and CC genotypes individuals.)

All double combinations for homozygote wild type (AA), heterozygote mutant (AC) and homozygote mutant genotypes (CC) were compared among the patients' own and among controls' own in terms of these parameters. In the comparisons of patients with AA and AC genotypes, only HDL-C level was found statistically higher in AC genotypes patients (1.35 ± 0.35 mmol/L; *p:* 0.043) than AA genotypes patients (1.23 ± 0.31 mmol/L), but after Bonferroni correction, this difference did not last important. There were no any other significant association in the rest of the parameters within this comparison group (their *p* values >0.01). In addition, the comparisons of AA and CC, and those of AC and CC patients in terms of mentioned parameters did not show any meaningful difference (all *p* values > 0.01). Likewise, no statistically significant differences were seen in all double combinations of controls (all *p* values > 0.01).

3.4.2. Analysis of Serum Lipids and Vitamin D Values with respect to Genotypes for CYP46A1 rs754203 T → C SNP

The possible relations of CYP46A1 rs754203 T → C SNP with plasma lipids and vitamin D concentrations in MS patients and healthy controls were examined in this section. This analysis was conducted according to dominant, recessive and additive model of this SNP, respectively.

3.4.2.1. Analysis of Serum Lipids and Vitamin D Values According to Dominant Model of CYP46A1 rs754203 T → C SNP

In dominant model of rs754203 T → C SNP, the mutant allele "C" was considered as having dominant character compared to wild type allele "T". Thus, total cholesterol, triglycerides, HDL-Cholesterol, LDL-Cholesterol and vitamin D levels were compared between TT genotype individuals and all of the TC and CC genotype individuals. In addition to that, the patient groups among themselves and the control groups among themselves were analyzed in terms of these parameters, and all results were summarized in **Table 3.14**.

Table 3. 14. *Serum lipid levels and Vitamin D values respect to genotypes of CYP46A1 rs754203 polymorphism with dominant model in MS patients and healthy controls.*

Genotype in Dominant Model		**TT**				**TC+CC**			**Comparison between PATIENTS of TT and TC+CC**	**Comparison between CONTROLS of TT and TC+CC**
	n	**Patients**	**Controls**	***P***	**n**	**Patients**	**Controls**	***P***	***p***	***p***
TC (mmol/L) [a]	P: 51 C: 58	mean ± SD: 4.44 ± 1.00 median ± IQR: 4.35 ± 1.04	mean ± SD: 4.77 ± 1.12 median ± IQR: 4.78 ± 1.30	0.048	P: 41 C: 42	mean ± SD: 4.77 ± 0.90 median ± IQR: 4.73 ± 1.02	mean ± SD: 5.21 ± 1.06 median ± IQR: 4.99 ± 1.15	0.028	0.057	0.068
TG (mmol/L) [a]	P: 52 C: 58	mean ± SD: 1.05 ± 0.47 median ± IQR: 0.97 ± 0.75	mean ± SD: 1.47 ± 0.94 median ± IQR: 1.33 ± 1.28	0.078	P: 41 C: 42	mean ± SD: 1.31 ± 0.63 median ± IQR: 1.19 ± 0.95	mean ± SD: 1.52 ± 0.91 median ± IQR: 1.20 ± 1.07	0.428	0.055	0.476
HDL-C (mmol/L) [a]	P: 44 C: 58	mean ± SD: 1.32 ± 0.30 median ± IQR: 1.26 ± 0.48	mean ± SD: 1.29 ± 0.30 median ± IQR: 1.27 ± 0.42	0.730	P: 40 C: 42	mean ± SD: 1.31 ± 0.30 median ± IQR: 1.26 ± 0.46	mean ± SD: 1.28 ± 0.31 median ± IQR: 1.23 ± 0.24	0.922	0.840	0.796
LDL-C (mmol/L) [a]	P: 44 C: 58	mean ± SD: 2.61 ± 0.90 median ± IQR: 2.51 ± 0.93	mean ± SD: 2.92 ± 0.90 median ± IQR: 2.87 ± 1.07	0.089	P: 40 C: 42	mean ± SD: 2.82 ± 0.71 median ± IQR: 2.68 ± 0.89	mean ± SD: 3.27 ± 0.96 median ± IQR: 3.08 ± 1.18	0.026	0.203	0.069
25-OH Vit D (nmol/L) [a]	P: 42 C: 58	mean ± SD: 58.98 ± 42.77 median ± IQR: 47.67 ± 52.42	mean ± SD: 61.63 ± 28.46 median ± IQR: 60.48 ± 41.23	0.202	P: 36 C: 42	mean ± SD: 47.83 ± 32.59 median ± IQR: 42.78 ± 52.16	mean ± SD: 66.42 ± 31.90 median ± IQR: 61.73 ± 32.68	**0.005**	0.321	0.716

([a] Mann Whitney U Test is applied; [b] Independent Samples T-test is performed; Bonferroni corrected p value threshold: 0.01).

In comparison of individuals with homozygote wild type TT genotype, the only difference found was that the patients had significantly lower total cholesterol (4.44 ± 1.00 mmol/L, *p:* 0.048) than controls (4.77 ± 1.12 mmol/L). But, when Bonferroni correction for multiple comparisons was done, this difference did not remain statistically significant. Similarly, the other lipid parameters and vitamin D levels did not show any statistically significant difference between them (their p values > 0.01).

In individuals having polymorphic "C" allele (TC+CC), meaningful differences were found at levels of TC and LDL-C from lipid parameters and vitamin D (*p:* 0.028, *p:* 0.026, *p:* 0.005, respectively), in which patients had lower levels of them (4.77 ± 0.90 mmol/L and 2.82 ± 0.71 mmol/L, 47.83 ± 32.59 nmol/L, respectively) than controls (5.21 ± 1.06 mmol/L and 3.27 ± 0.96 mmol/L, 66.42 ± 31.90 nmol/L, respectively). However, only vitamin D has continued its statistical significance after Bonferroni correction, for the others *p* values were higher than critical alpha 0.01.

When homozygous TT carries and C allele carriers from patients were compared between themselves regarding these values, no statistically important difference was observed between them (all *p* values > 0.01). Likewise, there were no significant differences in comparisons of controls with these two genotype groups (all *p* values > 0.01).

3.4.2.2. Analysis of Serum Lipids and Vitamin D Values According to Recessive Model of CYP46A1 rs754203 T → C SNP

In recessive model of rs754203 T → C SNP of CYP46A1 gene, the polymorphic "C" allele was assumed as having recessive character compared to wild type "T" allele. Therefore, TC and TT genotypes were pooled into one group. How lipid parameters and vitamin D values were changing among the patients and controls with these genotypes were investigated and the results were summarized in **Table 3.15**.

For lipid parameters, the patients from T allele carriers had significantly lower levels of total cholesterol, triglycerides and LDL-cholesterol (4.60 ± 0.98 mmol/L, 1.14 ± 0.53 mmol/L, 2.74 ± 0.84 mmol/L; *p:* 0.018, *p:* 0.016, and *p:* 0.021, respectively) than

controls from the same genotype (4.93 ± 1.13 mmol/L, 1.48 ± 0.94 mmol/L and 3.06 ± 0.94 mmol/L, respectively). Besides, the mean vitamin D level were 54.53 ± 39.63 nmol/L for patients and 63.28 ± 26.98 nmol/L for controls, which were statistically different among T allele carrier patients and controls (*p:* 0.005). But, when Bonferroni correction for multiple testing was implemented on them, only vitamin D kept its statistical importance. Any meaningful significance could not be found for lipid parameters and vitamin D levels in the comparison of patients and controls with homozygous CC genotypes (all *p* values > 0.01).

Classified according to recessive model of rs754203 SNP, both patients and controls groups were compared with their own group in terms of the mentioned parameters. However, no significant difference was not observed in them for both groups (all *p* values > 0.01).

3.4.2.3. Analysis of Serum Lipids and Vitamin D Values According to Additive Model of CYP46A1 rs754203 T → C SNP

In additive model of rs754203 SNP of CYP46A1 gene, there were not any dominant or recessive allele, and both wild type "T" allele and mutant "C" allele was assumed having equal effects on heterozygous individuals. Therefore, three different genotypes were formed and their possible associations on blood lipids and vitamin D levels were investigated among patients and controls. All double combinations between patient groups themselves and control groups themselves also were compared and their results were given in **Table 3.16**.

The only found difference in the homozygous TT carriers regarding these parameters was in the level of the total cholesterol, which was significantly lower in the patients (4.44 ± 1.00 mmol/L) than controls (4.77 ± 1.12 mmol/L), (*p:* 0.048) but, this difference didn't last after Bonferroni correction. The other lipid and vitamin D values were not significantly different among them (their p values > 0.01). Furthermore, in comparison of TC genotype individuals, the only distinction seen was in the vitamin

Table 3. 15. *Serum lipid levels and Vitamin D values respect to genotypes of CYP46A1 rs754203 polymorphism with recessive model in MS patients and healthy controls.*

Genotype in Recessive Model	TT + TC				CC				Comparison between PATIENTS of TT + TC vs CC	Comparison between CONTROLS of TT + TC vs CC
	n	Patients	Controls	*p*	n	Patients	Controls	*p*	*p*	*p*
TC (mmol/L) [a]	P: 86 C: 92	mean ± SD: 4.60 ± 0.98 median ± IQR: 4.49 ± 0.93	mean ± SD: 4.93 ± 1.13 median ± IQR: 4.83 ± 1.22	0.018	P: 6 C: 8	mean ± SD: 4.37 ± 0.77 median ± IQR: 4.56 ± 1.30	mean ± SD: 5.24 ± 0.96 median ± IQR: 5.12 ± 1.12	0.093	0.758	0.360
TG (mmol/L) *[b]	P: 87 C: 92	mean ± SD: 1.14 ± 0.53 median ± IQR: 0.99 ± 0.79	mean ± SD: 1.48 ± 0.94 median ± IQR: 1.20 ± 1.16	0.016	P: 6 C: 8	mean ± SD: 1.55 ± 0.80 median ± IQR: 1.22 ± 1.58	mean ± SD: 1.64 ± 0.76 median ± IQR: 1.55 ± 1.34	0.818	0.277	0.120
HDL-C (mmol/L) [a]	P: 78 C: 92	mean ± SD: 1.31 ± 0.30 median ± IQR: 1.26 ± 0.45	mean ± SD: 1.28 ± 0.31 median ± IQR: 1.23 ± 0.39	0.569	P: 6 C: 8	mean ± SD: 1.32 ± 0.36 median ± IQR: 1.24 ± 0.74	mean ± SD: 1.34 ± 0.10 median ± IQR: 1.35 ± 0.10	0.513	0.993	0.227
LDL-C (mmol/L) [b]	P: 78 C: 92	mean ± SD: 2.74 ± 0.84 median ± IQR: 2.65 ± 0.91	mean ± SD: 3.06 ± 0.94 median ± IQR: 2.97 ± 1.00	0.021	P: 6 C: 8	mean ± SD: 2.33 ± 0.41 median ± IQR: 2.29 ± 0.65	mean ± SD: 3.15 ± 0.90 median ± IQR: 3.15 ± 1.49	0.060	0.834	0.606
25-OH Vit D (nmol/L) [a]	P: 72 C: 92	mean ± SD: 54.53 ± 39.63 median ± IQR: 43.78 ± 53.70	mean ± SD: 63.28 ± 26.98 median ± IQR: 62.15 ± 34.08	**0.005**	P: 6 C: 8	mean ± SD: 45.44 ± 22.81 median ± IQR: 41.57 ± 45.30	mean ± SD: 67.80 ± 56.15 median ± IQR: 53.21 ± 70.64	0.606	0.888	0.602

*([a] Mann Whitney U Test is applied; [b] Independent Samples T-test; * Logarithmic transformation (lg10) into data is performed; Bonferroni corrected p value threshold: 0.01).*

D level (*p:* 0.006), even after doing Bonferroni correction, in which the mean value for the patient groups was 48.31 ± 34.50 nmol/L while it was 66.09 ± 24.39 nmol/L for the controls. No significant differences were found amongst the lipid parameters (their *p* values > 0.01). Similarly, there was no meaningful difference in these parameters between CC allele patients and controls (all *p* values > 0.01).

In comparison of the patients themselves in terms of being discussed parameters, three different couples were formed: individuals with TT versus TC, TT versus CC, and TC versus CC. Only total cholesterol level was found statistically different (p: 0.034) in the comparison of TT and TC genotype patients and its level was lower in the TT carriers (4.44 ± 1.00 mmol/L) than TC carriers (4.84 ± 0.91 mmol/L). Besides, in the comparisons for TC and CC genotypes patients, only LDL-C level was found significantly higher in the mutant heterozygous TC genotype ones (2.91 ± 0.72 mmol/L, *p*: 0.043) than mutant type CC ones (2.33 ± 0.41 mmol/L), However, after Bonferroni correction, both of them have been statistically insignificant. Similarly, there was no statistically important difference with respect to these parameters in the comparisons of TT and CC genotypes patients (all *p* values > 0.01).

In the comparisons of the controls themselves in terms of lipid parameters and vitamin D, three different combinations were analyzed like in patients: TT vs TC, TT vs CC, TC vs CC. However, no statistically significant association for these parameters was found among them (all *p* values > 0.01).

Table 3. 16. Serum lipid levels and Vitamin D values respect to genotypes of CYP46A1 rs754203 polymorphism with additive model in MS patients and healthy controls.

Genotype in Additive Model	TT				TC				CC				*TT vs TC [1]*		*TT vs CC [2]*		*TC vs CC [3]*	
													Patients	Controls	Patients	Controls	Patients	Controls
	n	Patients	Controls	*p*	n	Patients	Controls	*p*	n	Patients	Control	*p*	*p*	*p*	*p*	*p*	*p*	*p*
TC (mmol/L) [a]	P: 51 C: 58	mean ± SD: 4.44 ± 1.00 median ± IQR: 4.35 ± 1.04	mean ± SD: 4.77 ± 1.12 median ± IQR: 4.78 ± 1.30	0.048	P: 35 C: 34	mean ± SD: 4.84 ± 0.91 median ± IQR: 4.61 ± 1.04	mean ± SD: 5.20 ± 1.10 median ± IQR: 4.90 ± 1.17	0.100	P: 6 C: 8	mean ± SD: 4.37 ± 0.77 median ± IQR: 4.56 ± 1.30	mean ± SD: 5.24 ± 0.96 median ± IQR: 5.12 ± 1.12	0.093	0.034	0.111	0.959	0.209	0.428	0.798
TG (mmol/L) [a]	P: 52 C: 58	mean ± SD: 1.05 ± 0.47 median ± IQR: 0.97 ± 0.75	mean ± SD: 1.46 ± 0.94 median ± IQR: 1.33 ± 1.28	0.078	P: 35 C: 34	mean ± SD: 1.27 ± 0.60 median ± IQR: 1.19 ± 0.94	mean ± SD: 1.49 ± 0.95 median ± IQR: 1.20 ± 0.94	0.509	P: 6 C: 8	mean ± SD: 1.55 ± 0.80 median ± IQR: 1.22 ± 1.58	mean ± SD: 1.64 ± 0.76 median ± IQR: 1.55 ± 1.34	0.818	0.114	0.680	0.097	0.316	0.396	0.432
HDL-C (mmol/L) [a]	P: 44 C: 58	mean ± SD: 1.32 ± 0.30 median ± IQR: 1.26 ± 0.48	mean ± SD: 1.29 ± 0.30 median ± IQR: 1.27 ± 0.42	0.730	P: 34 C: 34	mean ± SD: 1.30 ± 0.30 median ± IQR: 1.26 ± 0.39	mean ± SD: 1.27 ± 0.34 median ± IQR: 1.19 ± 0.25	0.585	P: 6 C: 8	mean ± SD: 1.32 ± 0.36 median ± IQR: 1.24 ± 0.74	mean ± SD: 1.34 ± 0.10 median ± IQR: 1.35 ± 0.10	0.513	0.832	0.563	0.964	0.497	0.970	0.052
LDL-C (mmol/L) [a]	P: 44 C: 58	mean ± SD: 2.61 ± 0.90 median ± IQR: 2.51 ± 0.93	mean ± SD: 2.92 ± 0.90 median ± IQR: 2.87 ± 1.07	0.089	P: 34 C: 34	mean ± SD: 2.91 ± 0.72 median ± IQR: 2.81 ± 0.80	mean ± SD: 3.29 ± 0.98 median ± IQR: 3.02 ± 1.15	0.092	P: 6 C: 8	mean ± SD: 2.33 ± 0.41 median ± IQR: 2.29 ± 0.65	mean ± SD: 3.15 ± 0.90 median ± IQR: 3.15 ± 1.49	0.060	0.073	0.071	0.282	0.467	0.043	0.749
25-OH Vit D (nmol/L) [a]	P: 42 C: 58	mean ± SD: 58.98 ± 42.67 median ± IQR: 47.67 ± 52.42	mean ± SD: 61.63 ± 28.46 median ± IQR: 60.48 ± 41.23	0.202	P: 30 C: 34	mean ± SD: 48.31 ± 34.50 median ± IQR: 42.78 ± 58.21	mean ± SD: 66.09 ± 24.39 median ± IQR: 63.44 ± 31.68	**0.006**	P: 6 C: 8	mean ± SD: 45.44 ± 22.81 median ± IQR: 41.57 ± 45.30	mean ± SD: 67.80 ± 56.15 median ± IQR: 53.21 ± 70.64	0.606	0.340	0.571	0.629	0.724	0.734	0.461

([a] Mann Whitney U Test is applied; [b] Independent Samples T-test; * Logarithmic transformation (lg10) into data is performed; Bonferroni corrected p value threshold: 0.01).

