ASSOCIATION OF THE VITAMIN D METABOLIZING CYP24A1, CYP27A1, CYP27B1 AND VITAMIN D RECEPTOR GENETIC POLYMORPHISMS WITH MULTIPLE SCLEROSIS RISK IN TURKISH POPULATION

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ABSTRACT

ASSOCIATION OF THE VITAMIN D METABOLIZING CYP24A1, CYP27A1, CYP27B1 AND VITAMIN D RECEPTOR GENETIC POLYMORPHISMS WITH MULTIPLE SCLEROSIS RISK IN TURKISH POPULATION

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Multiple Sclerosis (MS) is the most common nontraumatic cause of young disability. There are many studies indicating the relationship between MS and vitamin D. Activation of vitamin D requires a series of hydroxylations that are conducted by cytochrome P450 enzymes (CYPs). Vitamin D shows its effects by binding to vitamin D receptor (VDR). In the present study, vitamin D metabolism related mitochondrial CYPs (CYP27A1, CYP27B1 and CYP24A1) and VDR gene polymorphisms were selected for investigation. 187 (130 female/57 male) MS patients and 140 (78 female/62 male) control subjects were involved in the study. DNA samples extracted from the blood of MS patients and control subjects were genotyped using RFLP. Three genetic models applied which were additive (wild type homozygous genotype vs heterozygous genotype and wild type genotype vs homozygous mutant genotype), dominant (wild type homozygous vs heterozygous+homozygous mutant) and recessive (wild type homozygous+heterozygous vs homozygous mutant). Also, allele frequencies (wild type vs mutant) were analyzed. All analyzes were applied to male and female subgroups of the samples. Analyzes were done with chi-square method and

frequencies of genotypes and alleles compared in between MS patients and the healthy control subjects. In TaqI (rs731236), “A” was the wild type and “G” was the mutant allele. In dominant model analysis result is borderline significant (p=0.051) suggesting that “G” allele reduces the risk (OR:0.642). None of the remaining models’ and subgroups’ analyzes were statistically significant. In CYP24A1 (rs1570669), “A” was the wild type and “G” was mutant allele. In statistical analyzes, AA vs AG additive model (p<0.001, OR:5.413) and dominant model (p<0.001, OR:4.429) results were significant and found to increase the susceptibility to the disease. For female subgroup, AA vs AG additive model (p<0.001, OR:6.693) and dominant model (p<0.001, OR:4.685) and for male subgroup, AA vs AG additive model (p<0.001, OR:4.387) and dominant model (p <0.001, OR:4.277) results were significant and found also to increase susceptibility to the disease. For female subgroup, recessive model result was also statistically significant (p=0.041, OR:0.957). Recessive model analyzes were not significant for general population and the male subgroup. AA vs GG additive model and allele frequency comparisons were not significant for general population and subgroups.

For VDR-ApaI (rs7975232), CYP27A1 (rs6709815), and CYP27B1 (rs4646536), none of the analyzes (additive, dominant, recessive models and allele frequency comparisons) were statistically significant (p>0.05) suggesting that there is no correlation between MS and the selected polymorphisms. However, for rs4646536, the population frequency of rare variant (“C” allele) was studied for the first time, and the frequency for patients was 2.41%, for control subjects was 3.31% and for total was 2.95%. By this study association of several polymorphisms (rs7975232, rs6709815, rs4646536) with MS risk were investigated for the first time in Turkish population. We hope our results contribute to understanding of genetic background of MS onset.

Keywords: Vitamin D, Multiple sclerosis, Vitamin D metabolizing CYPs, VDR, gene polymorphism

ÖZ

D VİTAMİNİ METABOLİZMASINDA ROL ALAN CYP24A1, CYP27A1, CYP27B1 VE D VİTAMİNİ RESEPTÖRÜNÜN GENETİK POLİMORFİZMLERİNİN TÜRK POPULASYONUNDA MULTİPL SKLEROZ RİSKİ İLE İLİŞKİSİ

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MS travmatik olmayan genç engelliliğin en yaygın nedenidir. Pek çok çalışma MS ile D vitamini araşındaki ilişkiye işaret etmektedir. D vitamininin aktivasyonu CYP'ler tarafından yürütülen bir dizi hidroksilasyon gerektirmektedir. D vitamini etkisini D vitamini reseptörüne (VDR) bağlanarak gösterir. Bu çalışmada D vitamini ile ilişkili mitokondriyal CYP'lerin (CYP27A1, CYP27B1 ve CYP24A1) ve VDR'nin polimorfizmleri incelenmek üzere seçilmiştir. 187 (130 kadın/57 erkek) hasta ve 140 (78 kadın/62 erkek) kontrol örneği ile çalışılmıştır. MS hastalarının ve kontrol katılımcılarının kanlarından izole edilen DNA örnekleri RFLP metodu kullanılarak genotiplenmiştir. Örneklerin istatistik analizi için üç genetik model uygulanmıştır. Bu modeller aditif (yabanıl tip homozigota karşı heterozigot genotip ve yabanıl tip homozigota karşı mutant homozigot genotip), dominant (yabanıl tip homozigota karşı heterozigot+homozigot mutant) ve resesif (yabanıl tip homozigot + heterozigota karşı homozigot mutant) modellerdir. Ayrıca alel frekansları da (yabanıl tip alele karşı mutant alel) analiz edilmiştir. Tüm analizler örneklerin erkek ve kadın alt gruplarına da uygulanmıştır. Analizler ki-kare yöntemi ile yapılmış, genotip ve alel frekansları MS hastaları ve sağlıklı kontroller arasında karşılaştırılmıştır.

TaqI (rs731236)'de "A" yabanıl tip ve "G" mutant aleldir. Dominant model analizinde sonuç sınırda anlamlı bulunmuş olup (p=0.051), bu "G" alelinin riski azalttığını düşündürmektedir (OR:0.642). Diğer model ve alt grup analizlerinin hiçbiri istatistiksel olarak anlamlı bulunmamıştır.

CYP24A1 (rs1570669)'de "A" yabanıl tip ve "G" mutant aleldir. İstatistik analizlerinde, AA'ya karşı AG'nin karşılaştırıldığı aditif model (p<0.001, OR:5.413) ve dominant model (p<0.001, OR:4.429) sonuçları anlamlı olup, hastalığa yatkınlığı arttırdığı belirlenmiştir. Kadın alt grubunda AA'ya karşı AG'nin karşılaştırıldığı aditif model (p<0.001, OR:6.693) ve dominant model (p<0.001, OR:4.685) ile erkek alt grubunda AA'ya karşı AG'nin karşılaştırıldığı aditif model (p<0.001, OR:4.387) ve dominant model (p <0.001, OR:4.277) sonuçları da anlamlı olup, hastalığa yatkınlığı arttırdığı belirlenmiştir. Kadın alt grubunda, resesif model sonuçları da anlamlı bulunmuştur (p=0.041, OR:0.957). Resesif model sonuçları genel örneklemde ve erkek alt grubunda anlamlı bulunmamıştır. AA'ya karşı GG aditif model ve alel frekansı analizleri genel örneklemde ve alt gruplarda anlamlı bulunmamıştır.

VDR-ApaI (rs7975232), CYP27A1 (rs6709815) ve CYP27B1 (rs4646536) için hiçbir analiz (aditif, dominant, resesif ve alelfrekans karşılaştırması) istatistiksel olarak anlamlı (p>0.05) bulunmamış olup, bu da MS ile seçilen bu polimorfizm arasında bir ilişki olmadığını düşündürmektedir. Bunların yanında rs4646536'da nadir varyantın ("C" aleli) popülasyon frekansı ilk defa belirlenmiş olup, frekanslar hastalarda %2.41, control örneklerinde %3.31 ve toplam %2.95'tir. Bu çalışma ile Türk popülasyonunda, bazı polimorfizmlerin (rs7975232, rs6709815, rs4646536) MS riski ile ilişkisi ilk defa araştırılmıştır. Umuyoruz ki çalışma sonuçlarımız MS gelişiminin genetik alt yapısının daha iyi anlaşılmasına katkıda bulunacaktır.

Anahtar Kelimeler: D vitamini, Multipl skleroz, D vitaminini metabolize eden CYP'ler, VDR, polimorfizm

This study is dedicated to my beloved wife and lovely son

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LIST OF ABBREVIATIONS

ABBREVIATIONS

bp	Base pair
BSA	Bovine serum albumin
CNS	Central nervous system
CYP	Cytochrome P450
DBD	DNA binding domain
DBP	Vitamin D binding protein
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DRIP	Vitamin D receptor-interacting protein complex
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FGF-23	Fibroblast growth factor-23
F-9	Ferriprotoporphyrin 9
gDNA	Genomic DNA
IL	Interleukin
IU	International unit
LBD	Ligand binding domain
MS	Multiple sclerosis
NADH	Nicotinamide adenine dinucleotide, reduced form

NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NCoR	Nuclear corepressor
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PTH	Parathyroid hormone
rpm	Revolutions per minute
RRMS	Relapsing-remitting multiple sclerosis
RXR	Retinoid X receptor
SDB	Sample dilution buffer
TBS	Tris-buffered saline
TBST	Tris-buffered saline and Tween 20
UVB	Ultraviolet B radiation
VDR	Vitamin D receptor
VDRE	Vitamin D response element

LIST OF SYMBOLS

SYMBOLS

Λ_{max}	Maximum absorption wavelength
^{o} C	Centigrade degree
D_2	Ergocalciferol
D_3	Cholecalciferol
g	Gravitational force
kDa	Kilo Dalton
K_m	Michaelis constant
µ	Micro
p	Probability
R^2	Coefficient of determination

CHAPTER 1

INTRODUCTION

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a complicated, relapsing, and frequently progressive inflammatory demyelinating autoimmune disease of the central nervous system (CNS) (Gaby, 2013). MS causes damage to the axons in addition to demyelination. Symptoms show relapses generally, yet sometimes gradual progression can be observed (Noseworthy et al., 2000). MS was described as a disease by French neurologist Jean Martin Charcot more than 150 years ago (Zalc, 2018). MS is the most common disabling disease among young adults that is not caused by any trauma (Dobson & Giovannoni, 2019).

The first symptoms of MS are generally blurred or lost vision and are experienced between the ages of 20-40. The disease is twofold common in women (Gaby, 2013). Recent studies showed a change in the ratio changed to 3:1 (Female: Male), which is tought to be related to women's changing smoking habits after world war two. Smoking became more common between women after great war (Dobson & Giovannoni, 2019). The exact cause of the disease is still ambiguous. However, several environmental factors like vitamin D levels, Epstein-Barr virus infection, and smoking are shown to be strongly related to disease development along with genetic factors (Ascherio et al., 2012).

Clinical subtypes of MS were defined as benign and malignant in 1996 by The US National Multiple Sclerosis Society (NMSS) Advisory Committee on Clinical Trials in Multiple Sclerosis (Lublin & Reingold, 1996). However, the lack of consensus on these definitions led to changes in time. (Lublin et al., 2014). MS can be sudden and severe, which might be fatal in months, or it can be

asymptomatic throughout all life (Sadovnick et al., 1997). Subtypes of MS are relapse–remit MS (RRMS), secondary progressive MS, and primary progressive MS (Silva et al., 2018). 85–90% of MS patients are suffering from RRMS. RRMS is defined as relapses that are observed as neurological symptoms without infection. Relapse is followed by a recovery period. This remission is generally partial. After 10 to 15 years of RRMS onset, the disease becomes progressive in most patients (around 70%). This type of progressive form of MS is called secondary progressive MS. Around 10-15% of the patients develop primary progressive MS which does not show any remission (Brownlee et al., 2017; Sadovnick et al., 1997; Ontaneda et al., 2017).

According to the incidence and prevalence studies, distance to the equatorial belt correlates with the prevalence of the disease (Goldberg, 1974; Kingwell et al., 2013). Moreover, the incidence of MS shows similar rates at different races, which are at the same latitudes. This result led to the emergence of the Vitamin D/MS hypothesis (Goldberg, 1974). The reason behind this correlation becomes clear through the years by the invention of the immunomodulatory effects of vitamin D. Vitamin D is not only important for the prevention of disease it also has a direct effect on the severity of the symptoms and progress of the disease (Pierrot-Deseilligny & Souberbielle, 2017).

Moreover, in the summer and spring, symptom severities show differences compared to winter. In the winter, more relapses are observed than in summer (Harding et al., 2017). This is tought to imply a plausible role of vitamin D on the disease's progression. The negative correlation between the Expanded Disability Status Scale (EDSS) score and vitamin D concentration in the patient's serum was shown (Smolders et al., 2008). In addition, another research showed that 10 nmol per liter increase in serum vitamin D level reduced the relapse risk by 10%, and a 12% reduction was observed in the hazard of relapse (Simpson et al., 2010).

Inflammation is important in all diseases, and MS's inflammatory components were well studied. In the inflammation process, many cytokines and chemokines

play a role. Even the exact cause of autoimmunity in MS is still not known; the process begins with an abnormal response of the immune system to CNS antigens, and particularly myelin is the target. This chronic immune response is led to the death of oligodendrocytes and demyelination progressively. That abnormal response can be caused by an external source like an infection. In that case, a peripheral antigen moves to the lymph node and starts an immune response. Alternatively, an inner source like oxidative stress might disrupt the integrity of neural cells and lead CNS antigens to move to the lymph node and start triggering immunity (Gharagozloo et al., 2018). Whether the source is inner or outer, both trigger adaptive and innate immunity (Figure 1.1), and cause progressive neural damage (Gharagozloo et al., 2018).

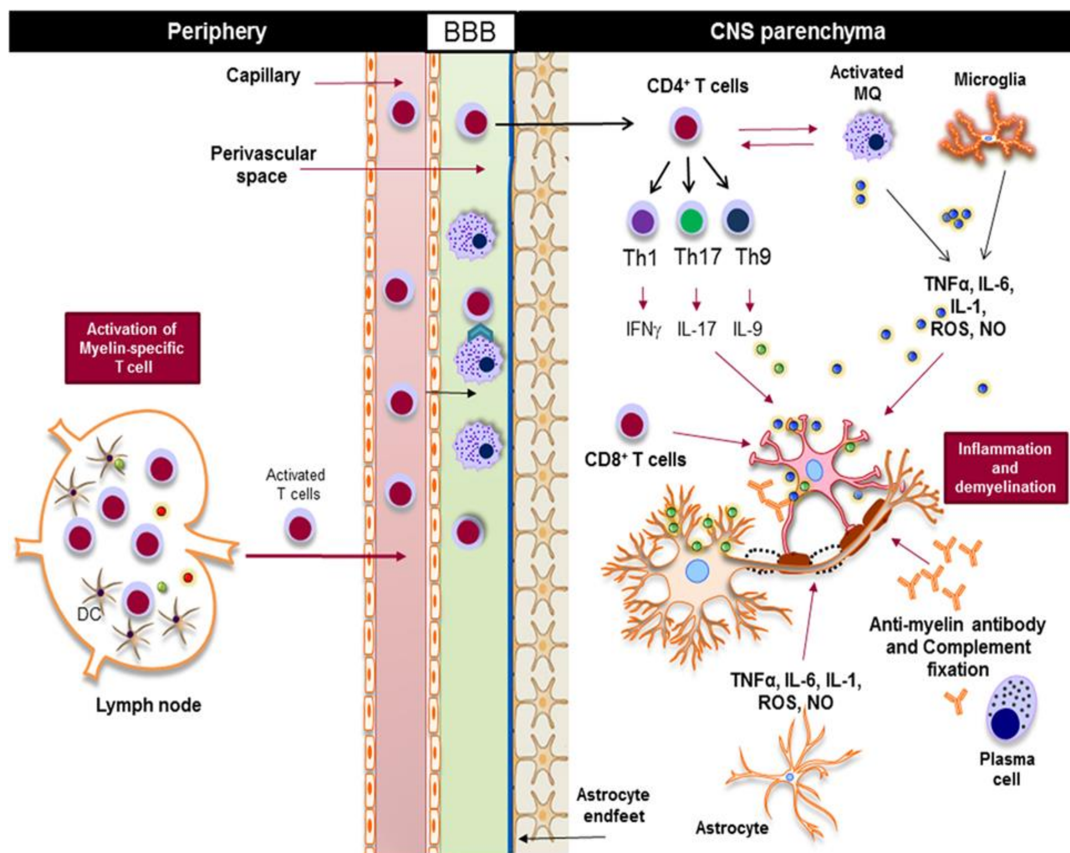


Figure 1. 1 Progress of MS after the primary immune system trigger (Gharagozloo et al., 2018).

MS affects more than 2.8 million people worldwide, and more than half live in Europe. Europe's total medical costs for treatment are around 15 billion Euros (Kingwell et al., 2013; Sawcer et al., 2014; Wijeratne, T., & Carroll, W., 2022). Under the light of this information, correct diagnosis for correct treatment becomes more important. Before the 1950s, there were no standardized methods for diagnosis, and only advanced cases with severe neurological symptoms could be diagnosed (Murray, 2022). First, "The Schumacher Criteria", then, "The Poser Criteria" and lastly, "The MacDonald Criteria" were used for diagnosis. Advances in imaging technologies, biochemistry, and genetics improved diagnosis criteria, and in the future technological improvements will positively affect diagnosis (Murray, 2022).

Until the early nineties, treatments were not effective on MS. Then immunomodulatory drugs like interferon, and glatiramer acetate were introduced, and they were reasonable because they had no severe side effects. Even if they reduce relapse frequency by around 30%, damage caused by disease remains, which decreases the patient's life quality (Dahbour et al., 2017). After that, other treatments were introduced to the field with better results. Yet they have limitations, and their effectiveness is still a question mark (Chedid et al., 2022; Dahbour et al., 2017).

1.2 Vitamin D

Vitamin D is a secosteroid, which means a steroid with a broken ring. There are two major types of the fat-soluble secosteroids; vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) (Figure 1.2) (Raveendran, 2022). They have slight structural differences, like a double bond at the position of C22-C23 and a methyl group at C24 (Jones, 2022). Both are activated via hydroxylation of their carbon atoms at the positions of 1 and 25 (Wanat et al., 2022). These hydroxylation reactions lead to the active form of vitamin D. Activation of vitamin D3 in the skin starts with the UVB exposure leads photolysis of 7-dehydroxycholesterol

which produce previtamin D and it isomerizes to vitamin D (Rochel, 2022; Wanat et al., 2022).