[1]: The analysis was performed between patients with TT and TC genotypes individuals in addition to controls with TT and TC genotypes individuals;

[2]: The analysis was performed between patients with TT and CC genotypes individuals in addition to controls with TT and CC genotypes individuals;

[3]: The analysis was performed between patients with TC and CC genotypes individuals in addition to controls with TC and CC genotypes individuals).

CHAPTER 4

DISCUSSION

Multiple sclerosis (MS) is a chronic demyelinating neuroinflammatory disease of the central nervous system with axonal degeneration. Inflammation in MS actively develops during the early stages of the disease, and is directed against the myelin sheath components in which there is proliferation and dysregulation of pro-inflammatory T lymphocytes (Th1 and Th 17), together with activation of B cells and secretion of inflammatory cytokines. Also, neurodegeneration has an important role in the progression of MS, which causes the destruction of oligodendrocytes, axons, and eventually, neurons (Kozin et al., 2018; Barcelos et al., 2019).

In about 85% - 90% patients, MS starts with a relapsing–remitting course (RRMS), which has periods of acute demyelination (relapses) and periods of neurological recovery and also affects women about twice as often as men. After 15–25 years, however, the relapses typically shift into secondary progressive MS which is characterized by a progression of worsened neurological function with few or no acute relapses. About 10%–15% of patients present with insidious disease onset and steady progression, named primary progressive MS which presents later in life, with a mean age of 45 years. It is not clear which factors are responsible for the different courses (Friese et al., 2014; Barcelos et al., 2019; Sospedra & Martin, 2005).

Even though the CNS has remyelination capacity, contributing to clinical remission even in the neuronal injury which can be repaired up to a certain point, if an axon is totally disturbed or the integrity of the neuronal cell body is severely broken, repair mechanisms will be inadequate (Ellwardt & Zipp, 2014).

Due to the deficits of sensation and of motor, autonomic, and neurocognitive function, it causes substantial disability. The disease is usually not life shortening, but its socioeconomic burden is huge due to its beginning at young ages (Sospedra & Martin, 2005).

The etiology of MS remains partly understood and it is considered a complex disease. According to current data the disease develops in genetically susceptible individuals and lifestyle and environmental factors potently influence this risk. Epstein–Barr virus (EBV) infection, smoking, limited sun exposure/ low vitamin D, and obesity in adolescent are thought as triggers for that. Industrialization, urban living, pollution, occupational exposures to solvents, changes in diet and breastfeeding also are linked to its incidence, for example, it is seen in low rate in Asia, but in Japan it has the highest frequency (Kozin et al., 2018; Alfredsson & Olsson, 2018; Sospedra & Martin, 2005).

Of the environmental risk factors for MS, vitamin D is the one of the most important ones. Having important role in calcium and bone metabolism, vitamin D regulates cell proliferation and differentiation and can regulate immune responses (Soilu-Hänninen et al., 2005). It has reported the people with low vitamin D intake or low circulating 25-hydroxyvitamin D [25(OH)D] levels have higher MS risk, suggesting that there is an inverse correlation between vitamin D levels and MS activity (Ascherio, et al., 2014). Also, vitamin D prevented experimental autoimmune encephalomyelitis (EAE), animal model of MS, by working as potent immunomodulator (Munger et al., 2006). This properties of vitamin D cover the induction of regulatory and anti-inflammatory T cells, downregulation of IgG synthesis by B cells and lessening of antigen presentation by dendritic cells (Holmøy et al., 2012).

Moreover, according to Ascherio and coworkers' study, individuals with average serum 25(OH)D levels less than 50.0 nmol/L in the 12 months following a first demyelinating event had worse clinical and imaging outcomes after 5 years than did

those with higher levels (Ascherio et al., 2014). And low level of it was also related with active disease in MS patients, involving higher relapse rate, new T2 lesion formation, and increased disability in another study (Graves et al., 2019).

It was showed by Simpson et al., that each 10 nmol/L rise in 25(OH)D resulted in up to a 12% reduction in risk of relapse in RRMS patients and increasing 25(OH)D levels by 50 nmol/L could halve the risk of a relapse (Simpson et al., 2010). In addition, Stewart and colleagues suggested that in IFN-β therapy which is used for treatment of MS, therapeutic effects of IFN-β on relapse in MS could be related with modulation of vitamin D metabolism and it was relatively more effective when vitamin D levels were high (Stewart et al., 2012).

It was found by Wergeland et al., that when vitamin D3 was provided as a dietary supplement in the cuprizone model, which is a toxicant-induced MS model, it protected against cuprizone-induced demyelination, and diminished microglia and macrophage activation (Wergeland et al., 2011).

The serum concentration of 25-hydroxyvitamin D reflects the intake of vitamin D in the food and its synthesis from provitamins in the skin due to circulating at 1000 times the concentration of $1.25(OH)_2D$ and having two weeks half-life (Soilu-Hänninen et al., 2004). The 25-hydroxyvitamin D level fluctuates throughout the year but according to Holmøy et al., measurements of the vitamin D in February most precisely predict its status throughout the year (Holmøy et al., 2012).

Vitamin D has two basic forms, D2 (ergocalciferol) and D3 (cholecalciferol). While both may be ingested, the former is used as a vitamin D supplement and the latter is produced naturally from 7-dehydrocholesterol in the skin of many vertebrate animals, including humans after exposure to ultraviolet B-light. Less than 5% of vitamin D is taken from the diet (dairy products and dark fish), since most is produced by the body and both of them are considered to be equivalent for vitamin D production. The *DHCR7* gene encodes an enzyme that acts on 7-dehydrocholesterol, and decreases of its amount will result in a significant reduction or the complete elimination of vitamin

D3 production in the skin. It is thought that this enzyme plays a critical role between cholesterol and vitamin D production (Holick, 2004; Rosen et al., 2016; Graves et al., 2019).

Vitamin D is biologically inactive with half-life of 12–16 h and it is converted to 25-hydroxyvitamin D [25(OH)D] or calcifediol, by a member of the CYP family, the 25-hydroxylases (CYP2R1, CYP27A1 and CYP3A4) in the liver to become biologically active. 25(OH)D is then converted to the biologically active form 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] or calcitriol, with another hydroxylation by 1-α-hydroxylase (CYP27B1). Activated vitamin D passes through the cytoplasmic membrane of cells and binds to the cytoplasmic vitamin D receptor and they make $1,25(OH)_2D$–VDR complex which is translocated into the nucleus to modulate expression of almost 500 genes by working as a transcriptional factor. As well as, excessive amounts of active vitamin D are catalyzed by 24-hydoxylase (CYP24A1) and excreted by the kidneys. In terms of immune system cells, resting monocytes and dendritic cells express the VDR as resting T and B lymphocytes express little to no VDR, but when activated, its expression in lymphocytes is increased (Peelen et al., 2011; Rosen et al., 2016). This inhibits transcription and secretion of pro-inflammatory cytokines, and skews CD4+ T-lymphocytes toward a Th2 cytokine profile that could achieve the suppression of undesirable autoimmunity and have a beneficial effect on the clinical course of the disease (Wergeland et al., 2011; Oreja-Guevara et al., 2012).

There are lots of evidences for associations of cholesterol with disease progression in MS. The brain is the most cholesterol-rich organ in the body and cholesterol composes of approximately 80% of the undamaged myelin. Lipids have important roles in the CNS and their transport through the BBB have been shown in healthy conditions and during breakdown of BBB in MS (Tettey et al., 2014). Some intermediate molecules like 8,9-unsaturated sterols in cholesterol biosynthesis pathway profoundly stimulate myelin formation and repair by promoting myelin-producing oligodendrocyte formation (Hubler et al., 2018).

Being primary target in the MS, myelin is formed in CNS by oligodendrocytes to insulate axons and represents a source of lipid antigens. In demyelination process, it is degraded and lipids are released from the membrane, this makes them a plausible target for an autoimmune reaction in MS (Jurewicz et al., 2017). Lipid metabolism anomalies are not only limited to the myelin sheet. Alterations affecting the plasma lipid profile can also be seen in MS patients (Çomoğlu et al., 2004). However, it is not certain whether these are causal or secondary to the disease process, treatment regimens or other factors (Sena et al., 2019).

It has been shown that HDL cholesterol has protective role in MS: affects immune cells counts, for instance, it is negatively related with monocyte numbers, and alters lesion causing interactions in the CNS by modulating immune cell phenotype in which HDL changes cellular cholesterol since it stimulates removal of cholesterol from macrophages and decreases plaque-forming foam cell production (Fellows et al., 2015). On the other hand, an early involvement of LDL in the development of MS lesions has been shown in many studies. Due to the BBB damage, high amount of plasma LDL enter the parenchyma of MS plaques and is oxidatively modified in the lesion. In the early stages of MS plaque, oxidized LDL uptake by infiltrating macrophages may have a significant role in demyelination (Giubilei et al., 2002).

A number of studies have compared lipid profiles between MS cases and healthy controls but there are some inconsistencies their results. It was found that there was critical connection between LDL-C and TC levels and worsening disability in which their levels were associated with new T2 lesions and grey matter atrophy, measured using the EDSS and the MS Severity Scale (MSSS) which are important to classify the severity and progression of MS (Mukhopadhyay et al., 2017).

In addition, when Jorissen et al., compared the lipoprotein profile of RRMS, PPMS patients and healthy controls, they found that RRMS patients had smaller LDL level from the others. They suggested that since LDL concentration increases with age and PPMS were significantly older than the HC and RRMS patients, this explained the

difference of LDL count between PPMS and RRMS patients. But age was not a cause for differences in HC and RRMS patients. They described that smaller LDL particles have an enhanced susceptibility to oxidation and a reduced LDL receptor affinity which can stimulate pro-inflammatory properties of LDL in RRMS patients (Jorissen et al., 2017[a]).

Also, in a study of Sena and coworkers, LDL levels were related with higher EDSS and MSSS in women RRMS patients while high TG levels with worsening disability in them (Sena et al., 2019). In another study, it was found higher TC and HDL levels while not significant differences in LDL and TG plasma levels in comparisons of MS patients with non-MS individuals (Sternberg et al., 2013). The amounts of TC and HDL cholesterol were significantly and LDL and TG levels were insignificantly higher in MS patients than in the controls. It was said that the level of total cholesterol was significantly affected by the number of increasing lesion (Giubilei et al., 2002). According to Çomoğlu et al., while total cholesterol and phospholipid content of myelin decreased in the demyelination area, demyelination of MS plaques leads to elevated plasma levels of TG, VLDL-C and TC (Çomoğluet al., 2004).

Cholesterol has major roles in many biological processes. It plays a structural role because of being an important component of cellular membranes. The precursors of steroid hormones and bile acids are produced from cholesterol. In addition to these, it is involved in formation of axons and dendrites during development, myelination during CNS maturation, neuro-steroid production, microtubule stability, neuronal repair and remodeling, learning, and memory. To maintain the cholesterol homeostasis, excess cholesterol in many tissues should be removed by different cytochrome P450 enzymes that hydroxylate cholesterol at specific positions, and bile acids are produced. Cholesterol 7α-hydroxylase (CYP7A1) in the liver and Cholesterol 24S-hydroxylase (CYP46A1) in the brain are responsible for cholesterol removal in human body.

CYP7A1 is the first and rate-limiting enzyme in the bile acids production pathway and it converts to cholesterol into 7 α-hydroxycholesterol. CYP46A1 converts the brain cholesterol into more water-soluble metabolite 24(S)-hydroxycholesterol (24OHC), levels of which has been thought being important in neurodegenerative disorders such as AD, PD and MS. It was shown that while level of 24(S)-hydroxycholesterol increased PPMS patients, 7 α-hydroxycholesterol decreased in RRMS patients over 5 years (Maxwell et al., 2019).

A single nucleotide polymorphism conferring A→C transition, named rs3808607, which is present at -204 location of form the transcriptional start site or -278 from the translation initiation codon, might play a critical role in gene expression and its enzyme activity (Srivastava et al., 2008; Cai et al., 2014). Associations between this polymorphic region and various metabolic disorders have been investigated in several studies due to the critical roles of the CYP7A1 in cholesterol and bile acid homeostasis (Qrafli et al., 2014; Xiang et al., 2012).

The A-to-C substitution in the CYP7A1 promoter have been associated with increased plasma total and LDL cholesterol concentrations, which was postulated that there was reduced CYP7A1 activity with carriers of the C allele (Wertheim et al., 2012; Xiang et al., 2012). However, -204CC genotype was associated with reduced risk of colorectal cancer, suggesting a protective gene effect due to the reduced bile acid synthesis in CC carriers (Wertheim et al., 2012). In another study the CC genotype may affect transcriptional regulation of the gene, causing CYP7A1 deficiency, which triggers several alterations in hepatic fatty acid metabolism and gallbladder bile composition, leading to more susceptibility to gallbladder cancer pathogenesis. In addition, while the CC genotype of rs3808607 was making a significant risk for gallstone disease in Indian people, AA genotype was discovered to be related with this disease in Chinese population (Srivastava et al., 2008; Jiang et al., 2004). Ethnic difference of the study population can exert a major effect on the disease predisposition.

CYP46A1 T → C single nucleotide polymorphism named rs754203 is investigated in terms of human health or disease (Papassotiropoulos et al., 2003). It does not affect the amino acids sequences or the structure of the protein expressed by gene (Papassotiropoulos et al., 2003; Jin et al., 2013). For example, it was related with increased risk of Alzheimer's disease (AD) in a Chinese population; however, there was not enough association about this polymorphism and AD in Hungarian and Italian populations (Rizvi et al., 2017; Fu et al., 2009). Besides, Mousavidehmordi et al., reported that TT genotype or T allele frequency of CYP46A1 may be a risk factor by increasing the susceptibility to AD in the Iranian population, whereas Borroni et al. described that CC genotype might act as a risk factor for LOAD (Late-Onset AD) in Italian people (Mousavidehmordi et al., 2016; Borroni et al., 2004). In addition to these studies, Juhász et al., could not find any relation between this SNPs of CYP46A1 and increasing susceptibility to LOAD in the Hungarian population (Juhász et al., 2005). As another example, T allele of its was correlated with a higher risk for primary open-angle glaucoma, which is a progressive optic neuropathy and the second leading cause of blindness, in French population; on the other hand, C allele of its was related to a higher risk for exudative age-related macular degeneration, which is the most common cause of irreversible visual loss in Western elderly populations (Fourgeux et al., 2009; Fourgeux et al., 2012). In type 2 Diabetes Mellitus (T2DM), CC and CT genotypes were found to increase the risk, however TT genotype was decreasing the risk of T2DM in Indian people (Rizvi et al., 2017). All these results suggest that predisposition of a disease related with a polymorphism may be distinct in different ethnic groups (Garcia et al., 2009).

The aim of the study is to investigate associations rs3808607 (A → C) in CYP7A1 and rs754203 (T → C) CYP46A1 single nucleotide polymorphic regions with multiple sclerosis in Turkish population and also their relations with lipid profiles (TC, TG, HDL, LDL) and vitamin D levels in MS cases and healthy control. It consisted of 138 MS patients and 100 symptom-free controls.

MS affects more than 2 million people worldwide and the mean age of beginning for MS is almost 30 in females while it was 33 years in males (Barcelos et al., 2019). The age of the study population ranged from 19 to 42 and the mean ages were 33.54 for MS patients while they were 19-70 and 32.79, respectively for controls. According to these results, no statistically significant difference was found in the mean age among them (**Table 3.1**).

Although it affects approximately three females per one male, men generally have quicker progression and more rapid disability. 95 female patients and 61 female controls were used within this study. In risk evaluation of being female, no significance was seen in the study population (*p*: 0.208) (**Table 3.1**).

When plasma lipids and Vitamin D levels were compared, MS patients had lower levels of all parameters than controls and these differences were statistically significant only for the total cholesterol (*p:* 0.005), LDL-cholesterol (*p:* 0.010) and Vitamin D (*p:* 0.003) and not for triglycerides (*p:* 0.054) and HDL- cholesterol (*p:* 0.725) levels (**Table 3.1**).

After determinations of genotype and allele frequencies of rs3808607 A→ C SNP of CYP7A1 and rs754203 T → C SNP of CYP46A1 genes in all groups, which were verified by sanger sequencing of six samples, it was wanted to be learned how their distributions were changing in MS patient and control groups and also to be determined whether these SNPs have any effects on risk of the MS. Therefore, the analyses were conducted by forming three genetic models of SNPs: Dominant, Recessive and Additive Model. In dominant model, the polymorphic allele was accepted as having dominant character compared to wild type allele. Therefore, the carries of mutant allele were pooled in a single group. On the other hand, in recessive model, it was assumed that for the polymorphic allele had recessive characteristic compared to wild type allele and therefore, wild type allele carriers were collected in a single group. And also, additive model was formed to be based upon the fact that both wild type allele and mutant allele have equal genetic effects on heterozygote

individuals. All comparisons for both SNPs were done within the all subjects, only male and only female subgroups, splitted according to these created models.

For CYP7A1 rs3808607 genotype analyses, 33.3% patients and 44.0% controls had homozygous wild type AA; 59.4% patients and 51.0% controls had heterozygous mutant type AC; and 7.2% patients and 5.0% controls had homozygous mutant genotype CC. In addition, the wild type allele (A) frequencies were 0.870 and 0.695 and the mutant allele (C) ones were 0.130 and 0.305 for patients and controls, respectively. Although the calculated odds ratios for all formed genetic models and alleles frequency were higher than 1.3, no statistically significant difference was found among them (all *p* values of them were higher than 0.01, **Table 3.2**). Similar consequences were obtained in subgroups analyses of 43 male patients and 39 male controls (**Table 3.3**) and 95 female patients and 61 female controls (**Table 3.4**) in terms of this SNP since all p values found for them were higher Bonferroni corrected-*p*-value threshold 0.01. Therefore, it was concluded that rs3808607 SNP could not be considered as a risk factor for multiple sclerosis.

The analyses of rs754203 SNP of CYP46A1 gene, there were 50.0% and 58% homozygote wild type TT, 42.8% and 34.0% heterozygote mutant TC, 7.2% and 8.0% homozygote mutant genotype CC individuals from patients and controls, respectively. Also, the distributions of both T and C allele were 0.714 and 0.286 for patients and 0.750 and 0.250 for controls. For genotype distributions, three different genetic models were formed based upon whether having additive, dominant and recessive characteristics of polymorphic allele compared to wild type allele like in the rs3808607. However, statistically significant relation was not found in any of them (all *p* values of them were higher than 0.01, **Table 3.5**). Similarly, male (**Table 3.6**) and female subgroups (**Table 3.7**) analyses of this SNP did not show any important relation for having MS. That's why, rs754203 SNP was not evaluated as a risk factor for multiple sclerosis.

To be a female with rs3808607 A → C or rs754203 T → C SNPs which were split according to additive, dominant, recessive models of selected SNPs was analyzed to learn whether it forms a risk factor for having MS among patients and controls or not. Summarized results in **Table 3.8** and **Table 3.9** showed that being female with AA or AC or CC and/or TT or TC or CC genotypes could not be considered as a risk factor for MS (all *p* values were higher than Bonferroni corrected-*p*-value threshold 0.01).

There were a relatively small number of patients with polymorphic allele for both SNPs. Therefore, all evaluations of genotypes and allele frequencies could be affected by this fact. 138 MS patients and 100 healthy controls were studied for double combinations of CYP7A1 rs3808607 A → C and CYP46A1 rs754203 T → C. In total, nine different double-combinations were created like AATT, AATC, AACC, ACTT, ACTC, ACCC, CCTT, CCTC, CCCC and only homozygote wild type allele combination of both alleles had almost two-fold protective role having MS (*p:* 0.039, **Table 3.10**), and no statistically significant associations were found in the other comparisons of double combinations (all p values of them > 0.05). In addition, any individual with CC+CC genotype was not seen in our study population.

We wanted to analyze possible relations of blood lipids and vitamin D with different genotype groups of CYP7A1 rs3808607 SNP and CYP46A1 rs754203 SNP, groups of which were split according to additive, dominant and recessive model of polymorphic alleles, in MS patients and healthy controls.

In assumed dominant model of rs3808607, these parameters were firstly compared between AA genotypes patients and controls, and between C allele carrier-patients and controls. Then, the comparisons were done between both patients and controls with AA and C allele carriers among themselves (**Table 3.11**).

In comparisons of the AA genotypes individuals, it was found that the patients had lower TC, TG, LDL-C and vitamin D levels than controls and HDL-C level was nearly similar between them. Before Bonferroni correction, TC and vitamin D levels were found statistically significant different (*p:* 0.022, *p:* 0.036, respectively) while levels

of TG, HDL-C, LDL-C were not (*p:* 0.096, *p:* 0.857, *p:* 0.100, respectively). On the other hand, in comparisons of C allele carriers' patients and controls, all parameters except for HDL-C were lower in patients and HDL-C was slightly higher in them. Similarly, no significant difference was found in these comparisons (all *p* values > 0.01, **Table 3.11**).

When patients from each genotype group were compared, AA genotypes patients were lower concentrations of TC, HDL-C, LDL-C and vitamin D and higher concentrations of TG than carrying mutant allele patients. On the other hand, controls with AA genotypes had almost same TC level, higher TG and vitamin D levels and lower HDL-C and LDL-C levels than controls with AC and CC genotypes. However, all these differences were not at statistically significant level (all *p* values > 0.01, **Table 3.11**). Therefore, it was concluded that founded distinctions could be related the difference between the diet of MS patients and healthy controls.

For recessive model of rs3808607, all comparisons were done similar to its dominant model. In comparisons of wild type-A allele- carriers, the patients had statistically significant lower levels of TC (*p:* 0.002) and vitamin D (*p:* 0.002) and insignificantly lower level of TG (*p:* 0.021) and LDL-C (*p:* 0.010), which was so close critical Bonferroni corrected *p* value but it could not be accepted as statistically significant. Even though HDL-C level was higher in patients, it was not meaningful difference (*p:* 0.690). Whereas, CC genotypes patients had higher levels of TC and vitamin D and lower levels of TG, HDL-C and LDL-C than controls with same genotypes. However, none of these differences were statistically important (all *p* values > 0.01, **Table 3.12**).

The comparisons of patients among themselves, CC genotypes individuals were insignificantly higher level of all parameters (all *p* values > 0.01). On the other hand, the comparisons of controls among themselves, A allele carriers individuals had higher levels of TC, LDL-C and vitamin D; and lower levels of TG and HDL-C than CC genotype individuals, but any of them was not statistically significant (all *p* values > 0.01, **Table 3.12**).

Since the number of homozygote mutant individuals was relatively small, this limited statistical power in our study. Diet differences of individuals could be related founded insignificant differences and also, we didn't know whether the patients used any lipid lowering drugs when the blood samples were taken.

For additive model of rs3808607, both wild type allele "A" and polymorphic allele "C" were assumed as having equal effects on heterozygous individuals. Therefore, how relations of three different genotypes on blood lipids and vitamin D concentrations had changed among patients and controls were investigated. Besides, patient groups and control groups among themselves were compared with all possible double combinations.

The comparisons of patients and controls with AA or CC genotypes were same in the dominant and recessive models, respectively. In addition, AC genotypes patients had lower levels of TC, TG, LDL-C, and vitamin D, and higher level of HDL-C. However, any of them was not statistically meaningful different (all *p* values > 0.01, **Table 3.13**).

In comparisons of with AA versus AC genotypes individuals, patients with AC genotypes had insignificantly higher levels of TC, HDL-C, LDL-C and vitamin D and lower level of TG than patients with AA, and the comparisons of controls with AA and AC genotypes showed almost same difference pattern (all *p* values > 0.01, **Table 3.13**). When the people with homozygote wild type and mutant type genotype were studied, the patients with CC genotype showed higher levels of all parameters than the patients with AA genotype. Whereas, the controls AA genotypes had higher levels of TC, TG, LDL-C and vitamin D and lower level of HDL-C. Exactly same pattern of differentness was seen in comparisons of both patients with AC and CC genotypes and controls with AC and CC genotypes. Any of founded differences were not statistically meaningful (all *p* values > 0.01, **Table 3.13**). It was concluded that there was no association between CYP7A1 rs3808607 genotype and serum and vitamin D level within its additive model.

Similar analyses were performed for CYP46A1 rs754203 T → C SNP. Firstly, the comparisons of plasma lipids and vitamin D concentrations in MS patients and healthy controls were done according to assumed dominant model of mutant allele. Amongst having TT genotypes individuals, the patients had lower levels of TC, TG, LDL-C and vitamin D, and higher level of HDL-C than the controls. However, any of these differences were not statistically important (all *p* values > 0.01, **Table 3.14**). As well as, when mutant allele carriers' patients and controls compared, they showed similar difference pattern like TT genotypes ones. However, only mean vitamin D content of patients statistically significantly lower than the controls (*p:* 0.005).

Higher levels of TC, TG, LDL-C and lower levels of HDL-C and vitamin D were seen in mutant C allele carriers' patients, compared to having homozygote wild type patients. Similarly, TT genotypes controls had lower levels of TC, TG, LDL-C and vitamin D and higher levels of HDL-C than C allele carriers' controls. Nevertheless, none of these differences were not statistically important (all *p* values > 0.01, **Table 3.14**). It can be said that mutant allele could be related with these parameters but it did not obtain enough statistical power. This could be due to the small sample size of the population.

In the analyses according to recessive model of rs754203, the patients with wild type allele had lower level of TC, TG, LDL-C and vitamin D and higher HDL content than controls with same genotypes. Of them, only founded difference of vitamin D was statistically significant (p: 0.005, **Table 3.15**). Although CC genotypes patients had lower level of all parameters than controls with CC genotypes, none of them were statistically meaningful (all *p* values > 0.01, **Table 3.15**).

Higher TC, LDL-C and vitamin D levels and lower TG and HDL-C levels were seen in the patients with TT and/or TC when they compared having CC genotype patients. However, in comparisons of controls among themselves with mentioned genotypes, all parameters were higher in CC genotypes individuals. There was no significant difference in any of them (all *p* values > 0.01, **Table 3.15**).

For additive model of rs754203, both wild type “T” allele and mutant “C” allele was assumed having equal effects on heterozygous individuals. Therefore, three different genotypes were formed and their possible associations on blood lipids and vitamin D levels were investigated among patients and controls. All double combinations between patient groups themselves and control groups themselves also were compared (**Table 3.16**). Of all the comparisons, only statistical significance was that vitamin D levels of patients with heterozygote mutant genotype was lower than controls with same genotype (*p:* 0.006).

It could be said that there was no relation between blood lipid parameters and rs754203 SNP of CYP46A1 gene within our population. However, vitamin D level was found especially lower in heterozygote genotypes patients than controls and this might be related this SNPs.

In the literature, there is no study investigating the associations of CYP7A1 rs3808607 and CYP46A1 rs754203 SNPs and multiple sclerosis and also their relations with lipid and vitamin D levels in Turkish population. Therefore, the present study is the first one to analyze these relations.

CHAPTER 5

CONCLUSION

Multiple Sclerosis is the most common autoimmune, inflammatory, demyelinating, neurodegenerative disorder of the central nervous system. Under the effects of certain environmental factors, it develops in genetically susceptible individuals. Obesity, smoking, Epstein-Barr virus and lower vitamin D levels have been associated with the development of MS. It generally starts in the most productive years of life, between 25-40 years, and decreases the life quality of those people.

Vitamin D has several important functions in human body such as in calcium homeostasis, apoptosis, cell proliferation, brain development and function, differentiation of immune cells and modulation of immune responses. Its level is increased in the human body by sun exposure, vitamin D supplementation and diet. UV-B radiation catalyzes the photo-conversion of 7-dehydrocholesterol, produced in Kandutsch–Russell pathway that is one of the cholesterol production pathways, to vitamin D (cholecalciferol) in the skin. This is a linker between Vitamin D and cholesterol pathways.

Cholesterol has major roles in many biological processes. It plays a structural role because of being an important component of cellular membranes. The precursors of steroid hormones and bile acids are produced from cholesterol. In addition to these, it is involved in formation of axons and dendrites during development, myelination during CNS maturation, neuro-steroid production, microtubule stability, neuronal repair and remodeling, learning, and memory. To maintain the cholesterol homeostasis, excess cholesterol in many tissues should be removed by different cytochrome P450 enzymes that hydroxylate cholesterol at specific positions, and bile acids are produced. Cholesterol 7α-hydroxylase (CYP7A1) in the liver and

Cholesterol 24S-hydroxylase (CYP46A1) in the brain are responsible for cholesterol removal in human body. CYP7A1 is the first and rate-limiting enzyme in the bile acids production pathway and it converts to cholesterol into 7 α-hydroxycholesterol. CYP46A1 converts the brain cholesterol into more water-soluble metabolite 24(S)-hydroxycholesterol (24OHC), levels of which has been thought being important in neurodegenerative disorders such as AD, PD and MS.

A single nucleotide polymorphism conferring A→C transition, named rs3808607 might play a critical role in gene expression and its enzyme activity. Associations between this polymorphic region and various metabolic disorders have been investigated in several studies due to the critical roles of the CYP7A1 in cholesterol and bile acid homeostasis. Also, CYP46A1 T → C single nucleotide polymorphism named rs754203 is investigated in terms of human health or disease although it does not affect the amino acids sequences or the structure of the protein expressed by gene.

In present study, rs3808607 A → C in CYP7A1 gene, and rs754203 T → C in CYP46A1 polymorphisms were investigated for the risk of multiple sclerosis. Because of their important roles in cholesterol metabolism, the associations between both cholesterol and vitamin D pathways and their effects in multiple sclerosis progression, these regions were selected for that study.

To investigate the associations rs3808607 and rs754203 SNP regions with multiple sclerosis in Turkish population and also their relations with lipid profiles (TC, TG, HDL, LDL) and vitamin D levels were aimed in 138 MS patients and 100 symptom-free controls. There was no significant difference between them with respect to their age and gender (*p:* 0.053, *p:* 0.208). When they compared in terms of lipid parameters and vitamin D levels, it was found that total cholesterol, LDL-cholesterol, and vitamin D levels were significantly lower in patients than controls (*p:* 0.005, *p:* 0.010, *p:* 0.003, respectively, Table 1).

After determination of genotype and allele frequencies of rs3808607 and rs754203 SNPs in all groups, it was wanted to be learned how their distributions were changing in MS patient and control groups and also to be determined whether these SNPs have any effects on risk of the MS. Therefore, the analyses were conducted by forming three genetic models of SNPs: Dominant, Recessive and Additive Model. In dominant model, the polymorphic allele was accepted as having dominant character compared to wild type allele. Therefore, the carries of mutant allele were pooled in a single group. On the other hand, in recessive model, it was assumed that for the polymorphic allele had recessive characteristic compared to wild type allele and therefore, wild type allele carriers were collected in a single group. And also, additive model was formed to be based upon the fact that both wild type allele and mutant allele have equal genetic effects on heterozygote individuals.

For CYP7A1 rs3808607 and CYP46A1 rs754203, all these analyses were performed with all subjects, only male and only female subgroups (Table 3.2, Table 3.3, Table 3.4, Table 3.5, Table 3.6, Table 3.7). However, any significant difference was not found in them since all *p* value of them were higher than Bonferroni corrected p value threshold 0.01.

Whether being a female with rs3808607 or rs754203 SNPs with assumed genetic models makes as a risk factor was investigated (Table 3.8 and table 3.9) within the population. To be a female with AA or AC or CC / TT or TC or CC genotypes could not be considered as a risk factor for MS (all *p* values were higher than 0.01).

As well as, whether double combinations of CYP7A1 rs3808607 A → C and CYP46A1 rs754203 T → C have any effect on MS risk were studied. In total, nine different double-combinations were created and only homozygote wild type allele combination of both alleles had almost two-fold protective role having MS (*p:* 0.039, Table 3.10). However, no statistically significant associations were found in comparisons of all the other double combinations (all p values of them > 0.05). In addition, any individual with CC+CC genotype was not seen in our study population.

It was wanted to learn whether there were any relations of blood lipids and vitamin D levels in MS patients and healthy controls with CYP7A1 rs3808607 and CYP46A1 rs754203 SNPs, split according to additive, dominant and recessive model of polymorphic alleles.

In assumed dominant and additive models of rs3808607, any statistically significant difference was not found between comparisons of both patients and controls with same genotype and patients or controls among themselves with different genotypes (Table 3.11 and Table 3.13; all p values of them > 0.01). However, in its recessive model, TC and vitamin D contents of patients, carrying wild type allele were statistically significantly lower than controls with same genotypes (*p:* 0.002, *p:* 0.002, respectively). The other similar comparisons didn't show any meaningful difference (Table 3.12). Therefore, it was concluded that founded distinctions could be related the difference between the diet of MS patients and healthy controls. In addition, since the number of homozygote mutant individuals was relatively small, this limited statistical power of our study.

As regard to CYP46A1 rs754203 SNP, same analyses were carried out to determine any possible relations between these parameters and patients and controls with this SNP. Of all the comparisons, only statistical significance was that vitamin D levels of patients with heterozygote mutant genotype was lower than controls with same genotype (*p:* < 0.01; Table 3.14, Table 3.15, Table 3.16). It could be said that there were no any relation blood lipid parameters and rs754203 SNP within our population. However, vitamin D level was found especially lower in heterozygote genotypes patients than controls and this might be related this SNPs.