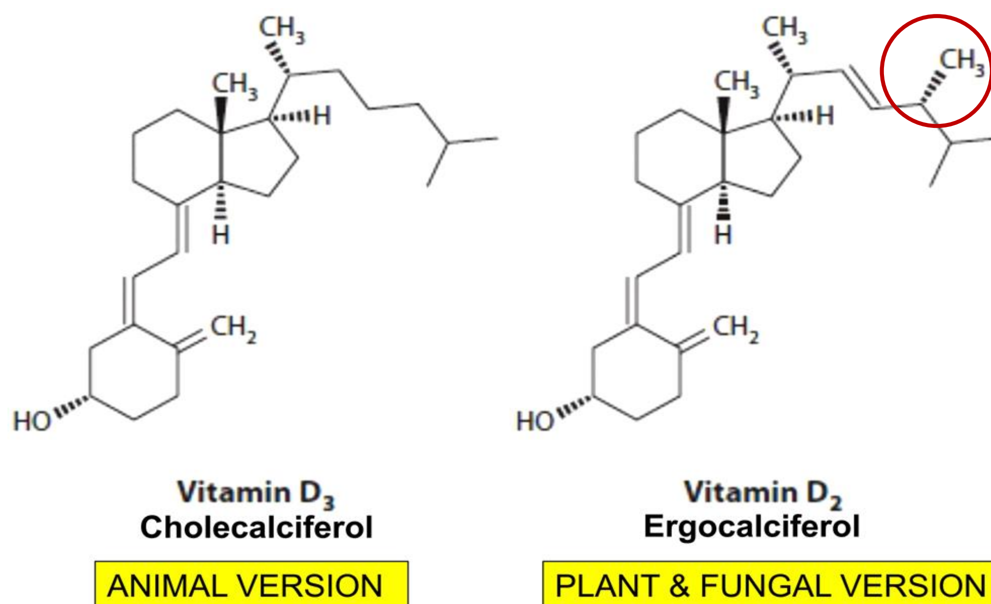


Figure 1. 2 Two major forms of vitamin D and their primary sources (Jones, 2022).

This isomerization is followed by a series of hydroxylation reactions conducted by CYPs. Vitamin D is carried in the body by vitamin D Binding Protein (VDBP/DBP). Independent from the source of vitamin D (synthesized from skin or dietary uptake), VDBP carries it. VDBP is capable of binding to different types and various forms of vitamin D (Bouillon et al., 2020). Hydroxylation reactions of vitamin D take place in the liver and kidneys. The complete path of vitamin D starts from the synthesis of previtamin D from 7-dehydroxycholesterol in the skin. Either taken by the diet or produced in the skin, cholecalciferol is carried by VDBP to the liver. In the liver 25-hydroxyvitamin D (25(OH)D) is synthesized from previtamin D via the activity of CYPs (CYP2R1/CYP27A1/CYP3A4). Then VDBP carries 25(OH)D from the liver to the kidney. In kidney 25(OH)D has two fates. Those are carried by two different CYPs (CYP27B1 or CYP 24A1). CYP27B1 hydroxylates it from its alpha carbon and 1.25(OH)2D (1,25 dihydroxyvitamin D) is produced. This form is biologically active and has the

ability to bind vitamin D receptor (VDR). This binding triggers some reactions and affects gene expression. CYP24A1 hydroxylates both 25(OH)D (inactive) and 1.25(OH)2D (active) from their 24th carbon and produce 24,25-dihydroxyvitamin D and 1,24,25-trihydroxyvitamin D (calcitrioic acid), respectively (Figure 1.3) (Agnello et al., 2018). Those forms are more soluble and secreted from the body via urine.

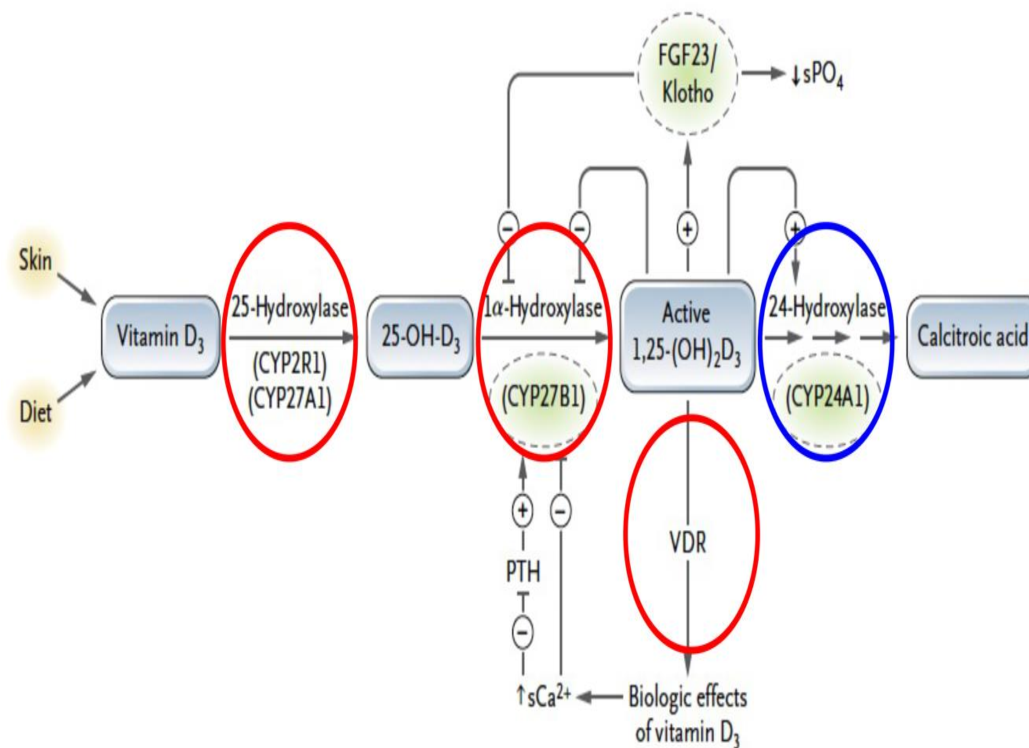


Figure 1. 3 Schematic representation of the path followed by vitamin D (Jones, 2022).

Vitamin D was named as a vitamin in the 1920s even though it is synthesized by the body. Because at that time, some trace compounds, which are taken by diet, seemed essential for health, and vitamin D was one of them (Jones, 2022). In the early 20th century role of vitamin D is mainly defined by its activity on phosphate and calcium homeostasis. After 1975, vitamin D was studied in various aspects. Those studies revealed vitamin D's important effects on the immune system

(Jones, 2022; Zorzella-Pezavento et al., 2022). These effects include the upregulation of macrophages, the polarization of T lymphocytes, and dendritic cell differentiation. Vitamin D leads those changes by binding to VDR and altering the gene transcription. Obviously, these properties make vitamin D important for not only phosphate and calcium homeostasis but also various diseases like cancer, diabetes, MS, Parkinson's Disease, Alzheimer's disease, cardiovascular diseases, and even Covid 19 infections (Jones, 2022; Pereira et al., 2022; Raveendran, 2022; Zorzella-Pezavento et al., 2022).

1.3 Vitamin D and MS

The hypothesis that vitamin D deficiency increases the susceptibility to MS is based on latitude and ecological studies. Studies show that the frequency of MS increases with the latitude increase and reaches nearly zero at the equatorial zone (Ascherio et al., 2010; Hayes et al., 1997). That change in the latitude leads to changes in the angle of sunlight and time of exposure to the sun. Therefore, the people around the equatorial zone are exposed to more UVB and produce more vitamin D. Yet, a controversial result obtained by Hayes et al. suggested decreased MS incidence at the Atlantic coasts of Norway. However, in that area, people consume more fish, which is a rich source of vitamin D (Hayes et al., 1997). In addition, according to some recent studies, MS onset was observed two years earlier among individuals who live in higher latitudes (Wood, 2017). All those results indicate a correlation between vitamin D and developing MS. That hypothesis and the relation between MS and vitamin D became more meaningful after the discovery of vitamin D's regulatory effects on the immune system (Ascherio et al., 2010). Patients with higher vitamin D concentration were found to develop fewer symptoms, and the devastating effects of the disease were relieved in some portion in this patients. However, still some people with lower blood vitamin D concentration were found not to develop MS. Those results indicate the genetic background as one of the major factors in MS onset. Also

vitamin D was found to have protective properties against not only MS onset but also MS symptoms (Hayes et al., 1997; Pierrot-Deseilligny & Souberbielle, 2017; Wood, 2017; Zorzella-Pezavento et al., 2022).

1.4 Vitamin D Receptor

Vitamin D receptor (VDR) is also known as calcitriol receptor. VDR is a member of the nuclear receptor family with high specificity to its ligand (Moore et al., 2006). The ligand of VDR is the active form of vitamin D (1,25(OD)2D), also known as calcitriol or 1,25 dihydroxycholecalciferol. VDR gene is positioned at chromosome 12q13.11 (geneID:7421) in humans. VDR protein has an amino acid length of 427 and a molecular mass of around 50-60 kDa, depending on the species. It has a ligand binding (LBD) domain and a nuclear binding (NBD) domain (Rochel, 2022). VDR is expressed in various tissues except liver, muscle, and brain tissues (Wang et al., 2012).

VDR activation starts with vitamin D entry to the cytoplasm. Vitamin D binds to VDR, and that leads to a conformational change in the binding pocket of VDR. This conformational change traps vitamin D in the pocket. This model is called "mouse trap". Then VDR phosphorylates, and this phosphorylation directs the complex to the nucleus. That complex heterodimerizes with the retinoid X receptor (RXR). After heterodimerization, VDR binds to vitamin D response elements (VDREs) on DNA in the promoter regions (Figure 1.4) (Issa et al., 1998). By that, stimulations or inhibitions on various genes occur. The regulatory function of VDR change due to which type of RXR heterodimerize with it. Even VDR could heterodimerize with many isoforms of RXR; its affinity to RXRγ is higher than other isoforms. Because of its effects on transcription, it attracted researchers' interest, and until now, many diseases are found to be related to VDR dysfunction. (Hayes et al., 1997; Issa et al., 1998; Raveendran, 2022; Rochel, 2022). MS is one of the most frequently studied of these diseases. Since vitamin D is a highly promising substance for preventing and decreasing the devastating

effects of the disease, today many researchers are investigating MS in the VDR axis (Agnello et al., 2018; Hayes et al., 1997; Jones, 2022; Smolders et al., 2008).

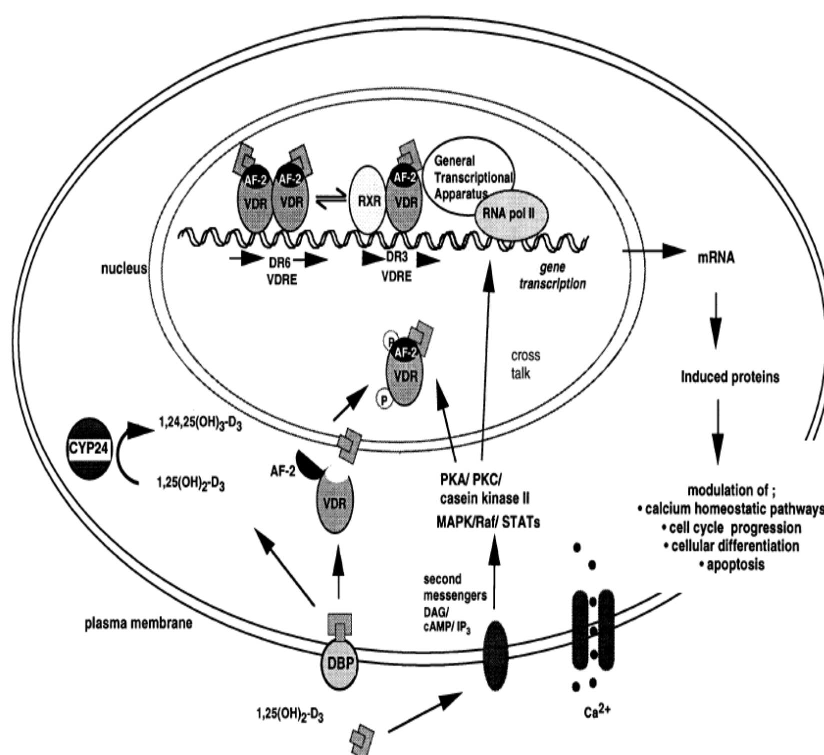


Figure 1. 4 Pathway of VDR. Its activation starts with vitamin D binding. It localizes to the nucleus, heterodimerize with RXR, and leads to gene expression changes (Issa et al., 1998).

1.5 Cytochrome P450s

Cytochrome P450s (CYPs or P450s) are the superfamily of heme-thiolate proteins that function in the metabolism of various endogenous and exogenous compounds. The number of CYPs is around 1000 in all species. In addition, around 57 different types of CYPs are identified in humans (Hasler et al., 1999; Uno et al., 2012; Nelson, 2009). CYP genes are identified in all species ranging from bacteria to animals. Lately, around 300.000 CYP sequences have been

identified with the help of bioinformatics tools (Nelson, 2018). CYPs are membrane-bound hemoproteins in eukaryotes, and their discovery occurred in 1958. The 450 in the name comes from the experiments that are conducted by Klingenberg, who is the discoverer of the spectrophotometric properties of CYPs. CYPs maximum absorbance is observed at 450nm, unique to that hemeproteins (Hasler et al., 1999). The structure of CYPs contains a conserved prosthetic group of heme called ferriprotoporphyrin IX at its active site (Figure 1.5) (Hasler et al., 1999).

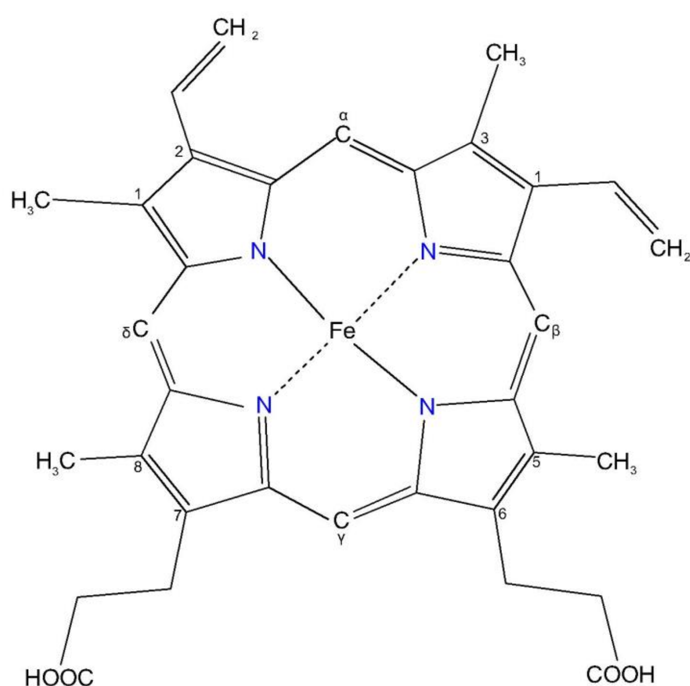


Figure 1. 5 Structure of ferriprotoporphyrin IX (adapted from Hasler et al., 1999).

The nomenclature of CYPs is based on number and letter classification. CYP, followed by a number, which stands for the family, then a letter, indicating the sub-family finally, another number for defining the individual CYP enzyme (Hasler et al., 1999; Nelson, 2009). In humans, 57 individual CYPs are defined (Table1.1) (Nelson, 2009). CYPs share increased percentages of amino acid sequences from family (40%) to sub-family (55%), and their differences play a role in their substrate selectivity (Coon et al., 1992; Lewis, 1999). CYPs are

membrane-spanning enzymes. They can accept molecules outside or inside. This property makes them essential for the metabolism of many substances, including drugs. They can play a role in whether activation or inactivation of drugs. Around 75% of drug metabolism is conducted by CYPs (Guengerich, 2008). Besides drug metabolism, they are also crucial for fatty acid metabolism, bile acid synthesis, steroid metabolism, and vitamin D metabolism (Nelson, 2009). In Table 1.1, CYP families and their roles in humans summarized. Vitamin D metabolizing ones are written in bold.

Table 1.1 Summary of human CYP genes and their functions (Nelson, 2009).

CYP Families	Individual Names	Function
CYP1 (3 genes) (1 pseudogene)	1A1, 1A2, 1B1	Xenobiotic Metabolism
CYP2 (16 genes) (16 pseudogenes)	2A6, 2A7, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 2R1, 2S1, 2U1, 2W1	Xenobiotic and Steroid Metabolism
CYP3 (4 genes) (4 pseudogenes)	3A4, 3A5, 3A7, 3A43	Xenobiotic and Steroid Metabolism
CYP4 (12 genes) (10 pseudogenes)	4A11, 4A22, 4B1, 4F2, 4F3, 4F8, 4F11, 4F12, 4F22, 4V2, 4X1, 4Z1	Fatty Acid Metabolism
CYP5 (1 gene)	5A1	Thromboxane A2 Synthesis
CYP7 (2 genes)	7A1, 7B1	Bile Acid Biosynthesis
CYP8 (2 genes)	8A1, 8B1	Prostacyclin Synthesis and Bile Acid Biosynthesis
CYP11 (3 genes)	11A1, 11B1, 11B2	Steroid Biosynthesis
CYP17 (1 gene)	17A1	Estrogen and Testosterone Biosynthesis
CYP19 (1 gene)	19A1	Estrogen Hormone Biosynthesis
CYP20 (1 gene)	20A1	Drug Metabolism and Cholesterol Biosynthesis
CYP21 (1 gene) (1 pseudogene)	21A2	Steroid Biosynthesis
CYP24 (1 gene)	**24A1**	**Vitamin D Degradation**
CYP26 (3 genes)	26A1, 26B1, 26C1	Retinoic Acid Metabolism
CYP27 (3 genes)	**27A1, 27B1**, 27C1	Bile Acid Biosynthesis and **Vitamin D3 Activation**
CYP39 (1 gene)	39A1	Cholesterol Biosynthesis
CYP46 (1 gene) (1 pseudogene)	46A1	Cholesterol Biosynthesis
CYP51 (1 gene) (3 pseudogenes)	51A1	Cholesterol Biosynthesis

The activity of CYPs is based on the reduction and oxidation of the iron placed in the enzyme's active site. The basic catalytic cycle of CYPs is shown in Figure 1.6. Nevertheless, this cycle is a simplified form of the pathway and is not necessarily followed by the substrate with the order. Substances can enter the cycle from different steps, and many reactions are reversible (Guengerich, 2008).