As a conclusion, MS has a highly variable inter and intra-personal clinical course, and there are no qualified blood biomarkers of disease progression. In this study, there were no significant associations observed between genotypes and allele frequencies of CYP7A1 rs3808607 and CYP46A1 rs754203 SNPs and MS risk. In terms of lipid parameters and vitamin D levels, these genes and lipid parameters were unrelated in

patients and controls, however, vitamin D levels were found lower in patients than controls within some subgroups. Since MS is a multifactorial disease, and the mechanisms underlying the associations of cholesterol biomarkers with its disease progression are not exactly known, more research is needed for clarification. Moreover, level of Vitamin D plays highly important role in MS, the right and convenient amount of vitamin D, which is specific for MS patient, should be determined in the future.

REFERENCES

Agnello, L., Scazzone, C., Lo Sasso, B., Ragonese, P., Milano, S., Salemi, G., & Ciaccio, M. (2018). CYP27A1, CYP24A1, and RXR-α Polymorphisms, Vitamin D, and Multiple Sclerosis: a Pilot Study. *Journal of Molecular Neuroscience, 66*(1), 77-84.

Agostini, S., Mancuso, R., Guerini, F. R., D'Alfonso, S., Agliardi, C., Hernis, A., . . . Clerici, M. (2018). HLA alleles modulate EBV viral load in multiple sclerosis. *Journal of Translational Medicine, 16*(1), 1-8.

Aikawa, T., Holm, M.-L., & Kanekiyo, T. (2018). ABCA7 and Pathogenic Pathways of Alzheimer's Disease. *Brain Sciences, 8*, 27-39.

Alfredsson, L., & Olsson, T. (2018). Lifestyle and Environmental Factors in Multiple Sclerosis. *Cold Spring Harbor Perspectives in Medicine, 9*(4), 1-13.

Alwi, Z. B. (2005). The Use of SNPs in Pharmacogenomics Studies. *Malaysian Journal of Medical Sciences, 12*(2), 4-12.

Andersen, C. J. (2018). Impact of Dietary Cholesterol on the Pathophysiology of Infectious and Autoimmune Disease. *Nutrients, 10*(6), 764-789.

Ascherio, A., Munger, K. L., & Simon, K. C. (2010). Vitamin D and multiple sclerosis. *Lancet Neurology, 9*, 599-612.

Ascherio, A., Munger, K. L., White, R., Köchert, K., Simon, K. C., &, .., & Pohl, C. (2014). Vitamin D as an Early Predictor of Multiple Sclerosis Activity and Progression. *JAMA Neurology, 71-91*(3), 306-.

Ayciriex, S., Djelti, F., Alves, S., Regazzetti, A., Gaudin, M., Varin, J., . . . Cartier, N. (2017). Neuronal Cholesterol Accumulation Induced by Cyp46a1 Down-Regulation in Mouse Hippocampus Disrupts Brain Lipid Homeostasis. *Frontiers in Molecular Neuroscience, 10*, 211-225.

Barcelos, I. P., Troxell, R. M., & Graves, J. S. (2019). Mitochondrial Dysfunction and Multiple Sclerosis. *Biology, 8*(2), 37-54.

Berghoff, S. A., Gerndt, N., Winchenbach, J., Stumpf, S. K., Hosang, L., (...), . . . Saher, G. (2017). Dietary cholesterol promotes repair of demyelinated lesions in the adult brain. *Nature Communications, 8*, 14241-14255.

Bernhardt, R. (2006). Cytochromes P450 as versatile biocatalysts. *Journal of Biotechnology, 124*, 128-145.

Bevilacqua, L., Navarra, C. O., Pirastu, N., Lenarda, R. D., Gasparini, P., & Robino, A. (2018). A genome- wide association study identifies an association between variants in EFCAB4B gene and periodontal disease in an Italian isolated population. *Journal of Periodontal Research.*, 1-7.

Björkhem, I., Lövgren-Sandblom, A., Leoni, V., Meaney, S., Brodin, L., (...), & Svenningsson, P. (2013). Oxysterols and Parkinson's disease: Evidence that levels of24S-hydroxycholesterol in cerebrospinal fluid correlateswith the duration of the disease. *Neuroscience Letters, 555*, 102-105.

Borroni, B., Archetti, S., Agosti, C., Akkawi, N., Brambilla, C., Caimi, L., . . . Padovani, A. (2004). Intronic CYP46 polymorphism along with ApoE genotype in sporadic Alzheimer Disease: from risk factors to disease modulators. *Neurobiology of Aging, 25*, 747–751.

Browne, R. W., Weinstock-Guttman, B., Zivadinov, R., Horakova, D., Bodziak, M. L., (...), & Ramanathan, M. (2014). Serum lipoprotein composition and vitamin D metabolite levels in clinically isolated syndromes: Results from a multi-center study. *Journal of Steroid Biochemistry & Molecular Biology, 143*, 424-433.

Brownlee, W. J., Hardy, T. A., Fazekas, F., & Miller, D. H. (2017). Diagnosis of multiple sclerosis: progress and challenges. *Lancet, 389*, 1336–1346.

Cai, Q., Wang, Z.-Q., Cai, Q., Li, C., Chen, E.-Z., & Jiang, Z.-Y. (2014). Relationship between CYP7A1 -204A > C polymorphism with gallbladder stone disease and serum lipid levels: a meta-analysis. *Lipids in Health and Disease, 13*(1), 126-132.

Can Demirdöğen, B. (2019). Potential role of calcifying nanoparticles in the etiology of Multiple Sclerosis. *Medical Hypotheses*, 1-12.

Charcot, J.-M. (1868). Histologie de la sclérose en plaques (leçon recueillie par Bourneville). *Gaz Hop Civ., 1051*(140).

Chiang, J. Y. (2013). Bile Acid Metabolism and Signaling. *Comprehensive Physiology*.

Ciaula, A. D., Garruti, G., Baccetto, R. L., Molina-Molina, E., Bonfrate, L., Wang, D. Q.-H., & Portincasa, P. (2017). Bile Acid Physiology. *Annals of Hepatology, 16*(0), 4-14.

Compston, A., & Coles, A. (2002). Multiple sclerosis. *Lancet, 359*, 1221-1231.

Cooper, A. D., Chen, J., Botelho-Yetkinler, M. J., Cao, Y., Taniguchi, T., & Levy-Wilson, B. (1997). Characterization of Hepatic-specific Regulatory Elements in the Promoter Region of the Human Cholesterol 7a-Hydroxylase Gene. *The Journal of Biological Chemistry, 272*(6), 3444-3452.

Cox, R. A., & García-Palmieri, M. R. (1990). Cholesterol, Triglycerides, and Associated Lipoproteins. In H. Walker, W. Hall, & J. Hurst, *Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd edition* (pp. 153-160). Baston .

Crick, P. J., Griffiths, W. J., Zhang, J., Beibel, M., Abdel-Khalik, J., (...), & Wang, Y. (2017). Reduced Plasma Levels of 25-Hydroxycholesterol and Increased Cerebrospinal Fluid Levels of Bile Acid Precursors in Multiple Sclerosis Patients. *Molecular Neurobiology, 54*, 8009-8020.

Çomoğlu, S., Serdar, Y., & Okçu, Z. (2004). Body Fat Distribution and Plasma Lipid Profiles of Patients with Multiple Sclerosis. *Turkish Journal of Medical Sciences, 34*(1), 43-48.

Dahbour, S., Jamali, F., Alhattab, D., Al-Radaideh, A., Ababneh, O., Al-Ryalat, N., . . . Awidi, A. (2017). Mesenchymal stem cells and conditioned media in the treatment of multiple sclerosis patients: Clinical, ophthalmological and radiological assessments of safety and efficacy. *CNS Neuroscience & Therapeutics, 23*, 866-874.

Dayger, C. A., Rosenberg, J. S., Winkler, C., Foster, S., Witkowski, E., (...), & Raber, J. (2013). Paradoxical effects of apolipoprotein E on cognitive function and

clinical progression in mice with experimental autoimmune encephalomyelitis. *Pharmacology, Biochemistry and Behavior, 103*, 860-868.

Deckx, N., Wens, I., Nuyts, A. H., Hens, N., De Winter, B. Y., Koppen, G., . . . Cools, N. (2016). Clinical Study: 12 Weeks of Combined Endurance and Resistance Training Reduces Innate Markers of Inflammation in a Randomized Controlled Clinical Trial in Patients with Multiple Sclerosis. *Mediators of Inflammation*, 1-13.

Dias, S., Pheiffer, C., Abrahams, Y., Rheeder, P., & Adam, S. (2018). Molecular Biomarkers for Gestational Diabetes Mellitus. *International Journal of Molecular Sciences, 19*, 2926-2952.

Dias, V., & Riberio, V. (2011). Ethnic differences in the prevalence of polymorphisms in CYP7A1, CYP7B1 AND CYP27A1 enzymes involved in cholesterol metabolism. *Journal of Pharmacy and Bioallied Sciences, 3*(3), 453-459.

Dionyssiotis, Y., Mavrogenis, A., Trovas, G., Skarantavos, G., Papathanasiou, J., & Papagelopoulos, P. (2014). Bone and Soft Tissue Changes in Patients with Spinal Cord Injury And Multiple Sclerosis. *Folia Medica, 56*(4), 237-244.

Dyment, D. A., Ebers, G. C., & Sadovnick, A. D. (2004). Genetics of multiple sclerosis. *Lancet Neurology, 3*, 104-110.

Elkama, A., & Karahalil, B. (2018). Role of gene polymorphisms in vitamin D metabolism and in multiple sclerosis. *Archives of Industrial Hygiene and Toxicology, 69*(1), 25-31.

Ellwardt, E., & Zipp, F. (2014). Molecular mechanisms linking neuroinflammation and neurodegeneration in MS. *Experimental Neurology, 262*, 8-17.

Engle, L., Simpson, C., & Landers, J. (2006). Using high-throughput SNP technologies to study cancer. *Oncogene, 25*, 1594-1601.

Fellows, K., Uher, T., Browne, R. W., Weinstock-Guttman, B., Horakova, D., Posova, H., & ...and Ramanathan, M. (2015). Protective associations of HDL with blood-brain barrier injury in multiple sclerosis patients. *Journal of Lipid Research, 56*, 2010-2018.

Feng, P., Zhao, L., Guo, F., Zhang, B., Fanga, L., Zhan, G., . . . Li, B. (2018). The enhancement of cardiotoxicity that results from inhibiton of CYP 3A4 activity and hERG channel by berberine in combination with statins. *Chemico-Biological Interactions, 293*, 115-123.

Ferdinandusse, S., & Houten, S. M. (2006). Peroxisomes and bile acid biosynthesis. *Biochimica et Biophysica Acta, 1763*, 1472-1440.

Fernández del Pozo, V., Álvarez Álvarez, M., Martínez, M. F., Alcelay, L. G., Busto, F. G., (...), & M. de Pancorbo, M. (2006). *Dementia and Geriatric Cognitive Disordes, 21*, 81-87.

Fourgeux, C., Dugas, B., Richard, F., Björkhem, I., Acar, N., Bron, A. M., & Bretillon, L. (2012). Single Nucleotide Polymorphism in the Cholesterol-24SHydroxylase (CYP46A1) Gene and Its Association with CFH and LOC387715 Gene Polymorphisms in Age-Related Macular Degeneration. *Investigative Ophthalmology & Visual Science, 53*(11), 7026-7033.

Fourgeux, C., Martine, L., Björkhem, I., Diczfalusy, U., Joffre, C., Acar, N., . . . Bretillon, L. (2009). Primary Open-Angle Glaucoma: Association with Cholesterol 24S-Hydroxylase (CYP46A1) Gene Polymorphism and Plasma 24-Hydroxycholesterol Levels. *Investigative Opthalmology & Visual Science, 50*(12), 5712-5717.

Fox, R. J., Coffey, C. S., Cudkowicz, M. E., Gleason, T., Goodman, A., Klawiter, E. C., . . . Zabeti, A. (2016). Design, Rationale, and Baseline Characteristics of the Randomized Double-Blind Phase II Clinical Trial of Ibudilast in Progressive Multiple Sclerosis. *Contemp Clin Trials, 50*, 166-177.

Freedman, M. S., Thompson, E. J., Giovannoni, G., Grimsley, G., Keir, G., (...), & Tourtellotte, W. W. (2005). Recommended Standard of Cerebrospinal Fluid Analysis in the Diagnosis of Multiple Sclerosis. *JAMA Neurology, 62*, 865-870.

Fridkis-Hareli, M. (2013). Design of Peptide Immunotherapies for MHC Class-II-Associated Autoimmune Disorders. *Clinical and Developmental Immunology*, 1-9.

Friese, M. A., Schattling, B., & Fugger, L. (2014). Mechanisms of neurodegeneration and axonal dysfunction in multiple sclerosis. *Nature Reviews Neurology, 10*(4), 225-238.

Fu, B. Y., Ma, S. L., Tang, N. L., Tam, C. W., Lui, V. W., Chiu, H. F., & Lam, L. C. (2009). Cholesterol 24-hydroxylase (CYP46A1) polymorphisms are associated with faster cognitive deterioration in Chinese older persons: a two-year follow up study. *International Journal of Geriatric Psychiatry, 24*, 921-926.

Fu, L., Zhao, Y., Wu, X., Liu, H., Shi, J., Lu, J., & Zhou, B. (2011). CYP7A1 genotypes and haplotypes associated with hypertension in an obese Han Chinese population. *Hypertension Research, 34*, 722–727.

Garcia, A. N., Muniz, M. T., Souza e Silva, H. R., Albuquerque da Silva, H., & Athayde-Junior, L. (2009). Cyp46 Polymorphisms in Alzheimer's Disease: A Review. *Journal of Molecular Neuroscience, 39*, 342-345.

Genetics Home Reference. (2018, Oct 23). Retrieved from NIH U.S. National Library of Medicine : https://ghr.nlm.nih.gov/gene/DHCR7

Gharagozloo, M., Gris, K. V., Mahvelati, T., Amrani, A., Lukens, J. R., & and Gris, D. (2018). NLR-Dependent Regulation of Inflammation in Multiple Sclerosis. *Frontiers in Immunology, 8*, 1-18.

Gil, Á., Plaza-Diaz, J., & Mesa, M. D. (2018). Vitamin D: Classic and Novel Actions. *Annals of Nutrition & Metabolism, 72*, 87-95.

Giubilei, F., Antonini, G., Di Legge, S., Sormani, M., Pantano, P., (...), & Pozzilli, C. (2002). Blood cholesterol and MRI activity in first clinical episode suggestive of multiple sclerosis. *Acta Neurologica Scandinavica, 106*, 109-112.

Golanska, E., Hulas-Bigoszewskaa, K., Wojcik, I., Rieske, P., Styczynska, M., Peplonska, B., & Liberski, P. P. (2005). CYP46: A risk factor for Alzheimer's disease or a coincidence? *Neuroscience Letters, 383*, 105–108.

Graves, J. S., Barcellos, L. F., Krupp, L., Belman, A., Shao, X., & ...& Waubant, E. (2019). Vitamin D genes influence MS relapses in children. *Multiple Sclerosis Journal*, 1-8.

Grouleff, J., Irudayam, S. J., Skeby, K. K., & Schiøtt, B. (2015). The influence of cholesterol on membrane protein structure, function, and dynamics studied by molecular dynamics simulations. *Biochimica et Biophysica Acta, 1848*(9), 1783-1795.

Group, T. I. (2001). A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature, 409*(6822), 928-933.

Harirchian, M. H., Fatehi, F., Sarraf, P., Honarvar, N. M., & Bitarafana, S. (2017). Worldwide prevalence of familial multiple sclerosis: A systematic review and meta-analysis. *Multiple Sclerosis and Related Disorders, 20*, 43-47.

He, X.-m., Zhang, Z.-x., Zhang, J.-w., Zhou, Y.-t., Wu, C.-b., Tang, M.-n., & Hon, Z. (2012). An Intronic CYP46A1 Polymorphism Is Associated with Alzheimer Disease in a Chinese Han Population. *Journal of Molecular Neuroscience, 47*, 514-518.

Hedström, A. K., Hössjer, O., Katsoulis, M., Kockum, I., Olsson, T., & Alfredsson, L. (2018). Organic solvents and MS susceptibility Interaction with MS risk HLA genes. *Neurology, 91*(5), 455-463.

Henriksson, A., Kam-Hansen, S., & Link, H. (1985). IgM, IgA and IgG producing cells in cerebrospinal fluid and peripheral blood in multiple sclerosis. *Clin. Exp. Immunol., 62*, 176-184.

Holick, M. F. (2003). Vitamin D: A Millenium Perspective. *Journal of Cellular Biochemistry, 88*, 296-307.

Holick, M. F. (2004). Vitamin D: importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis. *The American Journal of Clinical Nutrition, 79*(3), 362-371.

Hollenbach, J. A., & Oksenberg, J. R. (2015). The immunogenetics of multiple sclerosis: A comprehensive review. *Journal of Autoimmunity, 64*, 13-25.