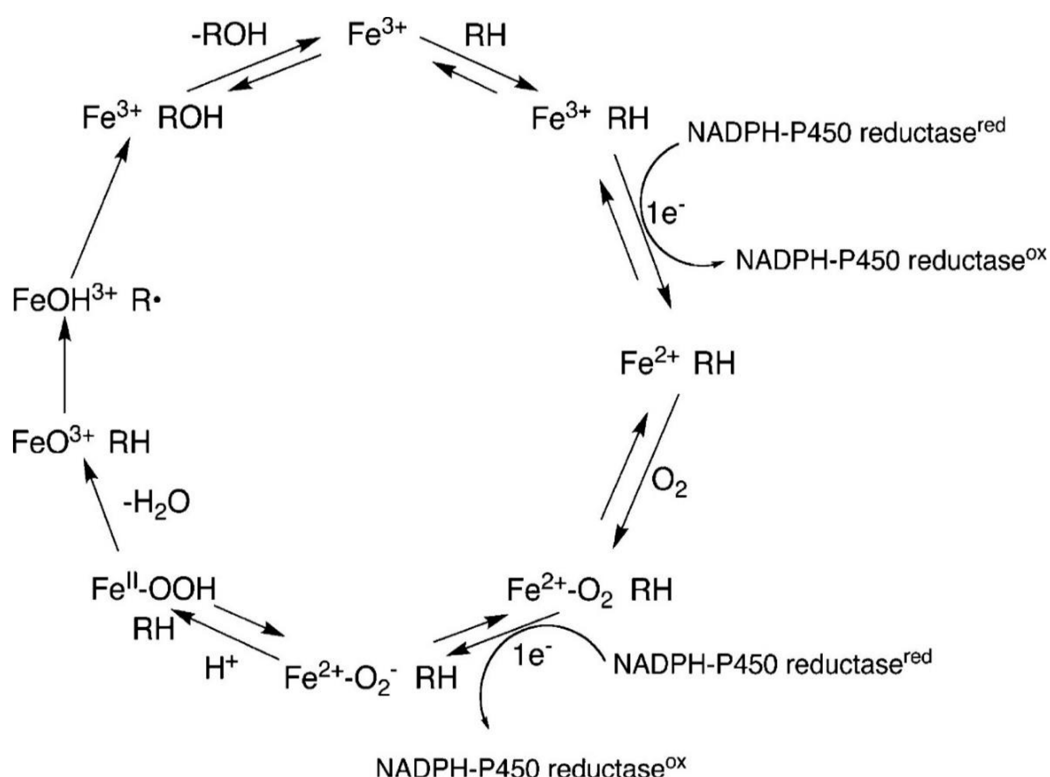


Figure 1. 6 Simplified CYP catalytic cycle (Guengerich, 2008).

CYPs are playing a critical role in vitamin D metabolism. Four isozymes that metabolize vitamin D was shown before in Figure 1.3. Those are CYP2R1, CYP27A1, CYP27B1, and CYP24A1. Except for cytoplasmic CYP2R1, the other three are mitochondrial enzymes (Jones, 2022; Nelson, 2009). In the mitochondria, CYP27A1 catalyzes 25-hydroxylation, CYP27B1 catalyzes 1α-hydroxylation, and CYP24A1 catalyzes 24-hydroxylation of vitamin D (Jones, 2022).

1.6 CYP 27A1

CYP27A1 is a mitochondrial monooxygenase enzyme. It catalyzes many reactions crucial for cholesterol metabolism, bile acid biosynthesis, and vitamin D activation. CYP27A1 is one of the key enzymes for bile acid production.

Dysfunction of CYP27A1 is related to some diseases like Cerebrotendinous xanthomatosis (CTX) (Koyama et al., 2022; Lumbreras et al., 2021; Sawada et al., 2000). CYP27A1 is a 531 amino acid long peptide. 33 of the amno acids are mitochondrial signal sequence, and the mature enzyme is 498 amino acid long. The molecular mass of CYP27A1 is 60kDa (Cali & Russell, 1991). The CYP27A1 gene is located on chromosome 2 (2q35) and contains nine exons (Figure 1.7). The length of that chromosome is 18.6 kb, and the mRNA length is between 1.8-2.2 kb (Björkhem & Leitersdorf, 2000).

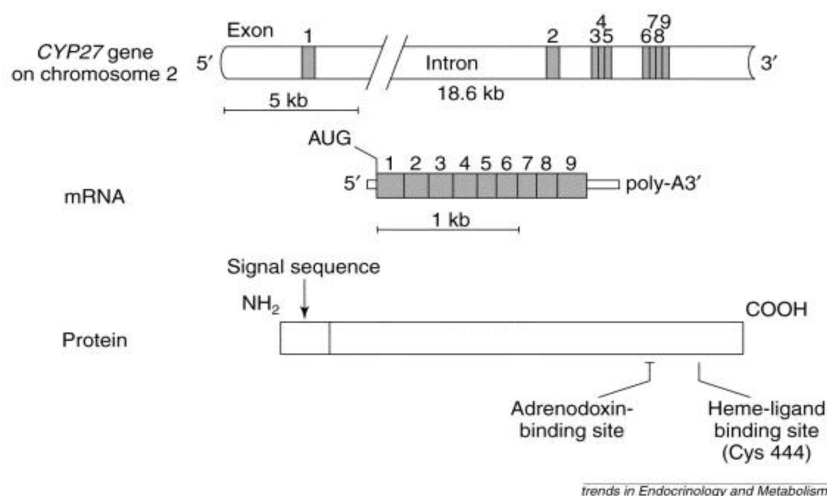


Figure 1. 7 Schematic representation of CYP27A1 from chromosome to protein (Björkhem & Leitersdorf, 2000).

CYP27A1 hydroxylates vitamin D in the liver, which is also essential for other cholesterol metabolism reactions. Because of that, CYP27A1 expression is observed in many extrahepatic tissues (Björkhem & Leitersdorf, 2000; Lumbreras et al., 2021). In the liver, it converts vitamin D to 25(OH) vitamin D (Figure 1.8) (Sakaki, 2005).

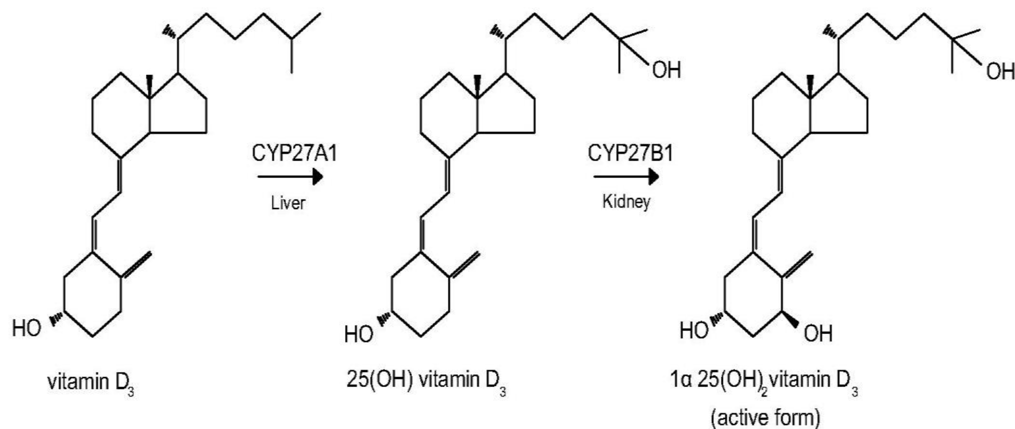


Figure 1. 8 Hydroxylation reactions of vitamin D from “pro” form to “active” form (adapted from Sakaki, 2005).

The expression of CYP27A1 is not affected by vitamin D levels. Nevertheless, insulin and bile acids decrease the expression levels in extrahepatic tissues like the intestine and kidney. In addition, the deletion of CYP27A1 is not affecting vitamin D levels in mice (Bikle, 2021). Many 3D structure prediction and mutation studies were conducted on CYP27A1 *in slico*. Those studies imply that some mutations at the active site lead the enzyme to lose its function, and many others lead to the instability of protein (Charvet et al., 2013; Sunkar & Neeharika, 2020).

1.7 CYP27B1

CYP27B1 is a mitochondrial monooxygenase enzyme that is mainly found in the kidney. However, it is also found in epithelial and some immune cells. It hydroxylates α carbon of 25(OH)D (Figure 1.8) in the kidney, and it is thought to be the key enzyme that determines $1,25(OH)_2D$ levels in the blood (Meyer et al., 2017; Sakaki, 2005). The CYP27B1 gene is found on chromosome 12 (12q14.1). Its length is around 6 kb and contains nine exons (Figure 1.9). The protein of CYP27B1 contains 508 amino acids with the molecular weight of 56.5 kDa (Monkawa et al., 1997).

Figure 1. 9 Schematic representation of human CYP27B1 gene. Boxes indicate the exons found on the gene. Boxes also indicate the relative sizes of exons (Monkawa et al., 1997).

Homology modeling studies revealed the structure of CYP27B1. It contains 17 helices and 6 β-sheets. This modeling also indicated that the arginines at positions 389 and 453 are critical for heme binding (Sakaki, 2005).

CYP27B1 is the key enzyme for producing the active form of vitamin D. It is crucial for maintaining calcium and phosphate homeostasis in the body. Excess $1,25(OH)_2D$ is related to many diseases, like stone formation in the kidney and hypercalcemia (Melo et al., 2020; Yimamu et al., 2022). Lower vitamin D levels are also related to Addison's disease, Hashimoto's disease, Grave's disease, diabetes, or systemic lupus (Lopez et al., 2004; Luo et al., 2022). These results are not surprising. The importance of vitamin D's on calcium homeostasis and immunoregulatory properties are obvious and all those diseases related with either calcium levels or autoimmunity. All these results indicate a strict regulation is needed for proper metabolism. However, even though vitamin D metabolizing enzymes are tightly regulated, seasonal changes occur in blood vitamin D levels (Lindh et al., 2012). Except for UVB radiation, blood levels of vitamin D depend on two controversy-working enzymes, one of which is CYP27B1. Expression of CYP27B1 gene regulated by $1,25(OH)_2D$, FGF23 (fibroblast growth factor 23), and PTH (parathyroid hormone). PTH induces the expression of CYP27B1 and, $1,25(OH)_2D$ and FGF23 reduce the expression of CYP27B1 (Adams et al., 2014; Meyer et al., 2017; Meyer & Pike, 2020).

1.8 CYP24A1

CYP24A1 is a renal, mitochondrial monooxygenase essential for the breakdown of vitamin D (Alyaa Hussein Hammadi & Shatha Hussein Ali, 2021). The primary function of CYP24A1 is catalyzing the 24 hydroxylation of active vitamin D to calcitroic acid by a five-step reaction. Besides 24 hydroxylation, it also has a 23-hydroxylation capacity, which produces lactone as the final product (Figure 1.10) (Jones et al., 2012; St-Arnaud & Jones, 2018).

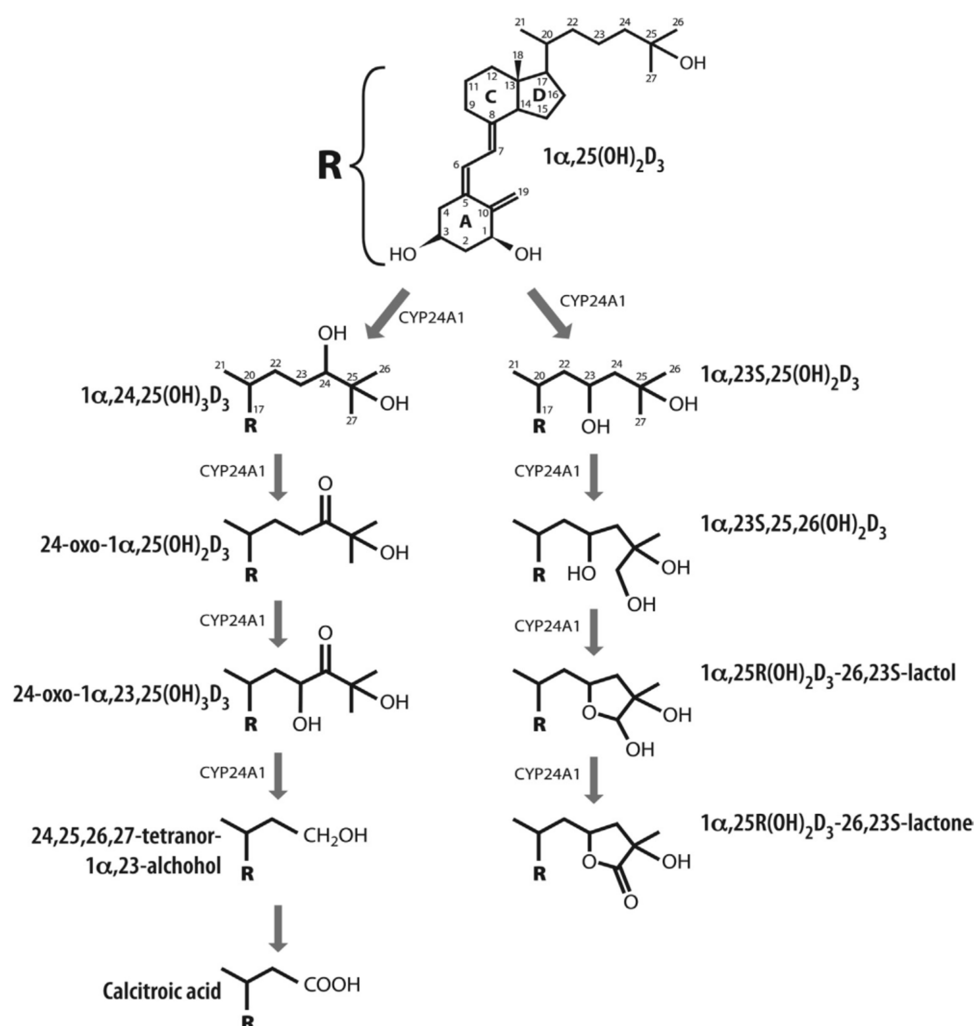


Figure 1. 10 Two different pathways of vitamin D catalyzed by CYP24A1 (St-Arnaud & Jones, 2018).

The Gene of CYP24A1 is located on the 20th chromosome (20q13.2). Gene contains 13 exons translated into 58.9 kDa, 514 amino acid long protein. 3D modeling and crystal structure studies show that it is formed of 12 α-helices, 4β-sheets, and five additional short helices (Jones et al., 2012; Sakaki, 2005; St-Arnaud & Jones, 2018).

CYP24A1 plays a critical role in the levels of active vitamin D in the body because 24 hydroxylated products are excreted from the body (Jones et al., 2012). Regulation of that enzyme is affected by the same substances with CYP27B1 but reciprocally. The primary regulators of the CYP24A1 are 1,25$(OH)_2$D and PTH, similar to CYP27B1. Two vitamin D response elements (VDREs) are essential for that regulation. Also, FGF, calcium, phosphate, insulin, and some sex steroids are the other regulators of CYP24A1 (Bikle, 2021). Increased vitamin D levels in the blood increase the expression of CYP24A1, and PTH negatively affects its expression (Bikle, 2021; Meyer & Pike, 2020). However, these regulations are tissue specific. For instance, PTH does not effect the expression of CYP24A1 in the intestines because there are no PTH receptors in the intestines (Bikle, 2021).

Expression levels of CYP24A1 are essential for healthy homeostasis. If the activity of CYP24A1 is low, it leads to stone formation. In addition, CYP24A1 knockout mice showed 50% lethality, hypercalcemia, hypercalciuria, and miscarriage (Melo et al., 2020; St-Arnaud & Jones, 2018). Elevated activity of CYP24A1 is also related to many other diseases, including MS, immune malfunctions, rickets, chronic kidney disease, and osteomalacia (Agnello et al., 2018; Alyaa Hussein Hammadi & Shatha Hussein Ali, 2021; St-Arnaud & Jones, 2018; Young et al., 2022).

1.9 Genetic Polymorphisms and Single Nucleotide Polymorphisms

Genetic polymorphism is the term used for defining the non-rare genetic difference in the gene that is typically seen in the population. In between humans, genetic similarity is 99.9%. Furthermore, this 0.1% difference, caused by polymorphisms, makes an individual unique (U. A. Meyer & Zanger, 1997). Different forms of the

gene are called an allele, and each allele has a different effect on the functional proteins, as it can increase or decrease the activity. Another option is polymorphism at a non-translated region; it can affect the protein's expression level. However, some changes show no effect on the final product (Bevilacqua et al., 2018; Kelada et al., 2003; U. A. Meyer & Zanger, 1997). Even some of the changes are not detectable; some others, especially in phase I enzymes, are the reason for different personal reactivity to drugs (U. A. Meyer & Zanger, 1997).

The interest in genetic studies in the medical field increased significantly with the completion of the human genome project. Yet, even the completion of the human genome project increased the interest; the scientists have been working on genetic polymorphisms for decades (Kelada et al., 2003; U. A. Meyer & Zanger, 1997). In the '80s, single nucleotide polymorphisms (SNP) were studied by the activities of restriction enzymes. Single nucleotide polymorphism is the polymorphic nucleotide in a specific position of the gene. It is seen around once in 500-1000 base pairs in the genome, making it the most abundant genetic variation (Smigielski et al., 2000). However, it lost popularity until the late '90s and regained its importance through the change in molecular biology studies (Gray et al., 2000). Even today, SNP studies preserve its importance and give scientists an idea about the disease's genetic background (Saengsiwaritt et al., 2022).

In the present study, five SNPs were selected for investigation. Two of them are on VDR gene (rs731236 (A→G) and rs7975232 (C→A)), others were on CYP24A1 gene (rs1570669 (A→G)), CYP27A1 gene (rs6709815 (G→T)) and CYP27B1 gene (rs4646536 (A→C)).

rs731236 (TaqI) and rs7975232 (ApaI) were two of the commonly studied polymorphisms on VDR. TaqI (rs731236) is located on the exon 9 but it is a synonymous polymorphism and lead no amino acid change. ApaI (rs7975232) is intronic polymorphism and located on intron 8. Both polymorphisms, mutant alleles have no effect on protein structure but their effect on blood vitamin D levels were shown (Ruiz-Ballesteros et al., 2020). Moreover, these two polymorphic regions'

relation with MS was also studied. Results of that studies are controversial. Some of them showed no relation between the disease and polymorphisms (Kamisli et al., 2018; Čierny et al., 2016). However, some studies displayed correlation between the disease and selected polymorphisms (Tizaoui et al., 2015; Zhang et al., 2018; Křenek et al., 2018; Yucel et al., 2018). As a result of that controversy, this field still needs more investigation in detail.

rs1570669 is a intronic polymorphism which is located on intron 9 of CYP24A1. rs1570669 was studied in many researches including MS studies. Some studies demonstrated mutant allele's protective effect on diseases like ischemic stroke and urinary cancers (Sun et al., 2021; Yang et al., 2020). Also, rs1570669 polymorphism was found to be related with increased risk of breast cancer (Fuhrman et al., 2013). In MS studies, results were also controversial as some researches claimed that rs1570669 was related with the increased risk and some other studies indicated no correlation between the polymorphism and MS (Orton et al., 2011; Scazzone et al., 2021). Those controversial results were mainly caused by the population sizes of the studies. Exact cause remains unknown but each new study leads researcher to more exact results.

rs6709815 is an intronic polymorphism of CYP27A1 which is located on intron 1 of the gene. This region's effect on blood vitamin D level were investigated in previous studies. Yet none of the studies revealed an interaction between polymorphism (T allele) and vitamin D levels (Bu et al., 2010; Waterhouse et al., 2014). In the present study, rs6709815 included because CYP27A1 is the only mitochondrial vitamin D oxidizing enzyme and rs6709815 was studied in the perspective of vitamin D levels.

rs4646536 is located on intron 6 of CYP27B1. CYP27B1 is important for vitamin D metabolism and rs4646536 related with many diseases like, diabetes mellitus type 1, lower vitamin D levels in blood and MS (Lopez et al., 2004; Sundqvist et al., 2010; Yu et al., 2019). rs4646536 is intronic and has no effect on the protein structure. Yet, some studies displayed that the rs4646536 represents a linkage disequilibrium with alleles related with diabetes mellitus type 1 (Lopez et al., 2004). Moreover,

rs4646536 also has a rare variant. Wild type allele is A and common mutant allele is G. Also it might be C in the rare mutant allele. In the present study, rare variant was selected for investigation. Since, the population size is small and higher frequencies might be hard to relate with a given disorder. Also, another advantage of that variant is the fact that there is no population data exist in databases.

1.10 Aim of the Study

MS is an autoimmune disease that affects CNS. The disease can be progressive, or relapses and remissions can be observed. In both cases, the life quality decreases dramatically. During the disease, immune cells attack the myelin of nerve cells, which reduces the conductive capacity of cells. Many factors, including; viral infections, vitamin D deficiency, polymorphism of vitamin D related genes, and many other environmental factors, are related to the disease development. However, none of them explains the background of the disease by itself because of the complexity of the disease. Twin studies show hereditary factors play a role in disease development, but it is not the only underlying reason. Some subjects do not develop the disease even with the same hereditary genetics. This point brings researchers to investigate environmental factors. Besides infections, studies about vitamin D and related genes seem strongly related to MS. Frequencies of the disease imply vitamin D deficiency might be a triggering factor for disease development. In addition, many studies show that vitamin D supplementation reduces the intensity and frequency of relapses or slows down disease progression. However, according to some other studies, vitamin D does not affect disease progression. Those controversial studies necessitate further investigations of the vitamin D and MS relationship.

rs6709815 (CYP27A1) and blood vitamin D relation was investigated in two different populations, but the results of that studies were statistically insignificant (Bu et al., 2010; Waterhouse et al., 2014). The correlation between rs4646536 (CYP27B1) and MS risk was examined in the Swedish population, and the polymorphic allele was found to increase MS risk (Sundqvist et al., 2010). According

to Orton et al., the mutant allele of rs1570669 (CYP24A1) was marked as a risk factor for MS, and Scazzone et al. claimed that the mutant allele was not shown any correlation with disease (Orton et al., 2011; Scazzone et al., 2021). rs731236 (TaqI) and rs7975232 (ApaI) of VDR were studied in many populations, including the Turkish population. The results of these two polymorphisms of VDR were controversial (Kamisli et al., 2018; Yucel et al., 2018; Čierny et al., 2016; Tizaoui et al., 2015). Therefore, the present study investigated the polymorphisms of selected genes that play a role in vitamin D metabolism in the Turkish population for the first time. In the Turkish population, two other studies investigated VDR's polymorphisms (rs731236 and rs7975232). However, the results of the Turkish population studies did not support each other (Kamisli et al., 2018; Yucel et al., 2018). In this study, our aim was investigation of the association between selected polymorphisms and MS. Except for VDR, selected other genes were CYPs (CYP27A1, CYP27B1, and CYP24A1) which are involved in the activation or deactivation of vitamin D. Our selected polymorphic regions and study population were the novelty of the research. The results of this study will be another brick in the wall for understanding the genetic background of MS. It is impossible to explain complete genetic factors of the disease with that complexity in a single study. However, by the research findings, it might be possible to turn the eyes of the researchers to the studied genes for in-depth investigations.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Blood Sampling

For this study, 187 MS patients and 140 control subjects' blood samples were collected with the collaboration of Gülhane Education and Research Hospital, Ankara, Turkey. Patients and control groups were selected from Central Anatolia, Turkey, and all were Caucasians. Patients were diagnosed according to 2010 McDonald Criteria. Those criteria include neurological examination, brain MRI scans, lumbar puncture, and blood tests. Patients in this study were selected preferably without any other significant disease, like carcinoma, failure in clotting, renal and hepatic failure, and no other neurological diseases. Control subjects were chosen randomly from a group of individuals who should not have any autoimmune or neurogenerative disease.

Patient and control group subjects were informed about our study, and consent forms were signed before any procedures were applied (Appendix A). Moreover, this study was approved by the Ethical Committee of Gülhane Education and Research Hospital (Appendix B) and was performed according to the principles of the Declaration of Helsinki (World Medical Association, 2014).

2.1.2 Chemicals

Agarose (A9539), ethidium bromide (E-7637), 2-amino-2(hydroxymethyl)-1,3-propanediol (Tris; T1378), Tris-Borate (348635), ethanol (24105) were purchased

from Sigma-Aldrich Chemical Company, Saint Louis, Missouri, USA. Ethylenediaminetetraacetic acid (EDTA; A5097) was acquired from Applichem GmbH, Germany. GeneJet whole blood genomic DNA isolation mini kit (#K0782), Taq DNA polymerase (provided with amplification buffer and MgCl2) (#EP0402), dNTP set (#R0181) GeneRuler 50bp DNA Ladder (#SM0371) were purchased from Thermo Fisher Scientific, Waltham, Massachusetts, USA. Sodium chloride (NaCl; 1.06400) and sodium hydroxide (NaOH; 06462) were the products of E. Merck, Darmstadt, Germany.

Restriction enzymes and buffers, TaqI, ApaI, BsmI, HphI, and BfuAI, were purchased from New England Biolabs, Ipswich, Massachusetts, USA. In this study, only analytical grade pure chemicals were used.

2.1.3 Primers

Primers were designed by using sequences of the genes at the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). SNPs were selected according to their allele frequencies. Selected polymorphic regions were shown in Table 2.1. Because the sample population was small and lower frequencies carry the risk of undetection, except CYP27B1, more than 20% minor allele frequencies (MAF) were selected. In CYP27B1, a rare variant was discovered at the rs4646536 which does not have any frequency information in databases.

Table 2. 1 SNPs, base change, frequencies and used restriction enzymes; *Region* identifies the selected rs numbers; *Polymorphism* indicates base changes; *MAF(%)* stands for minor allele frequency; *Restriction enzyme* defines the enzymes used for restriction endonuclease of specific base change at the position. (*) was used for marking the position that rare variant selection

Region	***Polymorphisms***	***MAF(%)***	***Restriction enzyme***
rs731236 (VDR)	A→G	%28	TaqI
rs7975232 (VDR)	C→A	%48	ApaI
rs1570669 (CYP24A1)	A→G	%47	HphI
rs6709815 (CYP27A1)	G→T	%37	BfuAI
rs4646536* (CYP27B1)	A→C	NA	BsmI

Primers for PCR were synthesized by Sentebiolab Ankara, Turkey. Stock primers diluted to 100 µM concentration. Stocks were stored at -20ºC. Before use, primers were aliquoted and diluted to 10 pmole/µl. Primer sequences are shown in Table 2.2.

Table 2. 2 Designed forward and reverse primer sequences for the selected five polymorphic regions and product sizes.

Region	***Primers***		***Product Size***
rs731236	Forward Primer	TCATGCTGCACTCAGGCTGGAAG	356 bp
(VDR)	Reverse Primer	CTCTATGACTGCTGACCGGTGATACC	
rs7975232	Forward Primer	GGTATCACCGGTCAGCAGTCATAGAG	748 bp
(VDR)	Reverse Primer	CCTGTCTGTTCCCTCAACATCAGTC	
rs1570669	Forward Primer	ACCTTGGAAGATACACTTTGC	430 bp
(CYP24A1)	Reverse Primer	CCAGAAGAGACCTTTGAATGG	
rs6709815	Forward Primer	GAATAGTACCCTCTGCATCCTCTGAGC	712 bp
(CYP27A1)	Reverse Primer	AGAACCCCAAACTTATCATGAGTTAGC	
rs4646536	Forward Primer	GCCTTTGTCCCTGTCTGAATATCCTC	641 bp
(CYP27B1)	Reverse Primer	GATCTGGAAAGGGAAGAAGGTGAGC	

2.2 Methods

2.2.1 Genomic DNA isolation, Spectrometric Quality and Quantity Controls

Genomic DNA isolation was performed by using GeneJet whole blood genomic DNA isolation mini kit according to the manufacturer's instructions supplied with the kit (Figure 2.1). Blood samples stored at -80ºC in EDTA-containing tubes were the starting material for DNA isolation.

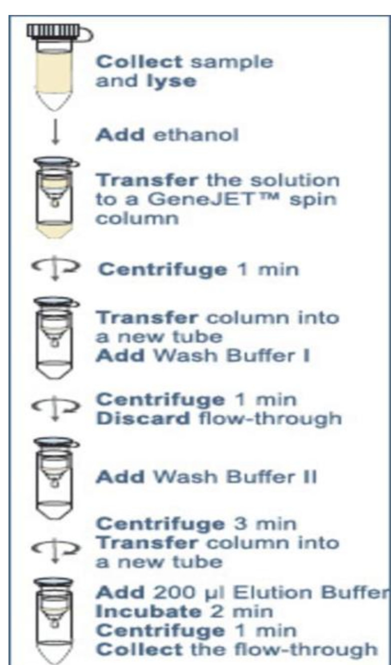


Figure 2.1 Genomic DNA isolation protocol provided by the manufacturer.

After isolation, the purity and concentrations of the samples were determined by Nanodrop™2000. According to spectrometric absorbance ratios, purity was detected. 260nm/280nm absorbance ratio is used for detecting the protein contaminant, and 1.8 is accepted as pure. 260nm/230nm ratio is also used for detecting phenolic contaminants, and the 2-2.2 range ratio is acceptable. The concentration of DNA is detected as µg/ml using the software of the instrument (Desjardins, P., & Conklin, D., 2010).

2.2.2 Qualification of DNA Samples by Agarose Gel Electrophoresis

Biogen horizontal gel electrophoresis system was used. 1% agarose gel was prepared for the system. The gel was prepared by dissolving 0.6 g agarose in 60 ml 0.5X TBE (Tris-Borate-EDTA) buffer at pH 8.3, which contains 45mM Tris, 45mM Boric Acid, and 1mM EDTA. The mixture was heated with a microwave oven until no solid particles remained in the solution. For viewing DNA under UV light, 3 µl ethidium bromide (0.5 µl/ml in the final mixture) was added to the solution while it was cooling down. While cooling, the mixture was continuously stirred to prevent unequal solidification. Then the solution was poured into a tray, and a comb was placed on it. All of the equipment was cleaned with 70% ethanol before use. While pouring the mixture, the air bubbles were avoid by help of a needle and then the gel let to wait at room temperature until it completely solidifies. The solid gel was placed in the chamber of the system afterwards and, 0.5X TBE was filled into the tank until it completely covered the gel.

10 µl of DNA samples was mixed with 1 µl 6X loading dye by pipetting and loaded into the wells of the gel. The first lane of the gel is separated for the DNA ladder. 100 V constant voltage was applied to the gel for running. Approximately 90 minutes of running was enough for clear visualization of samples. Next, the gel was viewed under UV light. Intact DNA was observed as a single band on the gel. If any degradation occurred, smear formation was seen.

2.2.3 Polymerase Chain Reactions

Polymerase chain reaction was conducted for amplification of the region of interest. By using specific primers defined in Table 2.2, reactions were conducted. Products of those reactions contain the specific SNPs (rs731236, rs7975232, rs1570669, rs6709815, rs4646536). PCR reactions were conducted with a final volume of 50 µl. The content of the PCR mix is shown in Table 2.3.

Table 2.3 Mixture content of PCR per tube.

Ingredient	Volume
Amplification Buffer	5 µl
25 mM $MgCl_2$	2 µl
dNTP Mix	1 µl
Forward Primer	1 µl
Reverse Primer	1 µl
Taq Polymerase (5U/µl)	0.5 µl
DNA template	2 µl
dH_2O	38.5 µl
Total	**50 µl**

Since each primer has different GC content, PCR reaction temperatures were adjusted accordingly (Table 2.4). Product size of each amplification reaction was checked by agarose gel electrophoresis, as defined in Section 2.2.2.

Table 2.4 PCR reaction conditions for 5 SNP regions VDR (rs731236, rs7975232), CYP27A1(rs6709815), CYP27B1(rs4646536), CYP24A1(rs1570669). “C” stands for the cycle part of the reaction. 35 repeats were done in the cycle.

	VDR rs731236 (TaqI)		VDR rs7975232 (ApaI)		CYP24A1 rs1570669 (HphI)		CYP27A1 rs6709815 (BfuAI) CYP27B1 rs6709815 (BsmI)	
	Temperature	Time	Temperature	Time	Temperature	Time	Temperature	Time
Pre-denaturation	95°C	3 min	95°C	3 min	95°C	3 min	95°C	5 min
Denaturation (C)	95°C	20 sec	95°C	30 sec	95°C	30 sec	95°C	30 sec
Annealing (C)	62°C	30 sec	60°C	30 sec	56°C	30 sec	61°C	30 sec
Extension (C)	72°C	30 sec	72°C	30 sec	72°C	30 sec	72°C	50 sec
Final Extension	72°C	5 min	72°C	10 min	72°C	5 min	72°C	5 min

2.2.4 Restriction Endonuclease Digestion Reactions

Restriction fragment length polymorphism (RFLP) was used to detect the SNPs on the target site of the gene. Five different restriction enzymes were used for five different polymorphisms. Those enzymes are specific for a sequence on the DNA and recognize that site, and cut DNA from a defined position. Manufacturer's orders were followed for the reactions. Their cut sites and product sizes after the reaction are summarized in Table 2.5.

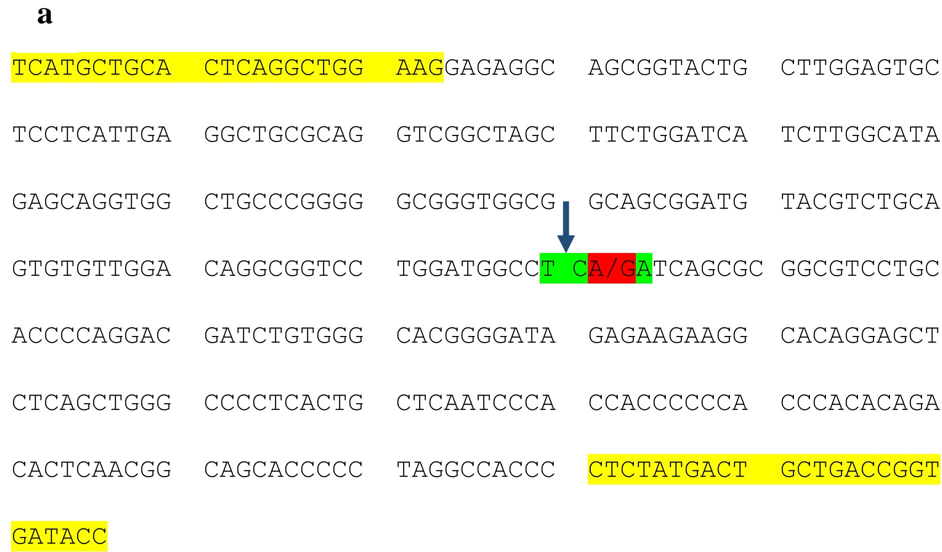
Table 2.5 Restriction enzymes used in RFLP studies, their recognition sites on the DNA, SNPs, alleles, fragment numbers after reaction, and the product sizes according to allele type.

Restriction Enzyme	Recognition Site	SNP	Allele	Number of Fragments	Sizes of Fragments
TaqI	5′…T▼CGA…3′ 3′…AGC▲T…5′	rs731236 (VDR)	A	1	356bp
			G	2	174bp/182bp
ApaI	5′…GGGCC▼C…3′ 3′…C▲CCGGG…5′	rs7975232 (VDR)	C	2	94bp/654bp
			A	1	748bp
HphI	5′…GGTGA(N)$_8$▼…3′ 3′…CCACT(N)$_7$▲…5′	rs1570669 (CYP24A1)	G	4	20bp/31bp/133bp/246bp
			A	3	20bp/31bp/379bp
BfuAI	5′…ACCTGC(N)$_4$▼…3′ 3′…TGGACG(N)$_8$▲…5′	rs6709815 (CYP27A1)	G	2	475/237bp
			T	1	712bp
BsmI	5′…GAATGCN▼…3′ 3′…CTTAC▲GN…5′	rs4646536 (CYP27B1)	A	1	641bp
			C	2	246bp/395bp

2.2.4.1 Restriction Endonuclease Digestion of rs731236 with TaqI and Genotyping

TaqI enzyme was used for detecting the base change of rs731236 on the VDR. In the ancestral form, wild type allele is A, and mutant allele G replaces it in the polymorphic individual. So the region of the cut site is TCAA in the ancestral form, and if the region is polymorphic, it becomes TCGA which is the recognition

sequence of the TaqI enzyme. Amplified region and schematic cut position was shown in Figure 2.2.