Holmøy, T., Torkildsen, Ø., Myhr, K.-M., & Løken-Amsrud, K. (2012). Vitamin D supplementation and monitoring in multiple sclerosis: who, when and wherefore. *Acta Neurologica Scandinavica, 216*, 63-69.

Houdebine, L., Gallelli, C. A., Rastelli, M., Sampathkumar, N. K., & Grenier, J. (2017). Effect of physical exercise on brain and lipid metabolism in mouse models of multiple sclerosis. *Chemistry and Physics of Lipids, 207*, 127-134.

Hubler, Z., Allimuthu, D., Bederman, I., Elitt, M. S., Madhavan, M., Allan, K. C., & ..., a. A. (2018). Accumulation of 8,9-unsaturated sterols drives oligodendrocyte formation and remyelination. *Nature, 560*, 372-399.

Huss, R. (2015). Biomarkers. *Translational Regenerative Medicine*, 235-241.

Iwanicki, T., Balcerzyk, A., Niemiec, P., Nowak, T., Ochalska-Tyka, A., (...), & Zak, I. (2015). CYP7A1 Gene Polymorphism Located in the 5' Upstream Region Modifies the Risk of Coronary Artery Disease. *Disease Markers, 2015*, 1-6.

Jackson, R. A., Nguyen, M. L., Barrett, A. N., Tan, Y. Y., Choolani, M. A., & Chen, E. S. (2016). Synthetic combinations of missense polymorphic genetic changes underlying Down syndrome susceptibility. *Cellular and Molecular Life Sciences, 73*, 4001-4017.

Jamroz-Wisniewska, A., Beltowski, J., Stelmasiak, Z., & Bartosik-Psujek, H. (2009). Paraoxonase 1 activity in different types of multiple sclerosis. *Multiple Sclerosis, 15*, 399–402.

Jelcic, I., Nimer, F. A., Wang, J., Lentsch, V., Planas, R., Jelcic, I., . . . Martin, R. (2018). Memory B Cells Activate Brain-Homing, Autoreactive CD4+ T Cells in Multiple Sclerosis. *Cell, 175*, 1-16.

Jiang, Z.-Y., Han, T.-Q., Suo, G.-J., Feng, D.-X., Chen, S., Cai, X.-X., . . . Zhang, S.-D. (2004). Polymorphisms at cholesterol 7a-hydroxylase, apolipoproteins B and E and low density lipoprotein receptor genes in patients with gallbladder stone disease. *World Journal of Gastroenterology, 10*(10), 1508-1512.

Jin, C., Zhang, F., Zhu, J., Yuan, J., Xia, M., Xu, Q., & Xu, W. (2013). Association of CYP46 gene polymorphism with sporadic Alzheimer's disease in Chinese Han populations: a meta-analysis. *International Journal of Neuroscience, 123*(4), 226-232.

Jorissen, W., Vanmierlo, T., Wens, I., Somers, V., Wijmeersch, B. V., (...), & Hendriks, J. J. (2018). Twelve Weeks of Medium-Intensity Exercise Therapy

Affects the Lipoprotein Profile of Multiple Sclerosis Patients. *International Journal of Molecular Sciences, 19*(1), 193-203.

Jorissen, W., Wouters, E., Bogie, J. F., Vanmierlo, T., Noben, J.-P., (...), & Hendriks, J. J. (2017). Relapsing-remitting multiple sclerosis patients display an altered lipoprotein profile with dysfunctional HDL. *Nature, 7*(1), 1-14.

Juhász, A., Rimanóczy, Á., Boda, K., Vincze, G., Szlávik, G., Zana, M., . . . Kálmán, J. (2005). CYP46 T/C Polymorphism is not Associated with Alzheimer's Dementia in a Population from Hungary. *Neurochemical Research, 30*(8), 943-948.

Jurewicz, A., Domowicz, M., Galazka, G., Raine, C. S., & Selmaj, K. (2017). Multiple Sclerosis: Presence of Serum Antibodies to Lipids and Predominance of Cholesterol Recognition. *Journal of Neuroscience Research, 95*, 1984-1992.

Kägi, L., Bettoni, C., Pastor-Arroyo, E. M., Schnitzbauer, U., Hernando, N., & Wagner, C. A. (2018). Regulation of vitamin D metabolizing enzymes in murine renal and extrarenal tissues by dietary phosphate, FGF23, and 1,25(OH)2D3. *PLoS ONE, 13*(5), 1-21.

Kamen, D. L., & Tangpricha, V. (2010). Vitamin D and molecular actions on the immune system: modulation of innate and autoimmunity. *Journal of Molecular Medicine, 88*(5), 441-450.

Kaminski, W. E., Ors'o, E., Diederich, W., Klucken, J., Drobnik, W., & Schmitz, G. (2000). Identification of a Novel Human Sterol-Sensitive ATP-Binding Cassette Transporter (ABCA7). *Biochemical and Biophysical Research Communications, 273*, 532-538.

Karmon, Y., Ramanathan, M., Minagar, A., Zivadinov, R., & Weinstock-Guttman, B. (2013). Arterial, venous and other vascular risk factors in multiple sclerosis. *Neurological Research, 34*(8), 754-760.

Koriem, K. M. (2016). Multiple sclerosis: New insights and trends. *Asian Pacific Journal of Tropical Biomedicine, 6*(5), 429-440.

Kozin, M. S., Kulakova, O. G., & Favorova, O. O. (2018). Involvement of Mitochondria in Neurodegeneration in Multiple Sclerosis. *Biochemistry (Moscow), 83*(7), 813-830.

Kölsch, H., Heun, R., Jessen, F., Popp, J., Hentschel, F., (...), & Lütjohann, D. (2010). Alterations of cholesterol precursor levels in Alzheimer's disease. *Biochimica et Biophysica Acta*(1801), 945-950.

Krementsov, D. N., Asarian, L., Fang, Q., Mcgill, M. M., & Teuscher, C. (2018). Sex-Specific Gene-by-Vitamin D Interactions Regulate Susceptibility to Central Nervous System Autoimmunity. *Frontiers in Immunology, 9*, 1-15.

Kular, L., Liu, Y., Ruhrmann, S., Zheleznyakova, G., Marabita, F., Gomez-Cabrero, D., . . . Jagodic, M. (2018). DNA methylation as a mediator of HLA-DRB1*15:01 and a protective variant in multiple sclerosis. *Nature Communications, 9*(1).

Kusumadewi, W., Imrana, D., Witjaksono, F., Pakasi, T. A., Rusmana, A. I., (...), & Estiasari, R. (2018). Low vitamin D-25(OH) level in Indonesian multiple sclerosis and neuromyelitis optic patients. *Multiple Sclerosis and Related Disorders, 25*, 329-333.

Labiano-Fontcuberta, A., Mato-Abad, V., A´lvarez-Linera, J., Herna´ndez-Tamames, J. A., Martı´nez-Gine´s, L., Aladro, Y., . . . Benito-Leo´n, J. (2016). Gray Matter Involvement in Radiologically Isolated Syndrome. *Medicine, 95*, 1-11.

Lassmann, H., & Horssen, J. v. (2011). The molecular basis of neurodegeneration in multiple sclerosis. *FEBS Letters, 585*, 3715-3723.

Laterra, J., Keep, R., Betz, L. A., & Goldstein, G. W. (1999). Blood—Brain Barrier. In G. J. Siegel, B. W. Agranoff, W. Albers, S. K. Fisher, & M. D. Uhler, *Basic Neurochemistry: Molecular, Cellular and Medical Aspects. 6th edition.*

Lavrnja, I., Smiljanic, K., Savic, D., Mladenovic-Djordjevic, A., Tesovic, K., Kanazir, S., & Pekovic, S. (2016). Expression profiles of cholesterol metabolism-related genes are altered during development of experimental autoimmune encephalomyelitis in the rat spinal cord. *Nature-Scientific Reports, 7*(1), 1-14.

Lee, B.-Y., Kim, D.-H., Kim, H.-S., Kim, B.-M., Han, J., & Lee, J.-S. (2018). Identification of 74 cytochrome P450 genes and co-localized cytochrome P450 genes of the CYP2K, CYP5A, and CYP46A subfamilies in the mangrove killifish Kryptolebias marmoratus. *BMC Genomics, 19*(7), 1-12.

Leoni, V. (2009). Oxysterols as markers of neurological disease – a review. *Scandinavian Journal of Clinical and Laboratory Investigation, 69*(1), 22-25.

Leoni, V., & Caccia, C. (2011). Oxysterols as biomarkers in neurodegenerative diseases. *Chemistry and Physics of Lipids, 164*, 515-524.

Leoni, V., & Caccia, C. (2013). 24S-hydroxycholesterol in plasma: A marker of cholesterol turnover in neurodegenerative diseases. *Biochimie, 95*, 595-612.

Leoni, V., & Caccia, C. (2014). Study of cholesterol metabolism in Huntington's disease. *Biochemical and Biophysical Research Communications, 446*, 697-701.

Leoni, V., & Caccia, C. (2015). The impairment of cholesterol metabolism in Huntington disease. *Biochimica et Biophysica Acta, 1851*, 1095-1105.

Leray, E., Moreau, T., Fromont, A., & Edan, G. (2016). Epidemiology of multiple sclerosis. *Revue Neurologique, 172*, 3-13.

Leray, E., Vukusic, S., Debouverie, M., Clanet, M., Brochet, B., Sèze, J. d., . . . Edan, G. (2015). Excess Mortality in Patients with Multiple Sclerosis Starts at 20 Years from Clinical Onset: Data from a Large-Scale French Observational Study. *Plos One, 10*(7), 1-12.

Li, L., Zeng, F., Liu, Y.-H., Li, H.-Y., Dong, S.-Y., Peng, Z.-Y., . . . Zhou, H.-D. (2018). CYP46A1 and the APOEε4 Allele Polymorphisms Correlate with the Risk of Alzheimer's Disease. *Molecular Neurobiology, 55*, 8179–8187.

Li, M., Wang, W., Li, Y., Wang, L., Shen, X., & Tang, Z. (2013). CYP46A1 intron-2T/C polymorphism and Alzheimer's disease: An updated meta-analysis of 16 studies including 3960 cases and 3828 controls. *Neuroscience Letters, 549*, 18-23.

Lin, J., Zhao, H.-S., Qin, L., Li, X.-N., Zhang, C., Xia, J., & Li, J.-L. (2018). Atrazine Triggers Mitochondrial Dysfunction and Oxidative Stress in Quail (Coturnix

C. coturnix) Cerebrum via Activating Xenobiotic- Sensing Nuclear Receptors and Modulating Cytochrome P450 Systems. *Journal of Agricultural and Food Chemistry, 66*(25), 6402-6413.

Lorbek, G., Lewinska, M., & Rozman, D. (2012). Cytochrome P450s in the synthesis of cholesterol and bile acids – from mouse models to human diseases. *FEBS Journal, 279*, 1516-1533.

Lu, F., Zhu, J., Guo, S., Wong, B. J., Chehab, F. F., Ferriero, D. M., & Jiang, X. (2018). Upregulation of cholesterol 24-hydroxylase following hypoxia–ischemia in neonatal mouse brain. *Pediatric Research, 83*(6), 1218-1227.

Lublin, F. D., & Reingold, S. C. (1996). Defining the clinical course of multiple sclerosis Results of an international survey. *Neurology Apr, 46*(4), 907-911.

Lublin, F. D., Reingold, S. C., Cohen, J. A., Cutter, G. R., Sørensen, P. S., Thompson, A. J., . . . Polman, C. H. (2014). Defining the clinical course of multiple sclerosis The 2013 revisions. *Neurology, 83*, 278-286.

Luque-Córdoba, D., & Luque de Castroa, M. D. (2017). Metabolomics: A potential way to know the role of vitamin D on multiple sclerosis. *Journal of Pharmaceutical and Biomedical Analysis, 136*, 22-31.

Lynch, T., & Price, A. (2007). The Effect of Cytochrome P450 Metabolism on Drug Response, Interactions, and Adverse Effects. *American Family Physician, 76*(3), 391-395.

Marqui, A. B. (2015). Turner syndrome and genetic polymorphism: a systematic review. *Revista Paulista de Pediatia, 33*(3), 363-370.

Mast, N., Anderson, K. W., Johnson, K. M., Phan, T. T., Guengerich, F. P., & Pikuleva, I. A. (2017[a]). In vitro cytochrome P450 46A1 (CYP46A1) activation by neuroactive compounds. *Journal of Biological Chemistry, 292*(31), 12934-12946.

Mast, N., Lin, J. B., Anderson, K. W., Bjorkhem, I., & Pikuleva, I. A. (2017[b]). Transcriptional and post-translational changes in the brain of mice deficient in cholesterol removal mediated by cytochrome P450 46A1 (CYP46A1). *PLoS ONE, 12*(10), 1-24.

Matsuda, K. (2017). PCR-Based Detection Methods for Single-Nucleotide Polymorphism or Mutation: Real-Time PCR and Its Substantial Contribution Toward Technological Refinement. *Advances in Clinical Chemistry*, 45-72.

Maxfield, F. R., & Tabas, I. (2005). Role of cholesterol and lipid organization in disease. *Nature, 438*, 36-45.

Maxwell, K. F., Bhattacharya, S., Bodziak, M. L., Jakimovski, D., Hagemeier, J., Browne, R. W., & ... and Ramanathan, M. (2019). Oxysterols and Apolipoproteins in Multiple Sclerosis: A 5-year Follow-up Study. *Journal of Lipid Research*, 1-33.

Méndez-Acevedo, K. M., Valdes, V. J., Asanov, A., & Vaca, L. (2017). A novel family of mammalian transmembrane proteins involved in cholesterol transport. *Nature, 7*, 7450-7461.

Milo, R., & Kahana, E. (2010). Multiple sclerosis: Geoepidemiology, genetics and the environment. *Autoimmunity Reviews, 9*, 387-394.

Mohajeri, M., Farazmand, A., Bonab, M. M., Nikbin, B., & Minagar, A. (2011). FOXP3 Gene Expression in Multiple Sclerosis Patients Pre- and Post Mesenchymal Stem Cell Therapy. *Iran J Allergy Asthma Immunol, 10*(3), 155-161.

Molowa, D. T., Chen, W. S., Cimis, G. M., & Tan, C. P. (1992). Transcriptional Regulation of the Human Cholesterol 7«-Hydroxylase Gene. *Biochemistry, 31*, 2539-2544.

Monte, M. J., Marin, J. J., Antelo, A., & Vazquez-Tato, J. (2009). Bile acids: Chemistry, physiology, and pathophysiology. *World Journal of Gastroenterology, 15*(7), 804-816.

Mossböck, G., Weger, M., Faschinger, C., Schmut, O., Renner, W., Wedrich, A., & El-Shabrawi, Y. (2011). Role of cholesterol 24S-hydroxylase gene polymorphism (rs754203) in primary open angle glaucoma. *Molecular Vision, 17*, 616-620.

Mousavidehmordi, R., Babaahmadi, H., Shalbafan, B., Mohammadzadeh, G., Afsharmanesh, M., & Kheirollah, A. (2016). Polymorphism of CYP46A1

Gene and Alzheimer's Disease in the Iranian Population. *Shiraz E-Medical Journal, 17*(9), 1-5.

Mukhopadhyay, S., Fellows, K., Browne, R. W., Khare, P., Radhakrishnan, S. K., & .. and Ramanathan, M. (2017). Interdependence of Oxysterols with Cholesterol Profiles in Multiple Sclerosis. *Multiple Sclerosis, 23*(6), 792-801.

Munger, K. L., Levin, L. I., Hollis, B. W., Howard, N. S., & Ascherio, A. (2006). Serum 25-Hydroxyvitamin D Levels and Risk of Multiple Sclerosis. *Jama, 296*(23), 2832-.

Munro, A. W., McLean, K. J., Grant, J. L., & Makris, T. M. (2018). Structure and function of the cytochrome P450 peroxygenase enzymes. *Biochemical Society Transactions, 46*, 183-196.

Nakamoto, K., Wang, S., Jenison, R. D., Guo, G. L., Klaassen, C. D., Yvonne, W. Y.-J., & Zhong, X.-b. (2006). Linkage disequilibrium blocks, haplotype structure, and htSNPs of human CYP7A1 gene. *BMC Genetics, 7*(1), 29-40.

Narayanaswamy, R., Iyer, V., Khare, P., Bodziak, M. L., Badgett, D., (...), & Browne, R. W. (2015). Simultaneous Determination of Oxysterols, Cholesterol and 25-Hydroxy-Vitamin D3 in Human Plasma by LC-UV-MS. *PLoS ONE, 10*(4), 1-15.

Nebert, D. W., & Russell, D. W. (2002). Clinical importance of the cytochromes P450. *The Lancet, 360*, 1155-1162.

Ohyama, Y., Meaney, S., Heverin, M., Ekström, L., Brafman, A., (...), & Björkhem, I. (2006). Studies on the Transcriptional Regulation of Cholesterol 24-Hydroxylase (CYP46A1) Marked Insensitivity Toward Different Regulatory Axes. *The Journal of Biological Chemistry , 281*(7), 3810-3820.