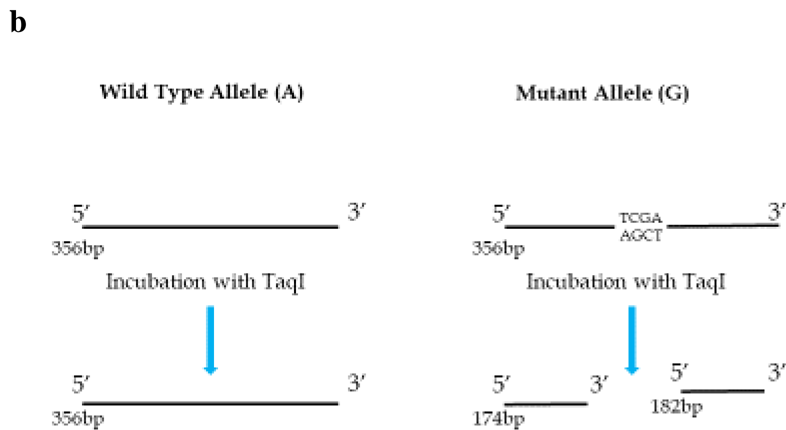


Figure 2.2 a) Amplified region after PCR reactions. Primers are indicated with yellow, restriction site is indicated with green and SNP is shown with red. Blue arrow points the cut position. b) Expected results after incubation with TaqI enzyme.

The homozygous wildtype (AA) genotype was expected to be as a single band, the homozygous polymorphic (GG) was expected to be seen as 2 bands, and the heterozygous (AG) genotype was expected to be seen as 3 bands on the gel (Figure 2.3). However, fragmented products were so close in size to each other. So they could seem like a thicker 1 band on the gel. For the reaction, 5 µl of PCR product, 9 µl dH2O, 1 µl 10X rCutSmart™ Buffer, and 0.5 µl TaqI enzyme were mixed and incubated at 65ºC for one hour. Then the products were loaded on the gel, and the results were visualized under the UV.

Figure 2.3 Expected gel images for 3 different genotypes of rs731236:

AA homozygous wild type, GG homozygous polymorphic and, AG heterozygous genotype.

2.2.4.2 Restriction Endonuclease Digestion of rs7975232 with ApaI and Genotyping

ApaI enzyme was used for detecting the base change of rs7975232 on the VDR. In the ancestral form, wild type allele is C, and mutant allele A replaces it in the polymorphic individual. So the region of the cut site is GGGCCC in the ancestral form, and if the region is polymorphic, it becomes GGGCAC which is not recognized by the enzyme. Amplified region and schematic cut position was shown in Figure 2.4.

a

```
CCTGTCTGTT  CCCTCAACAT  CAGTCAGCAG  CCACTTAGGC  AGCGGTGGAG
GCATCTCTGG  GCAAGGCCCT  GCCTCCAGCC  TCTGCCCTCT  GCCCCCACTT
GGGTTTCTTT  GTCAAACAAA  CAGCAACTCC  TCATGGCTGA  GGTCTCAAGG
GACCGGGGAA  AAGCCCGCAG  GAAAGGGGTT  AGGTTGGACA  GGAGAGAGAA
TGGGCTGGGT  GGATAGGGGA  GGTGGCAGAG  GAGGGGCTGA  ACCCCAGACG
GGGTGAGGAG  GGCTGCTGAG  TAGCCGCCAG  CCCCGGGCCT  GGCACGTGGC
CCTGGAGGAG  CAGCCCCACC  CAGGCACCGC  CACAGGCTGT  CCTAGTCAGG
AGATCTCATT  GCCAAACACT  TCGAGCACAA  GGGGCGTTAG  CTTCATGCTG
CACTCAGGCT  GGAAGGAGAG  GCAGCGGTAC  TGCTTGGAGT  GCTCCTCATT
GAGGCTGCGC  AGGTCGGCTA  GCTTCTGGAT  CATCTTGGCA  TAGAGCAGGT
GGCTGCCCGG  GGGCGGGTGG  CGGCAGCGGA  TGTACGTCTG  CAGTGTGTTG
GACAGGCGGT  CCTGGATGGC  CTCAATCAGC  GCGGCGTCCT  GCACCCCAGG
ACGATCTGTG  GGCACGGGGA  TAGAGAAGAA  GGCACAGGAG  CTCTCAGCTG
GGCC/ACCTCAC  TGCTCAATCC  CACCACCCCC  CACCCACACA  GACACTCAAC
GGCAGCACCC  CCTAGGCCAC  CCCTCTATGA  CTGCTGACCG  GTGATACC
```

b

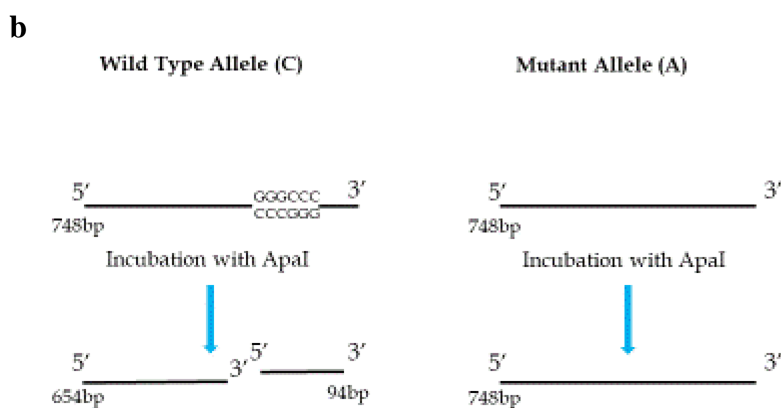


Figure 2.4 a) Amplified region after PCR reactions. Primers are indicated with yellow, restriction site is indicated with green and SNP is shown with red. Blue arrow points the cut position. b) Expected results after incubation of ApaI enzyme.

The homozygous wildtype (CC) genotype was expected to be seen as 2 bands, the homozygous polymorphic (AA) was expected to be seen as a single band, and the heterozygous (CA) genotype was expected to be seen as 3 bands on the gel (Figure 2.5). For the reaction, 5 µl of PCR product, 9 µl dH2O, 1 µl 10X rCutSmart™ Buffer, and 0.5 µl ApaI enzyme were mixed and incubated at 37ºC for 1-16 hours. In our studies, overnight incubation was preferred. Then the products were loaded on the gel, and the results are visualized under the UV.

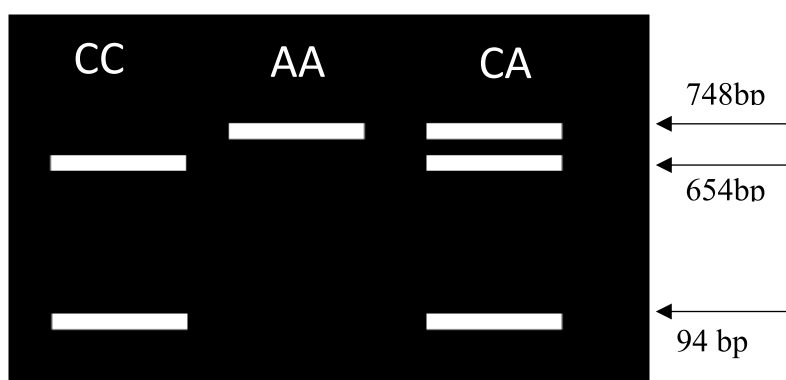


Figure 2.5 Expected gel images for 3 different genotypes of rs7975232:

CC homozygous wild type, AA homozygous polymorphic, and CA heterozygous genotype.

2.2.4.3 Restriction Endonuclease Digestion of rs1570669 with HphI and Genotyping

HphI enzyme was used for detecting the base change of rs1570669 on the CYP24A1. In the ancestral form, wild type allele is A, and mutant allele G replaces it in the polymorphic individual. So the region of the cut site is GATGA in the ancestral form, and if the region is polymorphic, it becomes GGTGA which is recognized by the enzyme. Amplified region and schematic cut position was shown in Figure 2.6.

a

```
ACCTTGGAAG  ATACACTTTG  CAAATATTCA  CCAGATCTCC  CTAGACTTTG
TACTGGAAAA  TATTCATTTT  GGGAATGCAG  AGATGGTAGA  GAATAGTTGA
CATGTTGCAT  AGTATAGAAT  TAGAATTCAC  GGCTATGGAG  ACAGAGACCT
GCATTCAGTT  TCAC/TCTTTTA TCTGTGTTGT  CTTGAGTGAG  TTACATAATC
TCTGTGAGCC  TCAACTTCGT  CTGTAAAAGG  GGTTTAATAA  CAGAATCTGC
TGCGCAGGGT  GGTTGTGAGT  ATGGTGCACA  ATTCTAAAGC  CCTTTACCTA
TTTATTACCT  GGCACATTTT  AAGCACTCAG  CGCATGTTGG  CAACCATCAT
TATTATAAAT  TTGTGGATTT  GGCTTTTGAT  TTGTAAGGTG  ACAACTTGTG
TCAATTTTGC  CATTCAAAGG  TCTCTTCTGG
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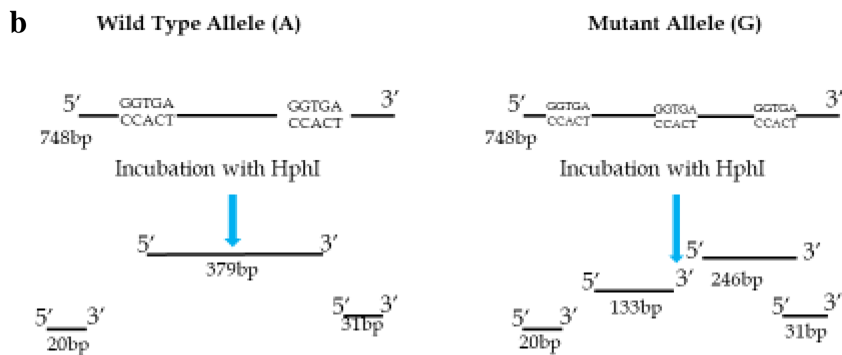

Figure 2.6 a) Amplified region after PCR reactions. Primers are indicated with yellow, restriction site is indicated with green and SNP is shown with red. Blue arrow points the cut position. b) Expected results after incubation of HphI enzyme.

The homozygous polymorphic (GG) genotype was expected to be seen as 2 bands, the homozygous wildtype (AA) was expected to be seen as 1 band, and the heterozygous (AG) genotype was expected to be seen as 3 bands on the gel (Figure 2.7). For the reaction, 5 µl of PCR product, 9 µl dH2O, 1 µl 10X rCutSmart™ Buffer, and 0.5 µl HphI enzyme were mixed and incubated at 37ºC for 1 hour. Then the products were loaded on the gel, and the results were visualized under the UV.

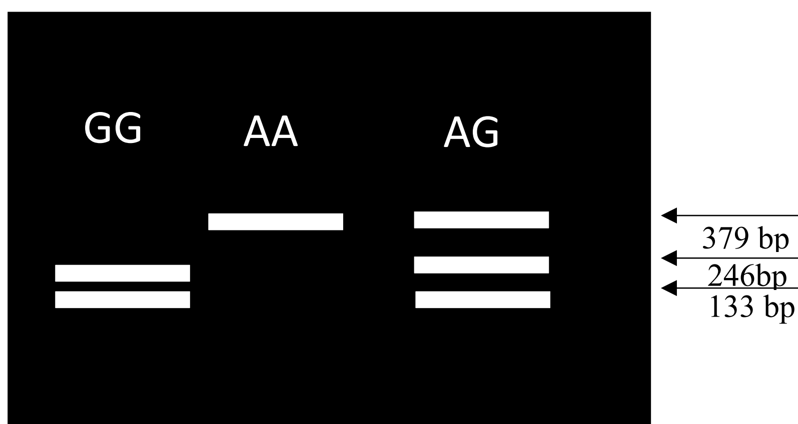

Figure 2.7 Expected gel images for 3 different genotypes of rs1570669:
GG homozygous polymorphic, AA homozygous wild type, and AG heterozygous genotype.

2.2.4.4 Restriction Endonuclease Digestion of rs6709815 with BfuAI and Genotyping

BfuAI enzyme was used for detecting the base change of rs6709815 on the CYP27A1. In the ancestral form, wild type allele is G, and mutant allele T replaces it in the polymorphic individual. So the region of the cut site is GCAGGT in the ancestral form, and if the region is polymorphic, it becomes GCATGT which is not recognized by the enzyme. Amplified region and schematic cut position was shown in Figure 2.8.

a

```
AGAACCCCAA  ACTTATCATG  AGTTAGCAGA  ATAGCTTTTT  TTCCCCAGTC
ATCTGACTCA  AAGATTCACA  AAAGAGCTTT  TAAATGCATG  CATATGATAT
CATCTATTCA  GGAAACTGGC  TTTAGAGAAA  AAAATGTAAA  TAGATTACAA
AGGGCTCTAC  ATAATGTTTA  GCTCCGATTC  TAAATCTGAG  CTGTCATGTC
ATAGGTGGCC  TGGCCCAGTG AAAACC/ATGCA ACCTAAATCT  TACCCTCAGT
GGCAGATGTC  AAGTTAAAAA  GTTTTTTTTT  TTTTCTTTTT  TGAGATGGAG
TCTCACTCTG  TTGCCCAGGC  TGGAGTGCAG  TGGCATGATC  TCGGCTCACT
GCAACCTCCA  CCTCCTGGGT  TCAAACGATC  CTCCTGCCTC  AGCCTCCCAA
GTAGCTGGGA  TTACAGGTGC  CCACCACCAC  ACCTGGCTAA  TTTTTGTGTT
TTTAGTAGAG  ACGGGGTTTC  ACCGTGTTGG  CCAAGCTGAT  CTCGAACCCC
TGAACTCAAG  TGATCCGCCC  ACCTCAGCCT  CCCAAAGTGC  TGGGATTACA
GGTGTGGTTT  TTGCCATAAT  TTTAAATGGT  GAAAACCACA  ATGACTTTTG
CACCAACCTA  ATATTTATTA  GTGGAGAGCC  AATGAATGGT  CCCTGGGCTT
CAGCACCTCC  CAACAACTGC  TCTCTAGACC  ATCAGGCTCA  GAGGATGCAG
AGGGTACTAT  TC
```

b

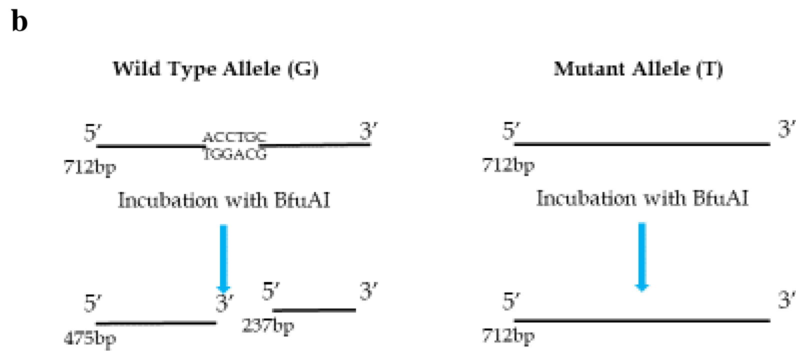

Figure 2.8 a) Amplified region after PCR reactions. Primers are indicated with yellow, restriction site is indicated with green and SNP is shown with red. Blue arrow points the cut position. b) Expected results after incubation of BfuAI enzyme.

The homozygous wildtype (GG) genotype was expected to be seen as a double band, the homozygous polymorphic (TT) was expected to be seen as 1 band, and the heterozygous (GT) genotype was expected to be seen as 3 bands on the gel (Figure 2.9). For the reaction, 5 µl of PCR product, 9 µl dH2O, 1 µl 10X

rCutSmart™ Buffer, and 0.5 µl BfuAI enzyme were mixed and incubated at 37ºC for 1 hour. Then the products were loaded on the gel, and the results were visualized under the UV.

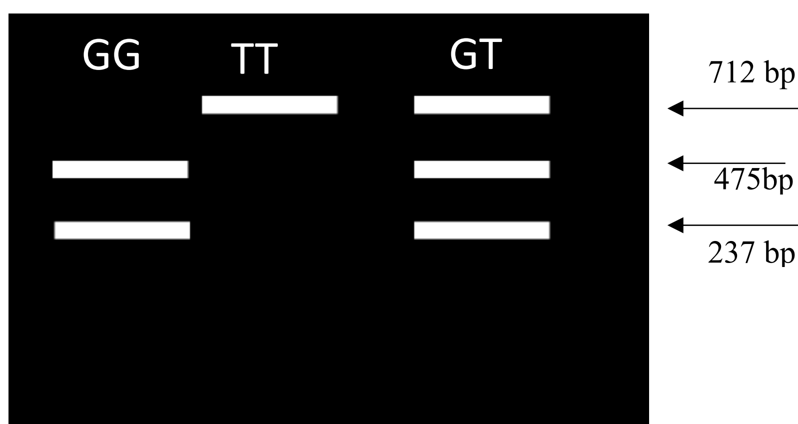

Figure 2.9 Expected gel images for 3 different genotypes of rs6709815:
GG homozygous wild type, TT homozygous polymorphic, and GT heterozygous genotype.

2.2.4.5 Restriction Endonuclease Digestion of rs4646536 with BsmI and Genotyping

BsmI enzyme was used for detecting the base change of rs4646536 on the CYP27B1. In the ancestral form, wild type allele is A, and mutant allele G replaces it in the polymorphic individual. In the rare variant A is replaced with C. The region of the recognition site is CTTACT in the ancestral form, and if the region is polymorphic, it becomes CTTACG which is recognized by the enzyme. Amplified region and schematic cut position was shown in Figure 2.10.

a

```
GCCTTTGTCC   CTGTCTGAAT   ATCCTCTTCT   TCATGCCTGC   CCTATTCTGA
GCCCAAACTG   ACAAGTTTGT   TTTCCTTTGC   ACCAGACGCT   GGTCACTCTG
TGTCACTATG   CCACTTCAAG   GGACCCTGCC   CAGTTCCCAG   AGCCAAATTC
TTTTCGTCCA   GCTCGCTGGC   TGGGGGAGGG   TCCCACCCCC   CACCCATTTG
CATCTCTTCC   CTTTGGCTTT   GGCAAGCGCA   GCTGTATGGG   GAGACGCCTG
GCAGAGCTTG   AATTGCAAAT   GGCTTTGGCC   CAGGTGAGTG   CTCTAGATTT
TATACCTTCC   CCAGACTGGA   GAGACCCTAA   CCCTCTAAAG   TTGTGAGCTC
TTTCCCCTGA   CAAGCATAGG   AAATCATATA   AGACCTGGTA   GAATGA/CATCT
TCTGAAATAT   GATAAGCCCA   TTATAGGCCT   GGAGTGTAAG   TGAGGGTATT
CAAACTATTT   TTTCCCTACC   ATAATCCCTC   ACCCTTATTA   ACCAAGAAGT
CCCCTACTGG   CCACAGGTGC   CACCCAATCA   TTGACCATTC   TAACACTAAT
AATGCATGCC   CTTTACCAAT   TGGATTACCA   ATGAATCCCC   CCATTATAGA
TATCTTTCAT   AGTAATGCTC   ACCTTCTTCC   CTTTCCAGAT   C
```

b

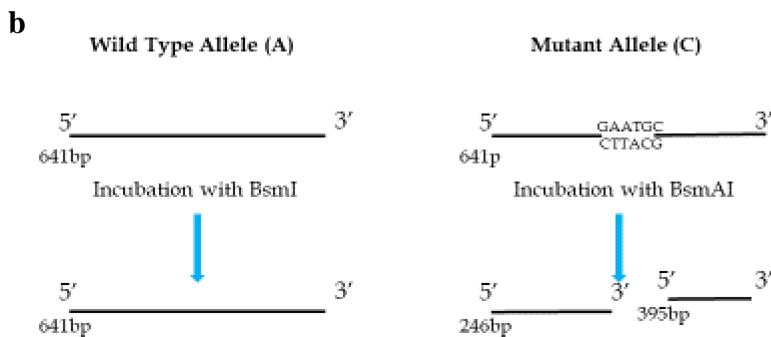

Figure 2.10 a) Amplified region after PCR reactions. Primers are indicated with yellow, restriction site is indicated with green and SNP is shown with red. Blue arrow points the cut position. b) Expected results after incubation of BsmI enzyme.

The homozygous wildtype (AA) genotype was expected to be seen as a single band, the homozygous polymorphic (CC) was expected to be seen as a double band, and the heterozygous (AC) genotype was expected to be seen as 3 bands on the gel (Figure 2.11). For the reaction, 5 µl of PCR product, 9 µl dH2O, 1 µl 10X

rCutSmart™ Buffer, and 0.5 µl BsmI enzyme were mixed and incubated at 65ºC for 1 hour. Then the products were loaded on the gel, and the results were visualized under the UV.

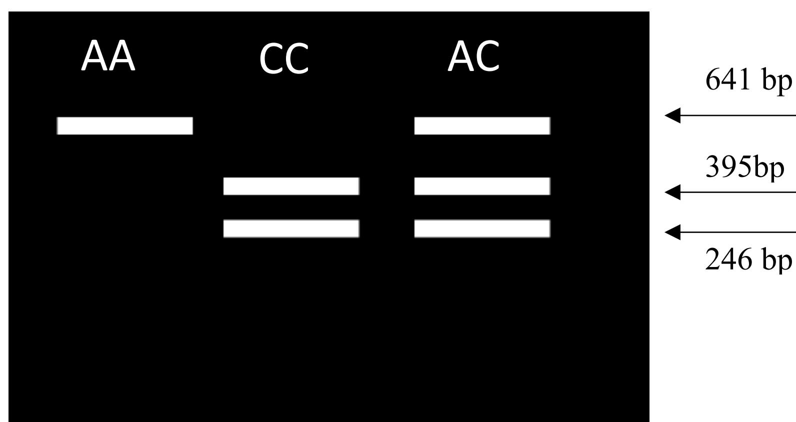

Figure 2.11 Expected gel images for 3 different genotypes of rs4646536:
AA homozygous wild type, CC homozygous polymorphic, and AC heterozygous genotype.

2.2.5 Statistical Analysis

Statistical analyses were performed using SPSS (Statistical Package for Social Sciences, IBM Corporation version 28.0.1). Allele frequencies were calculated by gene counting. Due to samples being categoric, chi-square was used to determine the departure from Hardy-Weinberg equilibrium and also in order to compare genotype distributions and allele frequencies. The selection of Pearson's chi-Square test or Fischer's exact test was based on the number of subjects. If the sample size was lesser than 5, Fischer's exact test was applied. Three hypothetical groups were formed for analysis. According to groups, three different models were used for comparison. Those models were additive, dominant, and recessive. In the additive model, the mutant allele was accepted as risky, and heterozygote and homozygote mutant individuals were compared to homozygote wild type individuals one by one. In the dominant model, mutant allele carrier individuals and in the recessive model, wild type allele carrier individuals were grouped and compared according to those

groups. Also, odds ratios (OR) were calculated according to that groups. Moreover, age-adjusted OR were calculated by SNPstat (Sole, X., et al., 2006). For age adjustment, only the samples with age information were used in analyzing.

p-value was used for the significance evaluation of the results. If the *p*-value was less than 0.05, the results were accepted as statistically significant, and vice versa; if the *p*-value was higher than 0.05, the results were accepted as statistically insignificant.

Power analysis of samples was done with the Genetic Association Study (GAS) Power Calculator (Home | GAS Power Calculator, 2017). Power was calculated for selected polymorphisms' highest and lowest minor allele frequencies. For the highest minor allele frequency (MAF), the calculated power was %80; for the lowest MAF, the calculated power was %76 with 187 patients and 140 control subjects.

CHAPTER 3

RESULTS

3.1 Study Population

187 patients and 140 healthy control subjects were included in this study. The age distribution of populations is varied from 23 to 74. Although age is important for the disease onset, in our study we worked on somatic cells and they carry the same genetic information from birth to death. So, age of the groups were not compared to each other, and the the variation by age among subjects is neglected. Genotyping of some samples could not be done in control group due to limitation of blood samples. Because some samples were taken to regular blood collection tubes and after first isolation it could not be possible to isolate DNA from the blood clots. This changed the group's sizes. After those miscollected samples, control groups shaped as 139 for VDR-TaqI, 137 for VDR-ApaI, 131 for CYP24A1, 140 for CYP27A1 and 136 for CYP 27B1. Gender distribution in patient group is 130 females and 57 males and in control group 78 females and 62 males. Demografic information given in Table 3.1

Table 3. 1 Demografic information of study population.

Parameter	Patients (n:163)	Controls (n:107)	*p*
Age (years)	mean ± SD: 43.8 ± 9.9 median ± IQR: 44.0 ± 14.0	mean ± SD: 36.5 ± 8.8 median ± IQR: 36.0 ± 11.0	<0.001
Gender, n (%)	Female: 130 (69.5%) Male: 57 (30.5%)	Female: 78 (55.7%) Male: 62 (44.3%)	0.01

3.2 Genotyping and Analysis of SNPs

All SNPs were genotyped by PCR-RFLP method. Regions containing the polymorphic base were amplified by PCR. Then PCR products were digested with restriction enzymes which are specific to SNPs. Next, products of digestion were investigated with agarose gel electrophoresis.

After visualization of band patterns on gel, genotypes were determined, and the results were analyzed with additive, dominant and recessive models. Additive model created by acceptance of each allele having equal contribution on heterozygous individuals. In dominant model, mutant allele was accepted as dominant to wild type allele and in recessive model, wild type allele was accepted as dominant. For testing these models different groups were formed. In additive model, each genotype was formed a group. In dominant model, according to acceptance of dominant character of mutant allele, carriers of mutant allele were grouped together. In contrast to dominant model, in recessive model, wild type carriers were grouped together. Odds ratio (OR) was calculated by using (a/b) / (c/d) formula where a is the number of patients with risky allele, b is the number of patients without risky allele, c is the number of control subjects with risky allele and d is the number of control subjects without risky allele. OR calculations were performed according to models because risky allele assumption was change according to model.

3.2.1 Genotyping and Analysis of VDR

On the VDR gene two SNPs, rs7975232 and rs731236 were analyzed. rs7975232 was digested with the ApaI enzyme so it was named as VDR-ApaI and rs731236 was digested with the TaqI enzyme and it was named as VDR-TaqI.

3.2.1.1 Genotyping and Analysis of VDR-ApaI

rs7975232 is one of the most studied SNP on VDR. It is an intron variant “C” that transforms to “A” and the MAF of “C” is 0.48. Initially genomic DNA was amplified

with specific primers and the product of that reaction was a 748 bp DNA fragment in the gel as shown in Figure 3.1.

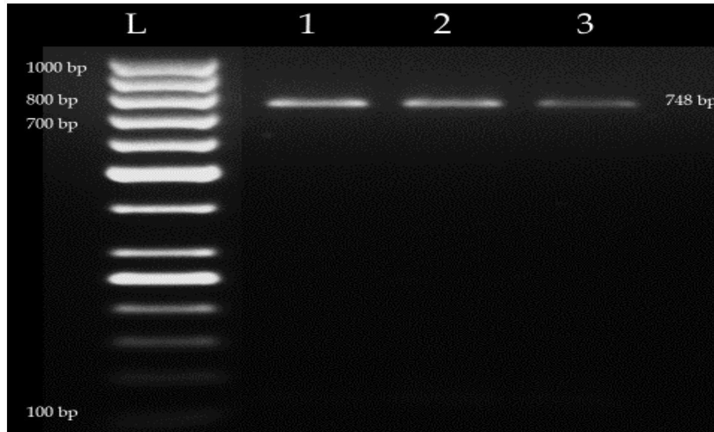

Figure 3. 1 Representative agarose gel (1%) image of rs7975232 PCR products. L is 100 bp ladder. Other Lanes (1-2-3) are the PCR products.

Then PCR products were digested with the ApaI enzyme. “C” is the wild type allele and provide a recognition site for the enzyme. It transforms to “A” in the polymorphic cases which disrupts the recognition site. In “C” allele 654 bp and 94 bp DNA fragments were produced. In “A” allele DNA remain non-digested due to lack of recognition site and only 748 bp DNA fragment was observed. Heterozygous samples are represented on the gel with the 748 bp, 654 bp and 94 bp DNA fragments. Three different genotypes are represented in the Figure 3.2.

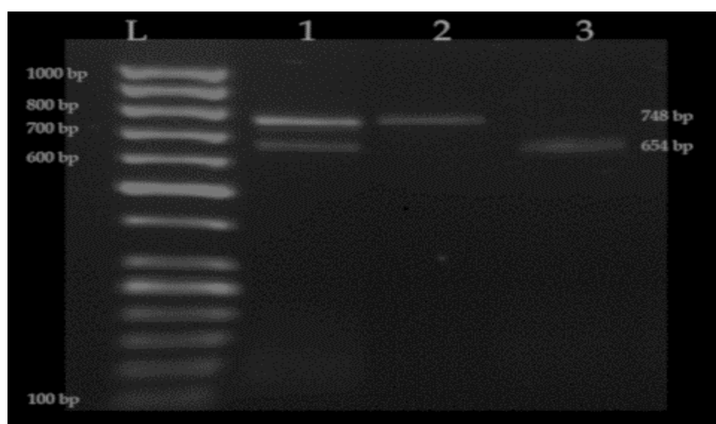

Figure 3. 2 Representative agarose gel (1%) image of rs7975232 restriction endonuclease digestion results. L is 100 bp ladder. Lanes 1, 2, and 3 represent CA, AA and CC genotypes, respectively.

By using gel images, genotyping of the samples were conducted. For rs7975232, 137 controls and 187 patients were studied. Allele distribution of samples and analysis results were shown in the Table 3.2. In patient group, 9 CC (5%), 109 CA (58%), 69 AA (37%) and, in control group, 5 CC (4%), 84 CA (61%), 48 AA (35%) individuals were observed. Frequencies are 0.340 for "C" allele and 0.660 for "A" allele in patient group, and 0.343 for "C" allele and 0.657 for "A" allele in control group.

In additive model, wild type (CC) compared with heterozygous (CA) and homozygous (AA) polymorphic individuals by Fischer's exact test. No statistically significant difference was observed (For CC vs CA, p=0.781, for CC vs AA, p=0.780). In dominant model, polymorphic allele carrier individuals (n:178 in patients and n:132 in control) were compared with the homozygous wild type individuals (n:9 in patients and n:5 in controls). According to the dominant model, comparisons were done with the Fischer's exact test, and no statistically significant difference was obtained (p=0.784). In recessive model, homozygous polymorphic individuals (n:69 in patients and n:48 in controls) compared with the dominant allele carrier individuals (n:118 in patients and n:89 in controls). According to recessive model, no statistically significant difference was obtained ($\chi2(1, N=324)=0.119$, $p=0.730$). Allele frequencies of both the groups were also compared and no statistically significant result was obtained ($\chi2(1, N=648)=0.09$, $p=0.926$).

Table 3. 2 Genotypes and allele frequency distribution of rs7975232 (VDR-ApaI) for patients and controls.

Genotype/Allele	**Patients (n:187) (n, %)**	**Controls (n:137) (n, %)**	**Model**	**OR (95% CI)**	***p*-values**	**Age Adjusted OR (95% CI) (n:270)**	**Adjusted *p*-values (n:270)**
CC	9 (5%)	5 (4%)	**Additive[a]**	Reference	Reference	Reference	Reference
CA	109 (58%)	84 (61%)		0.721 (0.233-2.231)	0.781 [2]	0.326 (0.068-1.561)	0.161
AA	69 (37%)	48 (35%)		0.799 (0.252-2.531)	0.780 [2]	0.325 (0.067-1.583)	0.164
CC	9 (5%)	5 (4%)	**Dominant[b]**	0.749 (0.245-2.287)	0.784 [2]	0.326 (0.069-1.539)	0.157
CA+AA	178 (95%)	132 (96%)					
CC+CA	118 (63%)	89 (65%)	**Recessive[c]**	1.084 (0.685-1.717)	0.730 [1]	0.9377 (0.5669-1.5510)	0.802
AA	69 (37%)	48 (35%)					
C	0.340	0.343	**Allele frequency[d]**	1.016 (0.731-1.411)	0.926 [1]	0.8899 (0.6162-1.2852)	0.534
A	0.660	0.657					

[a]: CC vs CA and CC vs AA, [b]: CC vs CA+AA, [c]: CC+CA vs AA, [d]: C vs A ([1]: Pearson's chi-square test, [2]: Fischer's exact test were applied)

3.2.1.1.1 Female Subgroup Analysis of VDR-ApaI

In VDR-ApaI female subgroup, 130 patients and 76 control subjects were analyzed. Allele distribution of samples and analysis results were shown in the Table 3.3. In patient group, 7 CC (5.4%), 78 CA (60%), 45 AA (34.9%) and, in control group, 2 CC (2.6%), 51 CA (67.1%), 23 AA (30.3%) individuals were observed. Frequencies are 0.354 for “C” allele and 0.646 for “A” allele in patient group, and 0.362 for “C” allele and 0.638 for “A” allele in control group.

In additive model, wild type (CC) compared with heterozygous (CA) and homozygous (AA) polymorphic individuals by Fischer’s exact test. No statistically significant difference was observed (For CC vs CA, p=0.482, for CC vs AA, p=0.710). In dominant model, polymorphic allele carrier individuals (n:123 in patients and n:74 in control) were compared with the homozygous wild type individuals (n:7 in patients and n:2 in controls). According to the dominant model, comparisons were done with the Fischer’s exact test, and no statistically significant difference was obtained (p=0.490). In recessive model, homozygous polymorphic individuals (n:45 in patients and n:23 in controls) compared with the dominant allele carrier individuals (n:85 in patients and n:53 in controls). According to recessive model, no statistically significant difference was obtained ($\chi 2(1, N=206)=0.411$, $p=0.522$). Allele frequencies of both group were also compared and no statistically significant result was obtained ($\chi 2(1, N=412)=0.027$, $p=0.870$).

Table 3.3 Genotypes and allele frequency distribution of female subgroup of rs7975232 (VDR-ApaI) for patients and controls.

Genotype/Allele	Patients (n:130) (n, %)	Controls (n:76) (n, %)	Model	OR (95% CI)	*p*-values
CC	7 (5.4%)	2 (2.6%)	**Additive**[a]	Reference	Reference
CA	78 (60%)	51 (67.1%)		0.437 (0.087-2.187)	0.482 [2]
AA	45 (37%)	23 (30.3%)		0.559 (0.107-2.910)	0.710 [2]
CC	7 (5.4%)	2 (2.6%)	**Dominant**[b]	0.475 (0.096-2.347)	0.490 [2]
CA+AA	123 (94.6%)	74 (97.4%)			
CC+CA	85(63%)	53 (69.7%)	**Recessive**[c]	1.220 (0.664-2.241)	0.522 [1]
AA	45 (34.6%)	23 (30.3%)			
C	0.354	0.362	**Allele frequency**[d]	1.035 (0.682-1.571)	0.870 [1]
A	0.646	0.638			

[a]: CC vs CA and CC vs AA, [b]: CC vs CA+AA, [c]: CC+CA vs AA, [d]: C vs A ([1]: Pearson's chi-square test, [2]: Fischer's exact test were applied)

3.2.1.1.2 Male Subgroup Analysis of VDR-ApaI

In VDR-ApaI male subgroup, 57 patients and 61 control subjects were analyzed. Allele distribution of samples and analysis results were shown in the Table 3.4. In patient group, 2 CC (3.5%), 31 CA (54.4%), 24 AA (42.1%) and, in control group, 3 CC (4.9%), 33 CA (54.1%), 25 AA (41%) individuals were observed. Frequencies are 0.307 for "C" allele and 0.693 for "A" allele in patient group, and 0.320 for "C" allele and 0.680 for "A" allele in control group.