Ontaneda, D., Thompson, A. J., Fox, R. J., & Cohen, J. A. (2017). Progressive multiple sclerosis: prospects for disease therapy, repair, and restoration of function. *Lancet, 389*, 1257-1366.

Oreja-Guevara, C., Ramos-Cejudo, J., Aroeira, L. S., Chamorro, B., & Diez-Tejedor, E. (2012). TH1/TH2 Cytokine profile in relapsing-remitting multiple sclerosis

patients treated with Glatiramer acetate or Natalizumab. *BMC Neurology, 12*(1), 1-6.

Ozgocmen, S., Bulut, S., Ilhan, N., Gulkesen, A., Ardıcoglu, O., & Ozkan, Y. (2005). Vitamin D deficiency and reduced bone mineral density in multiple sclerosis: effect of ambulatory status and functional capacity. *Journal of Bone and Mineral Metabolism, 23*, 309-313.

Papassotiropoulos, A., Streffer, J. R., Tsolaki, M., Schmid, S., Thal, D., Nicosia, F., . . . Hock, C. (2003). Increased brain beta-amyloid load, phosphorylated tau, and risk of Alzheimer disease associated with an intronic CYP46 polymorphism. *Archives of Neurology, 60*(1), 29-35.

Peelen, E., Knippenberg, S., Muris, A.-H., Thewissen, M., Smolders, J., Tervaert, J. W., . . . Damoiseaux, J. (2011). Effects of vitamin D on the peripheral adaptive immune system: A review. *Autoimmunity Reviews, 10*, 733-743.

Perez-Perez, S., Dominunguez-Mozo, M. G.-M., Aladro, Y., Martinez-Gines, M., (...), & Alvarez-Lafuente, R. (2018). Study of the possible link of 25-hydroxyvitamin D with Epstein–Barr virus and human herpesvirus 6 in patients with multiple sclerosis. *European Journal of Neurology*, 1-8.

Pfrieger, F. W., & Ungerer, N. (2011). Cholesterol metabolism in neurons and astrocytes. *Progress in Lipid Research, 50*, 357-371.

Pikuleva, I. A. (2006). Cholesterol-Metabolizing Cytochromes P450. *Drug Metabolism and Disposition, 34*, 513-520.

Polman, C. H., Reingold, S. C., Banwell, B., Clanet, M., Cohen, J. A., Filippi, M., . . . Wolinsky, J. S. (2011). Diagnostic Criteria for Multiple Sclerosis: 2010 Revisions to the McDonald Criteria. *ANNALS of Neurology, 69*, 292-302.

Przybek, J., Gniatkowska, I., Mirowska-Guzel, D., & Członkowska, A. (2015). Evolution of diagnostic criteria for multiple sclerosis. *Neurologia I Neurochirurgia Polska, 49*, 313-321.

Qrafli, M., Amar, Y., Bourkadi, J., Amor, J. B., Iraki, G., (...), & Sadki, K. (2014). The CYP7A1 gene rs3808607 variant is associated with susceptibility of tuberculosis in Moroccan population. *Pan African Medical Journal, 18*, 1-6.

Ramagopalan, S. V., Dobson, R., Meier, U. C., & Giovannoni, G. (2010). Multiple sclerosis: risk factors, prodromes, and potential causal pathways. *Lancet, 9*, 727-739.

Ramsuran, V., Ewy, R., Nguyen, H., & Kulkarni, S. (2018). Variation in the Untranslated Genome and Susceptibility to Infections. *Frontiers in Immunology, 9*, 2046-2053.

Riedhammer, C., & Weissert, R. (2015). Antigen presentation, autoantigens, and immune regulation in multiple sclerosis and other autoimmune diseases. *Frontiers in Immunology, 6*(322).

Rizvi, S., Raza, S. T., Mehdi, S. R., Siddiqi, Z., Eba, A., & Mahdi, F. (2017). The relationship between Multidrug Resistance Protein 1(rs1045642) and Cholesterol 24-hydroxylase (rs754203) genes polymorphism with type 2 diabetes mellitus. *British Journal of Biomedical Science, 74*(1), 30-35.

Rolf, L., Damoiseaux, J., Hupperts, R., Huitinga, I., & Smolders, J. (2016). Network of nuclear receptor ligands in multiple sclerosis: Common pathways and interactions of sex-steroids, corticosteroids and vitamin D3-derived molecules. *Autoimmunity Reviews*(15), 900-910.

Rosen, Y., Daich, J., Soliman, I., Brathwaite, E., & & Shoenfeld, Y. (2016). Vitamin D and autoimmunity. *Scandinavian Journal of Rheumatology, 45*(6), 439-447.

Sadovnick, A., Dyment, D., & Ebers, G. (1997). Genetic epidemiology of multiple sclerosis. *Epidemiol Rev., 19*(1), 99-106.

Santoro, M., Mirabella, M., De Fino, C., Bianco, A., Lucchini, M., Losavio, F., . . . Nociti, V. (2017). Sativex® effects on promotermethylation and on CNR1/CNR2 expression in peripheral blood mononuclear cells of progressive multiple sclerosis patients. *Journal of the Neurological Sciences, 379*, 298–303.

Sawcer, S., Franklin, R. J., & Ban, M. (2014). Multiple sclerosis genetics. *Lancet Neurol, 13*, 700-709.

Schroeder, H. W., & Cavacini, L. (2010). Structure and Function of Immunoglobulins. *J Allergy Clin Immunol., 125*, 41-52.

Sellner, J., Kraus, J., Awad, A., Milo, R., Hemmer, B., & Stüve, O. (2011). The increasing incidence and prevalence of female multiple sclerosis—A critical analysis of potential environmental factors. *Autoimmunity Reviews, 10*, 495-502.

Sena, A., Macedo, A., Ferret-Sena, V., Capela, C., & and Pedrosa, R. (2019). Serum Lipoprotein Profile Is Associated With Protective Effects of Oral Contraceptive Use on Multiple Sclerosis Severity: A Cross-Sectional Study. *Frontiers in Neurology, 10*(60), 1-7.

Shao, H., Stoecker, C., & Monnette, A. M. (2018). Health PolicyAnalysis Cost SharingofDisease-ModifyingTreatments(DMTs)asPolicy Lever toImproveDMTs' Access inMultipleSclerosis. *Value in Healty, 21*, 1083-1089.

Shen, J., Arnett, D. K., Parnell, L. D., Lai, C.-Q., Straka, R. J., Hopkins, P. N., . . . Ordovás, J. M. (2012). The effect of CYP7A1 polymorphisms on lipid responses to fenofibrate. *Journal of Cardiovascular Pharmacology, 59*(3), 254-259.

Silva, T. d., Silva, F. C., Gomes, A. O., Viana, A. O., Gonçalves, M. L., (...), . . . Rodrigues. (2018). Effect of photobiomodulation treatment in the sublingual, radial artery region, and along the spinal column in individuals with multiple sclerosis. *Medicine, 97*, 19-23.

Simpson, S., Taylor, B., Blizzard, L., Ponsonby, A.-L. P., Tremlett, H., & ..., M. I. (2010). Higher 25-hydroxyvitamin D Is Associated with Lower Relapse Risk in Multiple Sclerosis. *Annals of Neurology*, 1-11.

Sintzel, M. B., Rametta, M., & Reder, A. T. (2018). Vitamin D and Multiple Sclerosis: A Comprehensive Review. *Neurology and Therapy, 7*(1), 59-85.

Sobjanek, M., Zabłotna, M., Dobosz-Kawałko, M., Michajłowski, I., Mędrzycka-Dąbrowska, W., (...), & Sokołowska-Wojdyło, M. (2015). Polymorphisms in the cytochrome P-450 (CYP) 1A1 and 17 genes are not associated with acne vulgaris in the Polish population. *Postępy Dermatologii i Alergologii, 5*, 323-326.

Soilu-Hänninen, M., Airas, L., Mononen, I., Heikkilä, A., & Viljanen, M. a. (2005). 25-Hydroxyvitamin D levels in serum at the onset of multiple sclerosis. *Multiple Sclerosis Journal, 11*(3), 266-271.

Sospedra, M., & Martin, R. (2005). Immunology of Multiple Sclerosis. *Annual Review of Immunology, 23*(1), 683-747.

Srivastava, A., Pandey, S. N., Choudhuri, G., & Mittal, B. (2008). Role of genetic variant A-204C of cholesterol 7a-hydroxylase (CYP7A1) in susceptibility to gallbladder cancer. *Molecular Genetics and Metabolism, 94*, 83–89.

Sternberg, Z., Leung, C., Sternberg, D., Li, F., Karmon, Y., Chadha, K., & and Levy, E. (2013). The Prevalence of the Classical and Non-Classical Cardiovascular Risk Factors in Multiple Sclerosis Patients. *CNS & Neurological Disorders - Drug Targets, 12*(1), 104-111.

Stewart, N., Simpson, S., Mei, I. V., Ponsonby, A.-L., Blizzard, L., Dwyer, T., . . . Taylor, B. V. (2012). Interferon- and serum 25-hydroxyvitamin D interact to modulate relapse risk in MS. *Neurology, 79*(3), 254-260.

Tettey, P., Simpson, S., Taylor, B. V., & Mei, I. A. (2014). Vascular comorbidities in the onset and progression of multiple sclerosis. *Journal of the Neurological Sciences, 347*(1-2), 23-33.

Thompson, A. J., Banwell, B. L., Barkhof, F., Carroll, W. M., Coetzee, T., Comi, G., . . . Cohen, J. A. (2018). Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurology, 17*, 162-173.

Tiwari, S., Lapierre, J., Ojha, C. R., Martins, K., Parira, T., (...), & El-Hage, N. (2018). Signaling pathways and therapeutic perspectives related to environmental factors associated with multiple sclerosis. *Journal of Neuroscience Research*, 1-16.

Tracey, T. J., Steyn, F. J., Wolvetang, E. J., & Ngo, S. T. (2018). Neuronal Lipid Metabolism: Multiple Pathways Driving Functional Outcomes in Health and Disease. *Frontiers in Molecular Neuroscience, 11*, 1-25.

Valenza, M., & Cattaneo, E. (2011). Emerging roles for cholesterol in Huntington's disease. *Trends in Neurosciences, 34*(9), 474-486.

Vera, D., & Ribeiro, V. (2011). *Journal of Pharmacy and Bioallied Sciences, 3*(3), 453-459.

Vitali, C., Wellington, C. L., & Calabresi, L. (2014). HDL and cholesterol handling in the brain. *Cardiovascular Research, 103*, 405-413.

Vlachová, M., Blahová, T., Lánská, V., Leníček, M., Piťha, J., Vítek, L., & Kovář, J. (2016). Diurnal variation in cholesterol 7α-hydroxylase activity is determined by the -203A>C polymorphism of the CYP7A1 gene. *Croatian Medical Journal, 57*(2), 111-117.

Wang, J., Freeman, D. J., Grundy, S. M., Levine, D. M., Guerra, R., & Cohen, J. C. (1998). Linkage between cholesterol 7alpha-hydroxylase and high plasma low-density lipoprotein cholesterol concentrations. *Journal of Clinical Investigation, 101*(6), 1283-1291.

Wang, T.-T., Tavera-Mendoza, L. E., Laperriere, D., Libby, E., MacLeod, N. B., (...), & White, J. H. (2005). Large-Scale in Silico and Microarray-Based Identification of Direct 1,25-Dihydroxyvitamin D3 Target Genes. *Molecular Endocrinology, 19*(11), 2685–2695.

Wang, Y., Harding, S. V., Eck, P., Thandapilly, S. J., Gamel, T. H., Abdel-Aal, E.-S. M., . . . Ames, N. P. (2016). High-Molecular-Weight ß-Glucan Decreases Serum Cholesterol Differentially Based on the CYP7A1 rs3808607 Polymorphism in Mildly Hypercholesterolemic Adults. *The Journal of Nutrition - Nutrition and Disease, 146*(4), 720-727.

Weinstock-Guttman, B., Zivadinov, R., & Ramanathan, M. (2011). Weinstock-Guttman et al., 2011[a]). Inter-dependence of vitamin D levels with serum lipid profiles in multiple sclerosis. *Journal of the Neurological Sciences, 311*, 86-91.

Weinstock-Guttman, B., Zivadinov, R., Mahfooz, N., Carl, E., Drake, A., (...), & Ramanathan, M. (2011[b]). Serum lipid profiles are associated with disability and MRI outcomes in multiple sclerosis. *Journal of Neuroinflammation, 8*, 127-133.

Weng, S.-L., Wu, W.-J., Hsiao, Y.-H., Yang, S.-F., Hsu, C.-F., & Wang, P.-H. (2018). Significant association of long non-coding RNAs HOTAIR genetic polymorphisms with cancer recurrence and patient survival in patients with uterine cervical cancer. *International Journal of Medical Sciences, 15*(12), 1312-1319.

Wergeland, S., Torkildsen, Ø., Myhr, K.-M., Aksnes, L., Mørk, S. J., & Bø, L. (2011). Dietary Vitamin D3 Supplements Reduce Demyelination in the Cuprizone Model. *PloS ONE, 6*(10), 1-8.

Wertheim, B. C., Smith, J. W., Fang, C., Alberts, D. S., Lance, P., & Thompson, P. A. (2012). Risk modification of colorectal adenoma by CYP7A1 polymorphisms and the role of bile acid metabolism in carcinogenesis. *Cancer Prev Res (Phila), 5*(2), 197-204.

Xiang, X., Backman, J. T., Neuvonen, P. J., & Niemi, M. (2012). Gender, but not CYP7A1 or SLCO1B1 Polymorphism, Affects the Fasting Plasma Concentrations of Bile Acids in Human Beings. *Basic & Clinical Pharmacology & Toxicology, 110*, 245-252.

Yamamoto, R., Muroi, K., & Imaishi, H. (2018). Serum derived from ulcerative colitis mouse changes the metabolism of the fluorescent substrate by P450 depending on the degree of disease progression. *Chemico-Biological Interactions, 290*, 88-98.

Yamauchi, Y., & Rogers, M. A. (2018). Sterol Metabolism and Transport in Atherosclerosis and Cancer. *Frontiers in Endocrinology, 9*, 509-560.

Zaborska, K. E., & Cummings, B. P. (2018). Rethinking Bile Acid Metabolism and Signaling for Type 2 Diabetes Treatment. *Current Diabetes Reports, 18*, 109-118.

Zahoor, I., & Haq, E. (2017). Vitamin D and Multiple Sclerosis: An Update. In I. S. Zagon, & P. J. Mclaughlin, *Multiple Sclerosis Perspectives in Treatment and Pathogenesis* (pp. 71-84). Hershey, Pennsylvania, USA.

Zhang, X., Lv, C., An, Y., Liu, Q., Rong, H., (...), & Xiao, R. (2018). Increased Levels of 27-Hydroxycholesterol Induced by Dietary Cholesterol in Brain Contribute

to Learning and Memory Impairment in Rats. *Molecular Nutrition Food Research, 62*(3), 1-10.

Ziemssen, T. (2005). Modulating processes within the central nervous system is central to therapeutic control of multiple sclerosis. *Journal of Neurology, 252*, 38-45.

APPENDICES

A. INFORMED CONSENTS

A.1. INFORMED CONSENTS FOR PATIENTS

Multipler Skleroz (MS) hastalığı ile ilişkili olabileceğini düşündüğümüz iki gen üzerine araştırma yapmaktayız. Çalışmamızın adı "Türk Popülasyonunda Kolesterol 7-alfa Hidroksilaz (CYP7A1) ve Kolesterol 24-Hidroksilaz (CYP46A1) gen polimorfizimleri ile MS riski ilişkilerinin incelenmesi" olup sizin de bu çalışmaya katılmanızı öneriyoruz. Araştırmaya katılmak tamamen gönüllülük esasına dayalıdır ve nihai karar size aittir. Kararınızdan önce sizi araştırmamız hakkında bilgilendirmek istiyoruz. Bu bilgileri okuyup anladıktan sonra, çalışmamıza katılmak isterseniz formu imzalayınız. Araştırmaya davet edilmenizin nedeni sizde bu hastalığın bulunmasıdır. Size gerekli tetkikler yaptıktan sonra, bu hastalık için kabul görmüş klasik bir tedaviye başlayacağız. Eğer araştırmamıza katılmayı kabul ederseniz Prof. Dr. Şeref Demirkaya, Uz. Dr. Nuriye Bayraklı, Uz. Dr. Nedime Tuğçe Bilbay, ya da Uz. Dr. Semih Alay tarafından muayene edilecek ve bulgularınız kaydedilecektir. Bu çalışmayı yapabilmek için kolunuzdan 10 ml (2 tüp) kadar kan almamız gerekmektedir ve bu kanlardan araştırmamızda kullanılacak tetkikler çalışılacaktır. Kan alımı hastalığınız klinik takibi sırasında iken 2 tüp fazladan alınarak yapılacağından, size ekstra bir işlem yapılmayacaktır. Çalışmamıza katıldığınız için sizden herhangi bir ücret istenmeyecek ve size ek bir ödeme de yapılmayacaktır.