In the additive model, wild type (CC) compared with heterozygous (CA) and homozygous (AA) polymorphic individuals by Fischer's exact test. No statistically significant difference was observed (For CC vs CA, p=1, for CC vs AA, p=1). In dominant model, polymorphic allele carrier individuals (n:55 in patients and n:58 in control) were compared with the homozygous wild type individuals (n:2 in patients and n:3 in controls). According to the dominant model, comparisons were done with the Fischer's exact test, and no statistically significant difference was obtained (p=1). In recessive model homozygous polymorphic individuals (n:24 in patients and n:25 in controls) compared with the dominant allele carrier individuals (n:33 in patients and n:36 in controls). According to the recessive model, no statistically significant difference was observed ($\chi 2(1, N=118)=0.015$, $p=0.902$). Allele frequencies of both group were also compared and no statistically significant results were found ($\chi 2(1, N=236)=0.044$, $p=0.834$).

Table 3.4 Genotypes and allele frequency distribution of male subgroup of rs7975232 (VDR-ApaI) for patients and controls.

Genotype/Allele	**Patients (n:57) (n, %)**	**Controls (n:61) (n, %)**	**Model**	**OR (95% CI)**	***p*-values**
CC	2 (3.5%)	3 (4.9%)	**Additive**[a]	Reference	Reference
CA	31 (54.4%)	33 (54.1%)		1.409 (0.220-9.008)	1.000 [2]
AA	24 (42.1%)	25 (41%)		1.440 (0.221-9.388)	1.000 [2]
CC	2 (3.5%)	3 (4.9%)	**Dominant**[b]	1.422 (0.229-8.839)	1.000 [2]
CA+AA	55 (96.5%)	58 (95.1%)			
CC+CA	33 (57.9%)	36 (59%)	**Recessive**[c]	1.047 (0.503-2.179)	0.902 [1]
AA	24 (42.1%)	25 (41%)			
C	0.307	0.320	**Allele frequency**[d]	1.061 (0.612-1.839)	0.889 [1]
A	0.693	0.680			

[a]: CC vs CA and CC vs AA, [b]: CC vs CA+AA, [c]: CC+CA vs AA, [d]: C vs A ([1]: Pearson's chi-square test, [2]: Fischer's exact test were applied)

3.2.1.2 Genotyping and Analysis of VDR-TaqI

rs731236 is one of the highly studied SNP on VDR. It is an exon synonymous variant "A" that transforms to "G" and MAF of "G" is 0.28. Genomic DNA was amplified with specific primers and the product of PCR reaction was 356 bp DNA fragment in the gel as shown in Figure 3.3. Then PCR products were digested with the TaqI enzyme. "A" is the wild type allele and it transforms to "G" in the polymorphic cases which provide a recognition site for the enzyme. In "G" allele, 174 bp and 182 bp DNA fragments were produced. In "A" allele, DNA remain non-digested due to lack of recognition site and only 356 bp DNA fragment was observed. Heterozygous samples were represented on the gel with the 356 bp 174 bp and 182 bp DNA fragments.

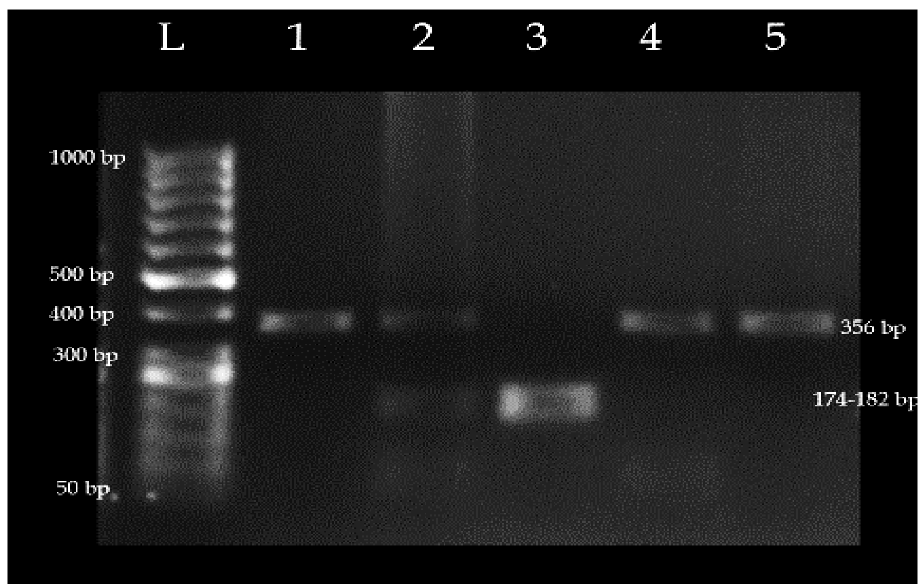

Figure 3.3 Representative agarose gel (1%) image of rs731236 restriction endonuclease digestion results and PCR products. L is 50 bp ladder. Lanes 1, 2 and 3 represent AA, AG and GG genotypes, respectively. Lanes 4 and 5 are the PCR products.

By using gel images, genotyping of the samples were conducted. For rs731236, 139 controls and 187 patients were studied. Allele distribution of samples and analysis results were shown in the Table 3.5. In patient group 79 AA (42.2%), 83 AG (44.4%), 25 GG (13.4%), and in control group 44 AA (31.7%), 72 AG (51.8%), 23 GG (16.5%) individuals were observed. Frequencies are 0.644 and 0.576 for “A” allele, and 0.356 and 0.424 for “C” allele in patients and controls, respectively.

In the additive model, wild type (AA) compared with heterozygous (AG) and homozygous (GG) polymorphic individuals by chi-square. AA vs AG comparison was found to be insignificant with the $\chi 2(1, N=278)=3.217$, $p=0.073$. AA vs GG comparison was also insignificant with the $\chi 2(1, N=171)=2.137$, $p=0.144$. In the dominant model, polymorphic allele carrier individuals (n:108 in patients and n:95 in controls) were compared with the homozygous wild type (n:79 in patients and n:44 in controls) individuals. According to the dominant model result was borderline significant ($\chi 2(1, N=326)=3.807$, $p=0.051$). In the recessive model, homozygous polymorphic individuals (n:25 in patients and n:23 in controls) were compared with the dominant allele carrier individuals (n:162 in patients and n:116 in controls). Result of the analysis was insignificant with the $\chi 2(1, N=326)=0.641$, $p=0.423$. Also, result of allele frequency comparison was insignificant with equation of $\chi 2(1, N=652)=3.192$, $p=0.074$.

Table 3. 5 Genotypes and allele frequency distribution of rs731236 (VDR-TaqI) for patients and controls.

Genotype/Allele	Patients (n:187) (n, %)	Controls (n:139) (n, %)	Model	OR (95% CI)	*p*-values	Age Adjusted OR (95% CI) (n:269)	Adjusted *p*-values (n:269)
AA	79 (42.2%)	44 (31.7%)	**Additive[a]**	Reference	Reference	Reference	Reference
AG	83 (44.4%)	72 (51.8%)		0.642 (0.395-1.043)	0.073 [1]	0.469 (0.268-0.821)	0.008*
GG	25 (13.4%)	23 (16.5%)		0.605 (0.308-1.190)	0.144 [1]	0.454 (0.216-0.9524)	0.037*
AA	79 (42.2%)	44 (31.7%)	**Dominant[b]**	0.633 (0.400-1.003)	0.051* [1]	0.465 (0.273-0.791)	0.0048*
AG+GG	108 (57.8%)	95 (68.3%)					
AA+AG	162 (86.6%)	116 (83.5%)	**Recessive[c]**	0.778 (0.421-1.439)	0.423 [1]	1.416 (0.734-2.730)	0.300
GG	25 (13.4%)	23 (16.5%)					
A	0.644	0.576	**Allele frequency[d]**	0.748 (0.544-1.029)	0.074 [1]	0.634 (0.446-0.902)	0.011*
G	0.356	0.424					

[a]: AA vs AG and AA vs GG, [b]: AA vs AG+GG, [c]: AA+AG vs GG, [d]: A vs G ([1]: Pearson's chi-square test, [2]: Fischer's exact test were applied)

3.2.1.2.1 Female Subgroup Analysis of VDR-TaqI

In VDR-TaqI female subgroup, 77 controls and 130 patients were studied. Allele distribution of samples and analysis results were shown in the Table 3.6. In patient group, 52 AA (40%), 62 AG (47.7%), 16 GG (12.3%), and in control group, 22 AA (28.6%), 43 AG (55.8%), 12 GG (15.6%) individuals were observed. Frequencies are 0.638 and 0.564 for "A" allele, and 0.362 and 0.436 for "G" allele in patients and controls, respectively.

In the additive model, wild type (AA) was compared with heterozygous (AG) and homozygous (GG) polymorphic individuals by chi-square. AA vs AG comparison was found to be insignificant with the $\chi 2(1, N=179)=2.364$, $p=0.124$. AA vs GG comparison was also insignificant with the $\chi 2(1, N=102)=1.575$, $p=0.209$. In the dominant model, polymorphic allele carrier individuals (n:78 in patients and n:55 in controls) were compared with the homozygous wild type (n:52 in patients and n:22 in controls) individuals. According to the dominant model, no statistically significant result was obtained ($\chi 2(1, N=207)=2.750$, p=0. 097). In the recessive model, homozygous polymorphic individuals (n:16 in patients and n:12 in controls) were compared with the dominant allele carrier individuals (n:114 in patients and n:65 in controls). Result of the analysis was insignificant with the $\chi 2(1, N=207)=0.444$, $p=0.505$. Allele frequencies of both group were also compared and result of that comparison was statistically insignificant with equation of $\chi 2(1, N=414)=2.200$, $p=0.134$.

Table 3. 6 Genotypes and allele frequency distribution of female subgroup of rs731236 (VDR-TaqI) for patients and controls.

Genotype/Allele	Patients (n:130) (n, %)	Controls (n:77) (n, %)	Model	OR (95% CI)	*p*-values
AA	52 (40%)	22 (28.6%)	**Additive**[a]	Reference	Reference
AG	62 (47.7%)	43 (55.8%)		0.610 (0.324-1.148)	0.124 [1]
GG	16 (12.3%)	12 (15.6%)		0.564 (0.230-1.386)	0.209 [1]
AA	52 (40%)	22 (28.6%)	**Dominant**[b]	0.600 (0.327-1.100)	0.097 [1]
AG+GG	78 (60.0%)	55 (71.4%)			
AA+AG	114 (87.7%)	65 (84.4%)	**Recessive**[c]	0.760 (0.339-1.706)	0.505 [1]
GG	16 (12.3%)	12 (15.6%)			
A	0.638	0.564	**Allele frequency**[d]	0.735 (0.490-1.104)	0.138 [1]
G	0.362	0.436			

[a]: AA vs AG and AA vs GG, [b]: AA vs AG+GG, [c]: AA+AG vs GG, [d]: A vs G ([1]: Pearson's chi-square test, [2]: Fischer's exact test were applied)

3.2.1.2.2 Male Subgroup Analysis of VDR-TaqI

In VDR-TaqI male subgroup, 62 controls and 57 patients were studied. Allele distribution of samples and analysis results were shown in the Table 3.7. In patient group, 27 AA (42.2%), 21 AG (44.4%), 9 GG (13.4%), and in control group, 22 AA (31.7%), 29 AG (51.8%), 11 GG (16.5%) individuals were observed. Frequencies are 0.658 and 0.589 for “A” allele, and 0.342 and 0.411 for “G” allele in patients and controls, respectively.

In additive model, wild type (AA) compared with heterozygous (AG) and homozygous (GG) polymorphic individuals by chi-square. AA vs AG comparison was found to be insignificant with the $\chi2(1, N=99)=1.701$, $p=0.192$. AA vs GG comparison was also insignificant with the $\chi2(1, N=69)=0.581$, $p=0.446$. In dominant model, polymorphic allele carrier individuals (n:30 in patients and n:40 in controls) were compared with the homozygous wild type (n:27 in patients and n:22 in controls) individuals. According to dominant model, no statistically significant result was obtained ($\chi2(1, N=119)=1.732$, $p=0.188$). In recessive model, homozygous polymorphic individuals (n:9 in patients and n:11 in controls) were compared with the dominant allele carrier individuals (n:48 in patients and n:51 in controls). Result of the analysis is in significant with the $\chi2(1, N=119)=0.081$, $p=0.776$. Allele frequencies of both group were also compared and with equation of $\chi2(1, N=238)=1.209$, $p=0.272$. Result of equation was statistically insignificant.

Table 3. 7 Genotypes and allele frequency distribution of male subgroup of rs731236 (VDR-TaqI) for patients and controls.

Genotype/Allele	Patients (n:57) (n, %)	Controls (n:62) (n, %)	Model	OR (95% CI)	*p*-values
AA	27 (47.4%)	22 (35.5%)	**Additive**[a]	Reference	Reference
AG	21 (36.8%)	29 (46.8%)		0.590 (0.234-1.896)	0.192 [1]
GG	9 (15.8%)	11 (17.7%)		0.667 (0.308-1.190)	0.446 [1]
AA	27 (47.4%)	22 (35.5%)	**Dominant**[b]	0.611 (0.293-1.275)	0.188 [1]
AG+GG	30 (52.6%)	40 (64.5%)			
AA+AG	48 (84.2%)	51 (82.3%)	**Recessive**[c]	0.869 (0.331-2.282)	0.776 [1]
GG	9 (15.8%)	11 (17.7%)			
A	0.658	0.589	**Allele frequency**[d]	0.744 (0.439-1.261)	0.272 [1]
G	0.342	0.411			

[a]: AA vs AG and AA vs GG, [b]: AA vs AG+GG, [c]: AA+AG vs GG, [d]: A vs G ([1]: Pearson's chi-square test, [2]: Fischer's exact test were applied)

3.2.2 Genotyping and Analysis of CYP24A1

rs1570669 is a SNP on CYP24A1. It is an intron variant "A" that transforms to "G" and MAF of "G" is 0.47. Genomic DNA was amplified with specific primers and the product of the PCR reaction was a 430 bp DNA fragment in the gel as shown in Figure 3.4. Then PCR products were digested with the HphI enzyme. "A" is the wild type allele and it transforms to "G" in the polymorphic cases which reveal the recognition site for the enzyme. In "G" allele, 133 bp and 246 bp DNA fragments were produced. In "A" allele, DNA fragment lost the restriction site and only 379 bp DNA fragment was observed. Heterozygous samples are represented on the gel with the 379 bp 246 bp and 133 bp DNA fragments.

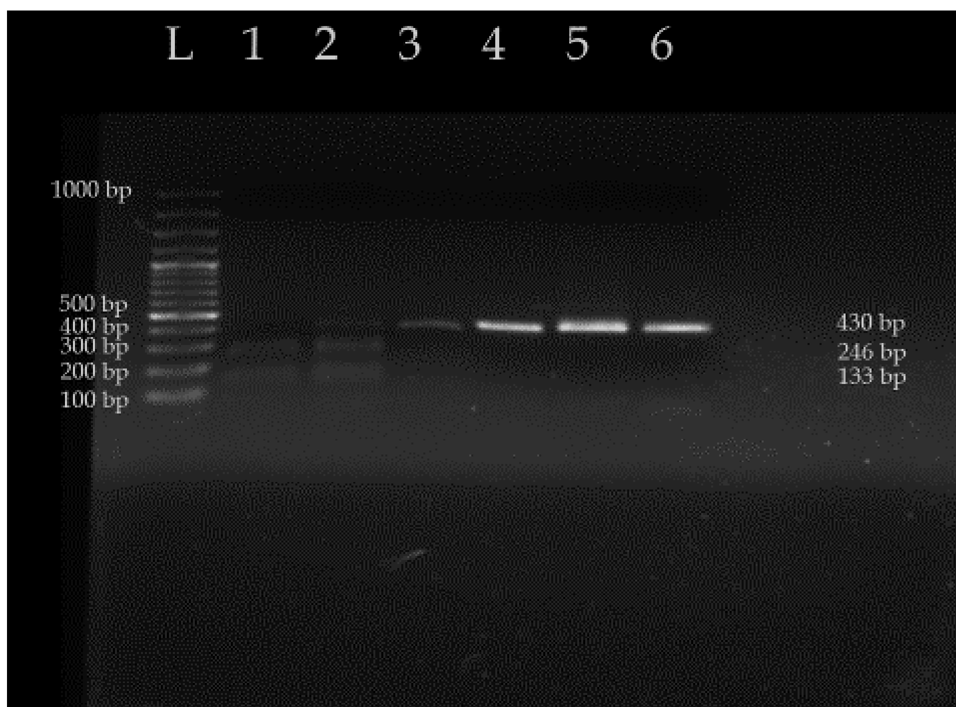

Figure 3. 4 Representative agarose gel (1%) image of rs1570669 restriction endonuclease digestion results and PCR products. L is 100 bp ladder. Lanes 1,2, and 3 represent GG, GA and AA genotypes, respectively. Lanes 4-6 are the PCR products.

By using gel images, genotyping of the samples was conducted. For rs1570669, 131 controls and 187 patients were studied. Allele distribution of samples and analysis results were shown in the Table 3.8. In patient group, 2 GG (1.1%), 81 GA (43.3%), 104 AA (55.6%), and in control group, 4 GG (3.1%), 16 GA (12.2%), 111 AA (84.7%) individuals were observed. Frequencies are 0.227 and 0.092 for “G” allele, and 0.773 and 0.908 for “A” allele in patients and controls, respectively.

In additive model, wild type (AA) compared with heterozygous (AG) with chi-square and homozygous (GG) polymorphic individuals by Fischer’s exact test. AA vs AG comparison was significant with the $p<0.001$. AA vs GG comparison was found to be insignificant with the $p=0.685$. In dominant model, polymorphic allele carrier individuals (n:83 in patients and n:20 in controls) were compared with the homozygous wild type (n:104 in patients and n:111 in controls) individuals. According to dominant model, result was significant with $p<0.001$. In recessive model, homozygous polymorphic individuals (n:2 in patients and n:4 in controls) were compared with the dominant allele carrier individuals (n:185 in patients and n:127 in controls). Result of the analysis was insignificant with the $p=0.234$. Allele frequencies of both group were also compared and with equation of $\chi2(1, N=636)=3.649$, $p=0.056$. Equation was calculated as statistically insignificant.

Table 3. 8 Genotypes and allele frequency distribution of rs1570669 (CYP24A1) for patients and controls.

Genotype/Allele	**Patients (n:187) (n, %)**	**Controls (n:131) (n, %)**	**Model**	**OR (95% CI)**	***p*-values**	**Age Adjusted OR (95% CI) (n:263)**	**Adjusted *p*-values (n:263)**
AA	104 (55.6%)	111 (84.7%)	**Additive**[a]	Reference	Reference	Reference	Reference
AG	81 (43.3%)	16 (12.2%)		5.413 (2.968-9.838)	<0.001* [1]	5.390 (2.731-10.634)	<0.001*
GG	2 (1.1%)	4 (3.1%)		0.534 (0.096-2.975)	0.685 [2]	0.308 (0.031-3.018)	0.312
AA	104 (55.6%)	111 (84.7%)	**Dominant**[b]	4.429 (2.538-7.729)	<0.001* [1]	4.373 (2.329-8.213)	<0.001*
AG+GG	83 (44.4%)	20 (15.3%)					
AA+AG	185 (44.4%)	127 (15.3%)	**Recessive**[c]	0.343 (0.062-1.902)	<0.234 [2]	0.200 (0.021-1.946)	0.165
GG	2 (1.1%)	4 (3.1%)					
A	0.773	0.908	**Allele frequency**[d]	0.669 (0.442-1.012)	0.056 [1]	2.866 (1.653-4.970)	0.0002*
G	0.227	0.092					

[a]: GG vs GA and GG vs AA, [b]: GG vs GA+AA, [c]: GG+GA vs AA, [d]: A vs G ([1]: Pearson's chi-square test, [2]: Fischer's exact test were applied)