Yapılacak araştırmanın getireceği olası yararlar:

Çalışmamızda, iki gen bölgesinin, MS hastalığı üzerinde risk faktörü olarak etki edip etmediğini ve MS ile ilişkisi olduğu düşünülen lipit profil ve Vitamin D değerlerinin de seçilen bu gen bölgeleri ile olan ilişkilerinin belirlenmesi amaçlanmaktadır. Şu anda bu çalışmanın hemen size bir fayda sağlayıp sağlamayacağını bilmiyoruz; fakat ilgili hastalığın temelinde yatan nedenlerin öğrenilmesinde ve gelecekte yeni tedavi

yaklaşımlarının geliştirilmesinde; bu hastalığın ortaya çıkabileceği kişileri önceden tespit edilmesinde ve hastalığın oluşumundan önce gerekli önlemlerin alınmasında fayda sağlayabileceğini düşünüyoruz. Bu çalışmaya katılmayı reddedebilirsiniz. Araştırmaya katılmak tamamen isteğe bağlıdır. Reddetmeniz halinde, size uygulanan tedavide ya da size karşı tavırlarımızda herhangi bir değişiklik olmayacaktır. Kabul etseniz bile, çalışmanın herhangi bir aşamasında onayınızı çekme hakkında sahip olduğunuzu da belirtmek isteriz.

Hastanın Beyanı:

Sayın Prof. Dr. Şeref Demirkaya ve ekibi tarafından Gülhane Eğitim ve Araştırma Hastanesi, Nöroloji Anabilim Dalı'nda tıbbi bir araştırma yapılacağı belirtilerek, bu araştırma ilgili yukarıdaki bilgiler bana aktarıldı. Bu bilgilerden sonra, böyle bir araştırmaya "katılımcı" olarak davet edildim.

Eğer bu araştırmaya katılırsam, hekim ile aramda kalması gereken bana ait bilgilerin gizliliğine bu araştırma sırasında da büyük özen ve saygı ile korunacağına inanıyorum. Araştırma sonuçlarının eğitim ve bilimsel amaçlarla kullanımı sırasında kişisel bilgilerimin ihtimamla korunacağı konusunda bana yeterli güven verildi.

Araştırma için yapılacak harcamalarla ilgili herhangi bir parasal sorumluluk altına girmiyorum. Bana da bir ödeme yapılmayacaktır. İster doğrudan ister dolaylı olsun, araştırma uygulamasından kaynaklanan nedenlerle meydana gelebilecek herhangi bir sağlık sorunumun ortaya çıkması halinde, her türlü tıbbi müdahalenin sağlanacağı konusunda gerekli güvence verildi (Bu tıbbi müdahalelerle ilgili olarak da parasal bir yük altına girmeyeceğim).

Bu araştırmaya katılmak zorunda değilim ve katılmayabilirim. Araştırmaya katılmam konusunda da zorlayıcı bir davranışla karşılaşmış değilim. Eğer katılmayı reddedersem, bu durumun tıbbi bakımıma ve hekim ile olan ilişkime herhangi bir zarar getirmeyeceğini de biliyorum.

Bana yapılan tüm açıklamaları ayrıntılarıyla anlamış bulunmaktayım. Kendi başıma belli bir düşünme süresi sonunda, adı geçen bu projede "katılımcı" olarak yer alma kararımı aldım. Bu konuda yapılan daveti büyük bir memnuniyet ve gönüllülük içerisinde kabul ediyorum.

Katılımcı:	**Görüşme Tanığı:**
Adı-Soyadı:	Adı-Soyadı:
Adres:	Adres:
Tel:	Tel
İmza:	İmza:

Katılımcı ile görüşen hekim:

Adı-Soyadı-Unvanı:

Adres:

Tel:

İmza:

A.2. INFORMED CONSENT FOR CONTROLS

Türk Popülasyonunda Kolesterol 7-alfa Hidroksilaz (CYP7A1) ve Kolesterol 24-Hidroksilaz (CYP46A1) gen polimorfizimleri ile MS riski ilişkilerinin incelenmesi" isimli çalışmamızda iki gen bölgesinin, MS üzerinde risk faktörü olarak etki edip etmediğinin belirlenmesi, bununla birlikte MS ile ilişkisi olduğu düşünülen lipit profil ve Vitamin D değerlerinin de seçilen bu gen bölgeleri ile olan ilişkilerinin, MS hasta ve sağlıklı kontrollerle de kıyaslaması yapılarak araştırılması amaçlanmaktadır. Tamamen gönüllülük esasına dayanan bu işlem için, sizden iki tüp kan (10 ml) alınacaktır, bunun haricinde size ek bir işlem yapılmayacaktır. Katılıp katılmama konusunda tamamen serbestsiniz. Katılmanız durumunda, bilgilerinizin gizliliği konusunda güvende olacağınızı belirtmek isteriz. Çalışmaya katılmanızdan dolayı sizden herhangi bir ücret talep edilmemekte ya da size herhangi bir ücret verilmemektedir.

Çalışmada yer aldığınız ve bilimsel gelişmelere katkılarınızdan dolayı teşekkür ediyoruz.

Çalışmaya Katılan Gönüllünün;

Adı-Soyadı:

Adres:

Tel:

İmza:

Açıklamayı Başından Sonuna Kadar Tanıklık Eden Kişinin;

Adı-Soyadı:

Adres:

Tel:

İmza:

Açıklamayı Yapan Araştırmacının;

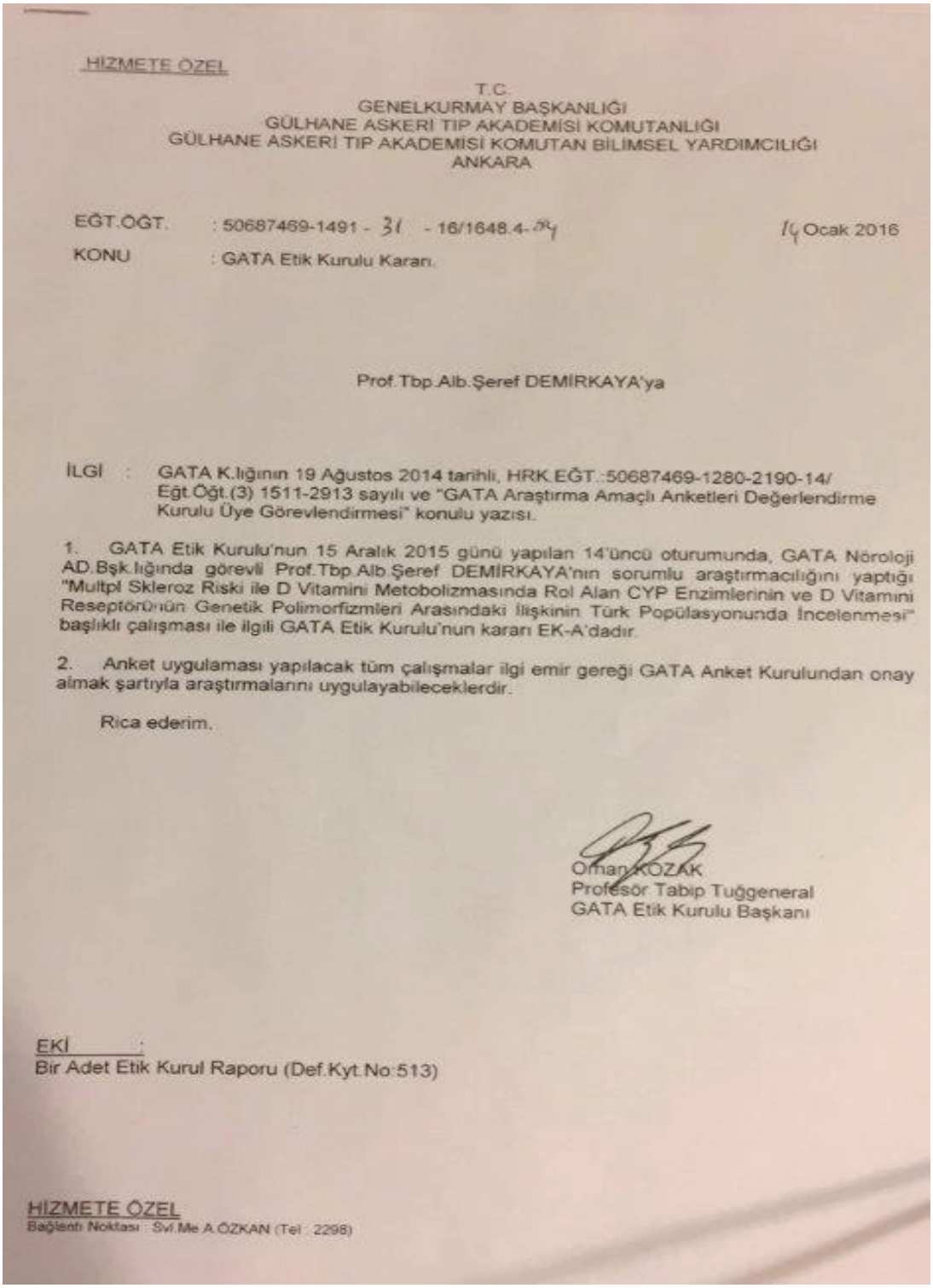
Adı-Soyadı:

Adres:

Tel:

İmza:

B. ETHICAL COMMITTEE APPROVAL FORM



HİZMETE ÖZEL

T.C.
GENELKURMAY BAŞKANLIĞI
GÜLHANE ASKERİ TIP AKADEMİSİ KOMUTANLIĞI
GÜLHANE ASKERİ TIP AKADEMİSİ KOMUTAN BİLİMSEL YARDIMCILIĞI
ANKARA

EĞT.ÖĞT. : 50687469-1491 - 31 - 16/1648.4-84 14 Ocak 2016

KONU : GATA Etik Kurulu Kararı.

Prof.Tbp.Alb.Şeref DEMİRKAYA'ya

İLGİ : GATA K.lığının 19 Ağustos 2014 tarihli, HRK.EĞT.:50687469-1280-2190-14/ Eğt.Öğt.(3) 1511-2913 sayılı ve "GATA Araştırma Amaçlı Anketleri Değerlendirme Kurulu Üye Görevlendirmesi" konulu yazısı.

1. GATA Etik Kurulu'nun 15 Aralık 2015 günü yapılan 14'üncü oturumunda, GATA Nöroloji AD.Bşk.lığında görevli Prof.Tbp.Alb.Şeref DEMİRKAYA'nın sorumlu araştırmacılığını yaptığı "Multpl Skleroz Riski ile D Vitamini Metobolizmasında Rol Alan CYP Enzimlerinin ve D Vitamini Reseptörü'nün Genetik Polimorfizmleri Arasındaki İlişkinin Türk Popülasyonunda İncelenmesi" başlıklı çalışması ile ilgili GATA Etik Kurulu'nun kararı EK-A'dadır.

2. Anket uygulaması yapılacak tüm çalışmalar ilgi emir gereği GATA Anket Kurulundan onay almak şartıyla araştırmalarını uygulayabileceklerdir.

Rica ederim.

Orhan KOZAK
Profesör Tabip Tuğgeneral
GATA Etik Kurulu Başkanı

EKİ :
Bir Adet Etik Kurul Raporu (Def.Kyt.No:513)

HİZMETE ÖZEL
Bağlantı Noktası : Sv.Me.A.ÖZKAN (Tel : 2298)

HİZMETE ÖZEL

T.C.
GENELKURMAY BAŞKANLIĞI
GÜLHANE ASKERİ TIP AKADEMİSİ KOMUTANLIĞI
ETİK KURUL TOPLANTI RAPORU

OTURUM NO : 14
OTURUM TARİHİ : 15 Aralık 2015
DEFTER KAYIT NO : 513
OTURUM BAŞKANI : Prof. Hv. Tbp. Tuğg. Orhan KOZAK
OTURUM SEKRETERİ : Prof. Tbp. Tuğg. Bülent BEŞİRBELLİOĞLU

GATA Etik Kurulu'nun 15 Aralık 2015 günü yapılan 14'üncü oturumunda, GATA Nöroloji AD Bşk.lığında görevli Prof.Tbp.Alb.Şeref DEMİRKAYA'nın sorumlu araştırmacılığını yaptığı "Multpl Skleroz Riski ile D Vitamini Metobolizmasında Rol Alan CYP Enzimlerinin ve D Vitamini Reseptörünün Genetik Polimorfizmleri Arasındaki İlişkinin Türk Popülasyonunda İncelenmesi" başlıklı çalışması değerlendirildi.

Araştırma dosyasının amaç, yöntem ve yaklaşım bakımından etik ilkelere UYGUN olduğuna karar verildi.

BAŞKAN — Orhan KOZAK, Prof.Hv.Tbp.Tuğg.

ÜYE — Bülent BEŞİRBELLİOĞLU, Prof.Tbp.Tuğg.

ÜYE — Necdet Doğan, Prof.Diş.Tbp.Alb.

ÜYE — Metin HASDE, Prof.Tbp.Alb.

ÜYE — Bülent GÜLEÇ, Prof.Tbp.Alb.

ÜYE — Puat TOSUN, Prof.Hv.Tbp.Alb.

ÜYE — Mahir GÜLEÇ, Prof.Tbp.Alb.

ÜYE — Ömer DENİZ, Prof.Tbp.Alb.

ÜYE — Suzi DEMİRBAĞ, Prof.Tbp.Alb.

ÜYE — Ahmet KORKMAZ, Prof.Hv.Tbp.Alb.

ÜYE — Harun TUĞCU, Prof.Tbp.Alb.

ÜYE — Muharrem UÇAR, Doç.Dr.J.Tbp.Alb.

ÜYE — Emine IYIGÜN, Doç.Dr.Hv.Sağ.

HİZMETE ÖZEL

C. REAGENTS

I. The preparation of reagents used in DNA Isolation

GenJET whole blood genomic DNA purification mini kit (#K0782) was used for DNA isolation and all reagents used for that purpose was included in kit. However, adding of ethanol (96-100 %) to Wash Buffer I and Wash Buffer II should be done prior to first used them. 120 ml Ethanol (96-100 %) was added to each concentrated wash solution (40 ml).

II. The reagents used in PCR

1. PCR Amplification Buffer (10X)

10X *Taq* Buffer with $(NH_4)_2SO_4$ was used in PCR and it was obtained commercially with the *Taq* DNA Polymerase (Thermo Scientific #EP0402) and 750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20. It is stored at -20°C.

2. dNTP Mixture

The solution was obtained commercially (Thermo Scientific #R0181) and included 100 mM of dATP, dGTP, dCTP, dTTP in aqueous solution. 10 mM dNTP mix was prepared by taking 10 µl from each of them and volume was arranged to 100 µl with 60 µl of nuclease-free water. It was stored at -20°C.

3. 25 mM $MgCl_2$

The solution was obtained commercially with the *Taq* DNA Polymerase (Thermo Scientific #EP0402) and was stored at -20°C.

III. The reagents used in Agarose Gel Electrophoresis

1. 5X TBE Buffer (Tris-Borate-EDTA, pH 8.3, 1000 ml)

54 g Trizma-Base, 27.5 g boric acid and 20 µl of 500 mM EDTA were mixed in a beaker and dissolved appropriate amount of distilled water. pH was adjusted to 8.3 and volume was completed to 1000 ml. After preparations, the solution was autoclaved.

2. Ethidium Bromide

0.1 g ethidium bromide was dissolved in 10 ml distilled water. The solution was stirred on magnetic stirrer for several hours to ensure that dye was completely dissolved. Due to the light sensitive characteristic of the solution, the bottle was covered with aluminum foil and stored at room temperature.

IV. The reagent used in restriction endonuclease digestion

1. 1X CutSmart Buffer

The buffer was obtained commercially with restriction enzymes Bsa I and Msp I. The buffer included 50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 100 µg/ml BSA pH 7.9. The solution was stored at -20°C.

D. LIST OF STUDY POPULATION

Table D.1: List of study population composed of 191 Multiple Sclerosis patients and 100 controls including demographic information, lipid parameters, allelic discrimination results of CYP7A1 rs3808607 and CYP46A1 rs754203 SNPs. The abbreviations mean:

P: Patient; C: Control; F: Female; M: Male; LDL-C: Low-Density Lipoprotein Cholesterol; HDL-C: High-Density Lipoprotein Cholesterol; TC: Total Cholesterol; TG: Triglycerides; Vit D: Vitamin D; AA: Wild type homozygous for rs3808607 polymorphism; AC: Heterozygous for rs3808607 polymorphism; CC: Homozygous mutant for rs3808607 polymorphism; TT: Wild type homozygous for rs754203 polymorphism; TC: Heterozygous for rs754203 polymorphism; CC: Homozygous mutant for rs754203 polymorphism.

Sample No	Patient/Control	CYP7A1 rs3808607 A:C	CYP46A1 rs754203 T:C	Sex	Age	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	Vitamin D (nmol/L)
1	P	AA	TC	F	19					17.39
2	P	AC	TT	F	20	4.22	0.63			9.98
3	P	AC	TT	F	20	4.35	1.49	1.19	2.49	35.69
4	P	AA	TT	F	21					
5	P	AC	TT	F	22	3.34	0.63	1.32	1.74	10.68
6	P	AC	TC	F	22	4.30	1.19	1.27	2.49	151.76
7	P	AC	TT	F	23	3.26	0.93	1.17	1.68	153.20
8	P	AC	TT	F	24	4.14	1.64	0.96	2.43	29.45
9	P	AA	TC	F	24	5.88	1.41	1.79	3.44	13.70
10	P	AA	TC	M	25	3.37	0.57	1.17	1.94	
11	P	AA	TT	F	25					
12	P	AC	TT	F	25	3.99	0.38	1.58	2.23	67.14
13	P	CC	TC	M	25	4.40	0.67	1.68	2.41	
14	P	AC	TT	M	25	2.38	0.97	1.01	0.93	91.90
15	P	AC	CC	F	26	4.58	1.29	1.81	2.18	51.69

Table D.1 (Continued)

16	P	AC	TT	M	26	3.13	0.61	1.61	1.24	50.92
17	P	AA	TT	F	26	3.96	1.14	1.06	2.38	35.44
18	P	AC	TC	F	27					
19	P	AC	TT	F	27	2.62	0.58	1.26	1.09	
20	P	AA	TT	F	27	4.79	0.33	1.42	3.21	
21	P	AA	TT	M	27	4.92	1.07	1.09	3.34	
22	P	AC	TT	M	27	4.58	0.84	0.96	3.24	21.22
23	P	CC	TT	F	28	4.90	1.51	1.27	2.93	
24	P	AA	TT	F	28					
25	P	AC	TT	F	28	4.74	0.35			
26	P	AC	TT	M	28	5.23	0.98	1.35	3.44	
27	P	AA	TC	F	29	4.04	0.75	1.14	2.56	12.48
28	P	AC	TT	F	29					38.19
29	P	AC	CC	F	30	3.21	0.75	1.19	1.68	31.45
30	P	AA	TT	F	30	3.19	0.78	1.22	1.61	
31	P	AC	TT	F	30	4.14	0.37	1.40	2.56	
32	P	AC	TC	M	30	3.73	0.73	1.40	1.92	30.95
33	P	AA	CC	F	30	3.76	1.01	0.96	2.25	21.96
34	P	AC	TC	F	30					
35	P	AA	TT	F	30	4.38	1.40			
36	P	CC	TT	F	30	5.23	0.51	1.71	3.29	125.72
37	P	AC	TC	M	31	6.06	1.67	1.09	4.27	37.96
38	P	AA	TT	M	31	4.20	0.72	0.98		
39	P	AC	TC	F	31					17.10
40	P	AC	TC	F	32	5.83	0.37	1.66	3.99	76.73
41	P	AC	TC	F	32	4.33	0.69	1.74	2.28	54.66
42	P	AC	TC	M	32	5.46	1.95	1.09	3.47	37.19
43	P	CC	TT	F	32	4.87	1.36	1.19	3.06	128.79
44	P	AC	CC	F	33	4.53	1.15	1.68	2.33	67.22
45	P	AA	TC	F	33					
46	P	AA	TC	F	33					
47	P	AC	TC	F	34	4.51	1.33	1,24	2.64	15.28

Table D.1 (Continued)

48	P	AC	TC	M	34	5.26	1.51	1,09	3.47	
49	P	AC	TC	F	34					
50	P	AC	TC	F	34					
51	P	AC	TT	F	34	3.73	0.85	1.48	1.86	24.56
52	P	AC	TT	F	34		0.67			
53	P	AC	TC	F	34	4.53	0.94	1.27	2.82	
54	P	AA	TT	F	35	3.78	0.62	1.19	2.31	60.15
55	P	AC	TC	F	35	5.00	1.85	1.27	2.90	22.46
56	P	AA	TT	F	35	3.70	0.88	1.61	1.68	35.19
57	P	AA	TC	F	35					78.87
58	P	AC	TC	M	35	4.12	0.97	1.17	2.51	
59	P	AC	TC	F	35					
60	P	AC	TT	F	35	5.39	1.28	1.04	3.76	
61	P	AC	TC	F	35					
62	P	AC	TT	M	35	3.11	2.06	1.09	1.09	54.66
63	P	AC	TT	F	35	4.87	0.99	1.89	2.51	41.38
64	P	AA	CC	M	36	5.36	2.62	1.30	2.87	75.63
65	P	AC	TC	F	36	6.19	1.05	1.45	4.25	
66	P	AA	TC	F	36	4.48	1.20	1.94	1.99	15.97
67	P	AA	TC	F	36	4.40	1.29	0.98	2.82	43.13
68	P	AC	TT	M	36	4.43	0.99	1.22	2.75	133.39
69	P	AC	TT	F	36	4.35	0.99	1.76	2.69	27.03
70	P	AA	CC	M	36	4.77	2.49	0.98	2.64	24.71
71	P	AA	TT	F	36	4.33	0.77	0.98	2.98	
72	P	AC	TT	F	36	3.83	0.93	1.06	2.36	60.90
73	P	AC	TT	F	37	5.62	0.97	1.74	3.44	22.31
74	P	AC	TT	M	37	6.58	2.00			108.83
75	P	AC	TT	M	37	4.27	1.63			
76	P	AA	TT	F	37	3.83	0.46	1.53	2.10	33.20
77	P	AA	TC	F	37	4.61	1.39	1.01	2.95	
78	P	AC	TT	F	37	4.53	1.42	1.40	2.49	84.99
79	P	AC	TT	F	37	4.87	0.68	2.12	2.43	7.94

Table D.1 (Continued)

80	P	AA	TC	F	38	4.92	0.47	1.55	3.16	
81	P	AA	TT	M	38	4.56	1.62			31.45
82	P	AC	TT	F	38	7.85	1.68	1.76	5.31	76.78
83	P	AA	TC	M	38	7.33	2.34	1.30	4.97	46.92
84	P	AA	TC	M	38					
85	P	AA	TT	M	38	3.63	1.10	0.83	2.31	15.50
86	P	AA	TC	M	38	4.25	1.73	0.96	2.49	61.50
87	P	AA	TC	F	38	5.10	1.95	0.93	3.26	
88	P	AC	TT	F	38					
89	P	AC	TC	M	38	4.77	0.93	1.19	3.16	
90	P	AC	TT	F	38					36.44
91	P	AC	TC	F	39	4.30	0.99	1.30	2.54	20.97
92	P	CC	TC	F	39	3.86	2.03	1.01	1.92	
93	P	AA	TC	F	39					47.67
94	P	AC	TC	M	39	5.34	1.63	1.32	3.26	47.17
95	P	AC	TC	F	39	4.95	0.76	1.24	3.36	21.07
96	P	AC	TT	M	40	5.78	0.50	1.50	4.04	168.73
97	P	AA	TC	F	40	4.51	1.68	0.93	2.80	75.13
98	P	AC	TC	M	40					
99	P	AC	TC	F	40	3.08	0.54	0.96	1.86	106.58
100	P	AA	TT	M	40	4.33	1.06	1.14	2.69	71.88
101	P	AA	TC	F	40	4.95	0.86	1.89	2.67	
102	P	AA	TT	F	40	4.74	1.62	1.55	2.43	7.49
103	P	CC	TC	F	40	6.86	2.27			12.48
104	P	AC	TT	F	40	3.81	2.20	1.09	1.71	
105	P	CC	TT	F	41	4.95	1.34	1.27	3.06	44.43
106	P	AC	TT	F	41					68.39
107	P	AC	TC	F	41	4.22	1.04	1.06	2.69	82.94
108	P	AC	TC	F	42	4.77	0.70	1.92	2.54	49.67
109	P	AC	TT	M	42	7.15	1.74	1.45	4.90	50.92
110	P	AC	TT	F	42	4.97	0.86	1.71	2.87	23.81
111	P	AA	TC	M	42	5.57	2.84	1.30	2.98	

Table D.1 (Continued)

112	P	AA	TT	M	42	3.89	0.95	0.93	2.51	69.39
113	P	AC	TT	F	42	4.84	0.89			23.14
114	P	AC	TT	M	42	4.27	1.62	0.88	2.64	54.49
115	P	AA	CC	F						
116	P	AC	CC	M						
117	P	AC	TC	M						
118	P	CC	TT	F						
119	P	AA	TT	F						
120	P	AC	CC	M						
121	P	AC	TC	M						
122	P	AC	TC	F						97.52
123	P	AC	TT	M						
124	P	CC	TT	F						
125	P	AA	TC	F						
126	P	AA	TC	F						
127	P	AC	TC	F						
128	P	CC	TT	F						
129	P	AC	TC	F						
130	P	AC	TC	F						
131	P	AC	CC	M						
132	P	AC	TT	M						
133	P	AC	TC	F						
134	P	AA	TT	F						
135	P	AC	TT	M						
136	P	AA	TT	M						
137	P	AC	TC	M						
138	P	AC	TT	F						
139	C	AA	TT	F	43	6.81	1.47	1.40	4.74	20.79
140	C	AC	TT	M	37	0.95	1.76	1.45	5.05	91.73
141	C	AA	TT	F	38	3.91	2.20	0.88	2.02	24.44
142	C	AA	TT	F	37	5.00	1.79	1.37	2.80	38.64
143	C	AA	TC	F	35	4.30	1.06	1.11	2.69	45.58

Table D.1 (Continued)

144	C	AC	TT	F	43	3.76	1.89	0.83	2.07	25.76
145	C	AA	TT	F	54	4.66	0.82	1.63	2.64	17.12
146	C	AC	TC	F	35	5.02	1.32	1.32	3.11	69.46
147	C	CC	TC	F	26	4.82	0.85	1.27	3.16	65.57
148	C	AA	TT	M	26	4.45	0.81	1.89	2.20	82.99
149	C	CC	TT	F	23	3.83	0.71	1.48	2.02	27.16
150	C	AA	TC	F	20	3.19	0.70	1.09	1.79	42.66
151	C	AA	TT	F	24	6.03	2.28	1.94	3.06	89.68
152	C	AC	TC	F	27	5.15	0.92	1.81	2.93	86.39
153	C	AA	TT	F	28	5.08	0.84	1.66	3.03	47.17
154	C	AC	TT	F	25	3.78	1.54	1.42	1.66	22.11
155	C	AA	CC	M	36	4.56	2.40	1.24	2.23	67.94
156	C	AC	TT	F	29	3.78	1.39	1.24	1.89	54.34
157	C	AC	TC	F	24	4.61	1.13	1.37	2.72	47.75
158	C	AC	CC	F	35	5.00	2.88	1.27	2.41	105.96
159	C	AC	TT	F	32	4.87	0.88	1.45	3.00	58.01
160	C	AC	TT	F	24	3.63	1.21	1.11	1.97	29.83
161	C	AC	TC	F	35	8.39	0.75	1.32	6.73	98.34
162	C	AC	TC	F	59	5.54	2.97	1.11	3.06	78.42
163	C	AC	TT	F	25	5.26	0.50	1.61	3.42	76.88
164	C	AA	TC	F	47	7.59	4.76	1.09	5.15	90.08
165	C	AC	TC	F	26	4.64	0.71	2.05	2.25	46.40
166	C	AC	CC	M	30	5.96	1.58	1.35	3.89	48.35
167	C	AC	TT	F	24	5.05	0.66	1.48	3.26	78.95
168	C	AC	TT	F	24	3.42	0.79	1.24	1.81	52.02
169	C	AA	CC	M	24	4.97	1.15	1.35	3.11	187.70
170	C	AA	TC	F	19	4.43	1.13	1.19	2.72	65.27
171	C	AC	TC	F	25	3.86	0.95	1.22	2.20	40.16
172	C	AC	TT	M	33	6.53	1.92	1.35	4.30	42.13
173	C	AA	TC	M	19	3.55	0.69	0.91	2.33	76.63
174	C	AC	TC	M	37	4.92	1.20	1.17	3.21	68.96
175	C	AA	CC	F	39	5.23	1.51	1.35	3.19	22.29

Table D.1 (Continued)

176	C	AC	TT	F	22	3.19	0.42	1.27	1.74	49.10
177	C	AC	TT	M	27	4.79	1.56	1.11	2.95	38.86
178	C	AC	TT	M	52	6.55	1.55	1.27	4.58	66.19
179	C	AC	TT	F	19	3.91	0.99	1.24	2.20	65.97
180	C	AA	TT	F	19	5.57	2.97	0.88	3.32	47.00
181	C	AC	CC	F	29	3.86	0.90	1.24	2.20	15.75
182	C	AC	TT	F	36	4.04	1.19	1.19	2.31	66.97
183	C	AC	TT	F	24	4.90	0.53	1.48	3.19	65.35
184	C	AC	TT	M	32	4.17	3.32	1.06	1.58	80.37
185	C	AC	TC	M	39	4.64	2.61	0.85	2.59	52.22
186	C	AA	TC	M	32	5.72	0.79	1.71	3.65	46.43
187	C	AC	TC	F	34	5.36	0.76	1.19	3.83	44.10
188	C	AA	TC	M	33	5.34	0.89	1.04	3.89	89.48
189	C	AA	TT	M	38	4.66	0.82	0.93	3.34	40.54
190	C	AC	CC	F	39	5.26	0.67	1.55	3.39	58.08
191	C	AA	TC	M	32	5.36	3.23	1.09	2.80	65.02
192	C	CC	TT	M	38	4.84	0.89	1.11	3.32	79.70
193	C	AA	TT	F	27	5.21	0.78	1.71	3.13	18.32
194	C	AC	TT	M	30	5.28	2.50	1.09	3.06	36.12
195	C	AA	TT	M	30	4.64	2.64	1.09	2.33	87.48
196	C	CC	TC	F	28	6.48	1.39	2.36	3.47	68.66
197	C	AC	TC	M	33	6.60	1.44	1.14	4.82	43.06
198	C	CC	TT	M	34	3.78	0.47	1.27	2.31	25.81
199	C	AA	TT	M	24	4.95	1.36	1.04	3.29	53.84
200	C	AC	TT	M	42	4.38	1.73	1.09	2.49	24.59
201	C	AA	TT	M	38	5.05	1.47	1.04	3.34	66.84
202	C	AC	TT	M	36	5.46	1.44	1.01	3.81	99.37
203	C	AA	TT	M	29	4.17	0.50	1.42	2.51	36.67
204	C	AA	TT	M	32	4.14	1.54	0.98	2.46	100.31
205	C	AC	TT	F	27	4.43	0.58	1.63	2.54	87.06
206	C	AC	TT	M	38	7.77	4.33	0.98	4.79	150.93
207	C	AA	CC	M	47	7.10	2.03	1.35	4.82	36.37

Table D.1 (Continued)

208	C	AA	TT	F	27	4.40	0.60	1.50	2.62	54.49
209	C	AC	TC	F	25	3.73	0.54	1.19	2.28	162.14
210	C	AC	TC	F	31	4.87	0.75	1.74	2.80	61.60
211	C	AA	TT	M	31	6.29	3.91	1.04	3.47	63.15
212	C	AA	TT	F	38	3.68	2.10	0.80	1.92	105.83
213	C	AA	TT	F	21	3.16	0.63	1.30	1.58	17.70
214	C	AC	TC	F	35	5.67	1.28	1.22	3.86	48.15
215	C	AA	TC	F	31	4.66	1.20	1.30	3.63	56.41
216	C	AC	TC	F	41	6.19	1.20	1.53	4.12	72.13
217	C	AC	TC	F	70	6.01	173	1.30	3.91	81.12
218	C	AA	TT	F	43	5.31	2.55	0.78	3.37	79.92
219	C	AA	TC	F	42	4.77	1.92	1.09	2.80	33.70
220	C	AA	TC	M	46	4.69	1.58	0.98	2.98	82.12
221	C	AA	TT	F	27	4.30	0.69	1.35	2.64	117.50
222	C	AA	TT	F	29	5.18	1.30	1.94	2.64	76.85
223	C	AC	TC	M	29	4.69	2.29	0.88	2.75	53.06
224	C	AA	TT	F	38	4.35	0.57	1.32	2.77	54.46
225	C	AC	TT	F	26	4.64	0.57	1.86	2.51	58.51
226	C	AA	TT	F	29	3.26	2.51	0.70	1.42	94.50
227	C	AC	TC	F	35	4.74	1.18	1.48	2.72	47.75
228	C	AC	TT	M	26	6.73	0.69	1.11	5.31	54.74
229	C	AA	TT	M	36	5.70	0.54	1.50	3.94	95.27
230	C	AA	TC	M	37	6.71	3.34	1.11	4.07	97.64
231	C	AC	TT	F	30	4.87	1.36	1.22	3.03	48.62
232	C	AA	TC	M	35	6.01	1.20	1.14	4.33	58.78
233	C	AC	TT	F	58	6.68	2.80	1.50	3.89	95.82
234	C	AA	TC	M	32	4.56	2.35	0.67	2.67	61.85
235	C	AC	TT	M	28	5.65	2.35	1.09	3.47	78.25
236	C	AC	TT	F	34	4.77	0.59	1.53	2.98	77.13
237	C	AC	TT	F	29	5.65	0.75	1.61	3.70	62.45
238	C	AA	TT	M	33	5.31	3.54	1.06	2.62	72.38