3.2.2.1 Female Subgroup Analysis of CYP24A1

In female subgroup of rs1570669, 70 controls and 130 patients were studied. Allele distribution of samples and analysis results were shown in the Table 3.9. In patient group, 0 GG (0.0%), 57 GA (43.8%), 73 AA (56.2%), and in control group, 3 GG (4.3%), 7 GA (10.0%), 60 AA (85.7%) individuals were observed. Frequencies are 0.219 and 0.093 for “G” allele, and 0.781 and 0.907 for “A” allele in patients and controls, respectively.

In the additive model, wild type (AA) compared with heterozygous (AG) with chi-square and homozygous (GG) polymorphic individuals by Fischer’s exact test. AA vs AG comparison was significant with the $p<0.001$. AA vs GG comparison was found to be insignificant with the $p=0.097$. In the dominant model, polymorphic allele carrier individuals (n:57 in patients and n:10 in controls) were compared with the homozygous wild type (n:73 in patients and n:60 in controls) individuals. According to the dominant model, result is significant with $p<0.001$. In the recessive model, homozygous polymorphic individuals (n:0 in patients and n:3 in controls) were compared with the dominant allele carrier individuals (n:130 in patients and n:67 in controls). Result of the analysis was insignificant with the $p=0.042$. Allele frequencies of both group were also compared and with equation of $\chi2(1, N=400)=1.139$, $p=0.286$, it was calculated as insignificant.

Table 3. 9 Genotypes and allele frequency distribution of female subgroup of rs1570669 (CYP24A1) for patients and controls.

Genotype/Allele	Patients (n:130) (n, %)	Controls (n:70) (n, %)	Model	OR (95% CI)	*p*-values
AA	73 (56.2%)	60 (85.7%)	**Additive[a]**	Reference	Reference
AG	57 (43.8%)	7 (10.0%)		6.693 (2.843-15.754)	<0.001* [1]
GG	0 (0.0%)	3 (4.3%)		0.952 (0.901-1.006)	0.097 [2]
AA	73 (56.2%)	60 (85.7%)	**Dominant[b]**	4.685 (2.205-9.956)	<0.001* [1]
AG+GG	57 (43.8%)	10 (14.3%)			
AA+AG	185 (98.9%)	67 (95.7%)	**Recessive[c]**	0.957 (0.911-1.006)	0.042[2]
GG	0 (0.0%)	3 (4.3%)			
A	0.781	0.093	**Allele frequency[d]**	0.740 (0.426-1.288)	0.286 [1]
G	0.219	0.907			

[a]: GG vs GA and GG vs AA, [b]: GG vs GA+AA, [c]: GG+GA vs AA, [d]: A vs G ([1]: Pearson's chi-square test, [2]: Fischer's exact test were applied)

3.2.2.2 Male Subgroup Analysis of CYP24A1

In male subgroup of rs1570669, 61 controls and 57 patients were studied. Allele distribution of samples and analysis results were shown in the Table 3.10. In patient group, 2 GG (3.5%), 24 GA (42.1%), 31 AA (54.4%), and in control group, 1 GG (1.6%), 9 GA (14.8%), 51 AA (83.6%) individuals were observed. Frequencies are 0.246 and 0.090 for “G” allele, and 0.754 and 0.910 for “A” allele in patients and controls, respectively.

In the additive model, wild type (AA) compared with heterozygous (AG) with chi-square and homozygous (GG) polymorphic individuals by Fischer’s exact test. AA vs AG comparison was significant with the $p<0.001$. AA vs GG comparison was found to be insignificant with the $p=0.557$. In the dominant model, polymorphic allele carrier individuals (n:26 in patients and n:10 in controls) were compared with the homozygous wild type (n:31 in patients and n:51 in controls) individuals. According to the dominant model, result was significant with $p<0.001$. In the recessive model, homozygous polymorphic individuals (n:2 in patients and n:1 in controls) were compared with the wild type allele carrier individuals (n:55 in patients and n:60 in controls). Result of the analysis was insignificant with the $p=0.609$. Allele frequencies of both group were also compared and result of this comparison was insignificant with equation of $\chi 2(1, N=234)=1.686$, $p=0.194$.

Table 3. 10 Genotypes and allele frequency distribution of male subgroup of rs1570669 (CYP24A1) for patients and controls.

Genotype/Allele	Patients (n:57) (n, %)	Controls (n:61) (n, %)	Model	OR (95% CI)	*p*-values
AA	31 (54.4%)	51 (83.6%)		Reference	Reference
AG	24 (42.1%)	9 (14.8%)	**Additive**[a]	4.387 (1.808-10.647)	<0.001* [1]
GG	2 (3.5%)	1 (1.6%)		3.290 (0.286-37.811)	0.557 [2]
AA	31 (54.4%)	51 (83.6%)	**Dominant**[b]	4.277 (1.819-10.058)	<0.001* [1]
AG+GG	26 (45.6%)	10 (16.4%)			
AA+AG	55 (96.5%)	60 (98.4%)	**Recessive**[c]	2.182 (0.1929-24.738)	0.609 [2]
GG	2 (3.5%)	1 (1.6%)			
A	0.754	0.910	**Allele frequency**[d]	0.654 (0.344-1.244)	0.194 [1]
G	0.246	0.090			

[a]: GG vs GA and GG vs AA, [b]: GG vs GA+AA, [c]: GG+GA vs AA, [d]: A vs G ([1]: Pearson's chi-square test, [2]: Fischer's exact test were applied)

3.2.3 Genotyping and Analysis of CYP 27A1

rs6709815 is a SNP on CYP27A1. It is an intron variant "G" that transforms to "T" and MAF of "T" is 0.37. Genomic DNA was amplified with specific primers and the product of the PCR reaction was 712 bp DNA fragment in the gel as shown in Figure 3.5. Then PCR products were digested with the BfuAI enzyme. "G" is the wild type allele and it transforms to "T" in the mutant allele which disrupts the recognition site for the enzyme. In "G" allele, 475 bp and 237 bp DNA fragments were produced. In "T" allele, DNA fragment remain non-digested. Heterozygous samples were represented on the gel with the 712 bp, 475 bp and 237 bp DNA fragments.

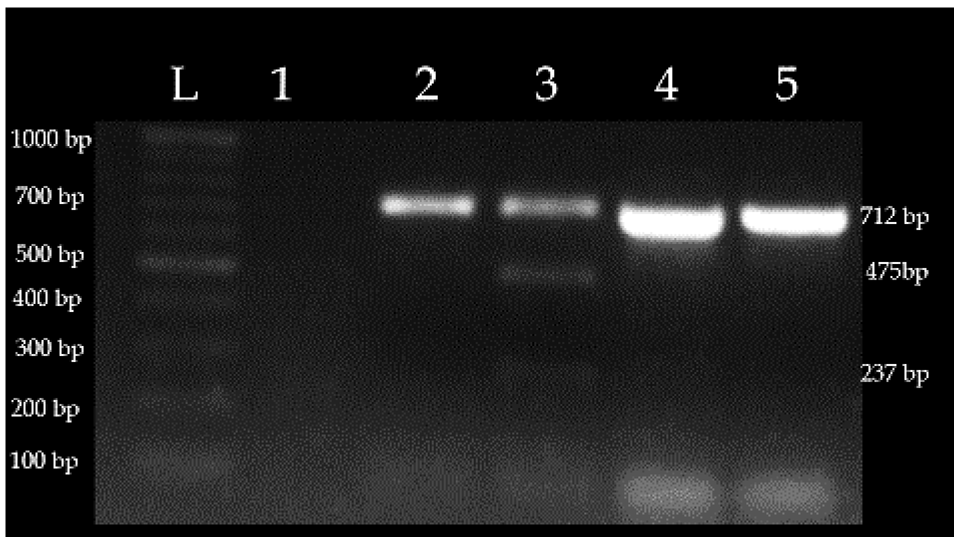

Figure 3. 5 Representative agarose gel (1%) image of rs6709815 restriction endonuclease digestion results and PCR products. L is 100 bp ladder. Lane 1 is empty Lanes 2, and 3 are represent TT and GT genotypes, respectively. Lane 4 and 5 are the PCR products.

By using gel images, genotyping of the samples was conducted. For rs6709815, 140 controls and 187 patients were studied. Allele distribution of samples and analysis results were shown in the Table 3.11. In patient group, 74 GT (39.6%), 113 TT (60.4%), and in control group, 47 GT (33.6%), 93 TT (66.4%) individuals were

observed. Frequencies are 0.198 and 0.168 for “G” allele, and 0.802 and 0.832 for “T” allele in patients and controls, respectively.

Additive, dominant and recessive models were not applied due to lack of GG allele. In the additive model, GT and TT comparison was made by chi-square ($\chi 2(1, N=327)=1.237$, $p=0.266$), and the result was statistically insignificant. Allele frequencies of both group were also compared and with equation of $\chi 2(1, N=654)=0.956$, $p=0.328$. The result of equation was calculated as insignificant.

Table 3. 11 Genotypes and allele frequency distribution of rs6709815 (CYP27A1) for patients and controls.

Genotype/Allele	**Patients (n:187) (n, %)**	**Controls (n:140) (n, %)**	**Model**	**OR (95% CI)**	***p*-values**	**Age Adjusted OR (95% CI) (n:270)**	**Adjusted *p*-values (n:270)**
GG	0 (0.0%)	0 (0.0%)	**Additive[a]**	NA	NA	NA	NA
GT	74 (39.6%)	47 (33.6%)		Reference	Reference	Reference	Reference
TT	113 (60.4%)	93 (66.4%)		0.772 (0.489-1.297)	0.266	1.094 (0.667-1.796)	0.722
GG **GT+TT**	NA		**Dominant**	NA			
GG+GT **TT**	NA		**Recessive**	NA			
G	0.198	0.168	**Allele frequency[b]**	1.223 (0.817-1.831)	0.328	1.069 (0.698-1.638)	0.759
T	0.802	0.832					

[a]: GT vs GG and, [b]: G vs T (Pearson's chi-square test applied)

3.2.3.1 Female Subgroup Analysis of CYP27A1

In female subgroup of rs6709815, 78 controls and 130 patients were studied. Allele distribution of samples and analysis results were shown in the Table 3.12. In patient group, 53 GT (40.8%), 77 TT (59.2%), and in control group, 28 GT (35.9%), 50 TT (64.1%) individuals were observed. Frequencies are 0.204 and 0.179 for “G” allele, and 0.796 and 0.821 for “T” allele in patients and controls, respectively.

Additive, dominant and recessive models were not applied in here also due to the lack of GG allele. In additive model, GT and TT comparison was made by chi-square ($\chi2(1, N=208)=0.487$, $p=0.485$), and the result was found to be statistically insignificant. Allele frequencies of both group were also compared and with equation of $\chi2(1, N=416)=0.369$, $p=0.544$. It is calculated as insignificant.

Table 3. 12 Genotypes and allele frequency distribution of female subgroup of rs6709815 (CYP27A1) for patients and controls.

Genotype/Allele	Patients (n:130) (n, %)	Controls (n:78) (n, %)	Model	OR (95% CI)	*p*-values
GG	0 (0.0%)	0 (0.0%)	**Additive[a]**	NA	NA
GT	53 (40.8%)	28 (35.9%)		Reference	Reference
TT	77 (59.2%)	50 (64.1%)		0.814 (0.456-1.453)	0.485
GG / **GT+TT**	NA		**Dominant**	NA	
GG+GT / **TT**	NA		**Recessive**	NA	
G	0.198	0.168	**Allele frequency[b]**	1.170 (0.704-1.946)	0.544
T	0.802	0.832			

[a]: GT vs GG and, [b]: G vs T (Pearson's chi-square test applied)

3.2.3.2 Male Subgroup Analysis of CYP27A1

In male subgroup of rs6709815, 62 controls and 57 patients were studied. Allele distribution of samples and analysis results were shown in the Table 3.13. In patient group, 21 GT (36.8%), 36 TT (63.2%), and in control group, 19 GT (30.6%), 43 TT (69.4%) individuals were observed. Frequencies are 0.184 and 0.153 for "G" allele, and 0.816 and 0.847 for "T" allele in patients and controls, respectively.

Additive, dominant and recessive models were also not applied in here. In the additive model, GT and TT comparison was made by chi-square ($\chi2(1, N=119)=0.757$, $p=0.475$), and result was again found to be statistically insignificant. Allele frequencies of both group were also compared and with equation of $\chi2(1, N=238)=0.408$, $p=0.523$. Result was insignificant.

Table 3. 13 Genotypes and allele frequency distribution of male subgroup of rs6709815 (CYP27A1) for patients and controls.

Genotype/Allele	**Patients (n:57) (n, %)**	**Controls (n:62) (n, %)**	**Model**	**OR (95% CI)**	***p*-values**
GG	0 (0.0%)	0 (0.0%)	**Additive**[a]	NA	NA
GT	21 (36.8%)	19 (30.6%)		Reference	Reference
TT	36 (63.2%)	43 (69.4%)		0.757 (0.353-1.623)	0.475
GG **GT+TT**	NA		**Dominant**	NA	
GG+GT **TT**	NA		**Recessive**	NA	
G	0.184	0.153	**Allele frequency**[b]	1.248 (0.632-2.464)	0.523
T	0.816	0.847			

[a]: GT vs GG and, [b]: G vs T (Pearson's chi-square test applied)

3.2.4 Genotyping and Analysis of CYP27B1

rs4646536 is a SNP on CYP27B1. It is an intron variant “A” that transforms to “C” and MAF of “A” is 0.59 and no population frequency defined for “C”. Genomic DNA was amplified with specific primers and the product of the PCR reaction was 641 bp DNA fragment in the gel as shown in Figure 3.6. Then PCR products were digested with the BsmI enzyme. “A” is the wild type allele and it transforms to “C” in the polymorphic cases which provide a recognition site for the enzyme. In “C” allele, 246 bp and 395 bp DNA fragments were produced. In “A” allele, DNA fragment remain non-digested. Heterozygous samples were represented on the gel with the 641 bp, 246 bp and 395 bp DNA fragments.

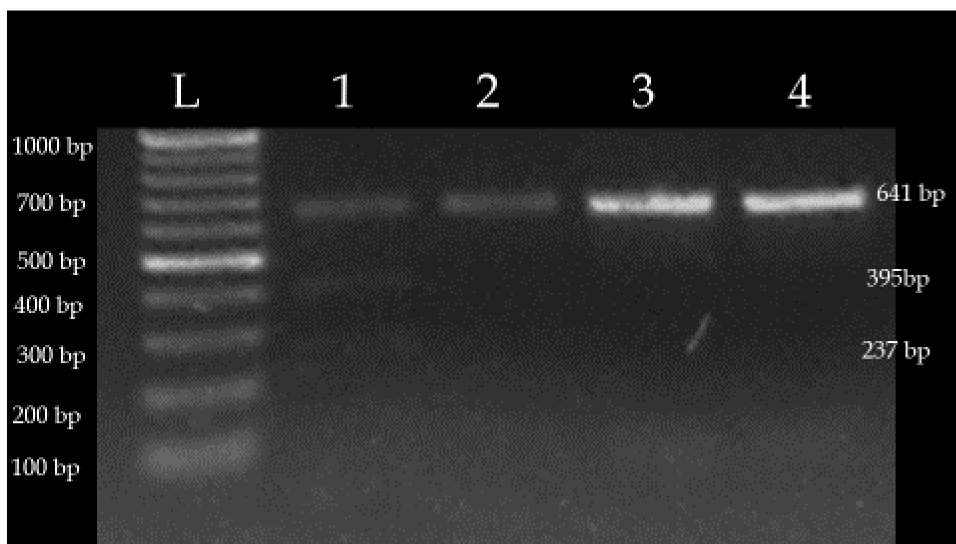

Figure 3. 6 Representative agarose gel (1%) image of rs4646536 restriction endonuclease digestion results and PCR products. L is 100 bp ladder. Lanes 1 and 2 represent AA and AC genotypes, respectively. Lanes 3 and 4 are the PCR products.

By using gel images, genotyping of the samples was conducted. For rs4646536, 136 controls and 187 patients were studied. Allele distribution of samples and analysis results were shown in the Table 3.14. In patient group, 178 AA (95%), 9 AC (5%),

and in control group, 127 AA (93%), 9 AC (7%) individuals were observed. Frequencies are 0.976 and 0.967 for “A” allele, and 0.024 and 0.033 for “C” allele in patients and controls, respectively.

The lack of CC allele prevented the use of dominant and recessive models. In the additive model, AA and AC comparison was made by chi-square ($\chi 2(1, N=323)=0.487$, $p=0.485$), and the result was statistically insignificant. Allele frequencies of both group were also compared and with equation of $\chi 2(1, N=646)=0.473$, $p=0.491$, the result of comparison also calculated as insignificant.

Table 3. 14 Genotypes and allele frequency distribution of rs4646536 (CYP27B1) for patients and controls.

Genotype/Allele	Patients (n:187) (n, %)	Controls (n:137) (n, %)	Model	OR (95% CI)	*p*-values	Age Adjusted OR (95% CI) (n:266)	Adjusted *p*-values (n:270)
AA	178 (95%)	127 (93%)	**Additive[a]**	Reference	Reference	Reference	Reference
AC	9 (5%)	9 (7%)		0.713 (0.276-1.848)	0.485	0.802 (0.289-2.223)	0.671
CC	0 (0.0%)	0 (0.0%)		NA	NA	NA	NA
AA / **AC+CC**	NA		**Dominant**	NA			
AA+AC / **CC**	NA		**Recessive**	NA			
A	0.976	0.967	**Allele frequency[b]**	0.721 (0.282-1.840)	0.491	0.807 (0.296-2.202)	0.676
C	0.024	0.033					

[a]: AA vs AC and, [b]: A vs C (Pearson's chi-square test applied)

3.2.4.1 Female Subgroup Analysis of CYP27B1

74 controls and 130 patients were examined in the female subgroup of rs4646536. Allele distribution of samples and analysis results were shown in the Table 3.15. In the patient group, 125AA (96.2%), 5 AC (3.8%), and in the control group, 69 AA (93.2%), 5 AC (6.8%) individuals were observed. Frequencies are 0.981 and 0.966 for "A" allele, and 0.019 and 0.034 for "C" allele in patients and controls, respectively.

As mentioned above, dominant and recessive models were not applied to the female subgroup due to the lack of CC allele. AA and AC comparison made by chi-square ($\chi2(1, N=204)=0.857$, $p=0.355$), under additive model application. The result of that comparison was statistically insignificant. Allele frequencies of both group were also compared and calculated as insignificant with equation of $\chi2(1, N=408)=0.835$, $p=0.361$.

Table 3. 15 Genotypes and allele frequency distribution of female subgroup of rs4646536 (CYP27B1) for patients and controls.

Genotype/Allele	**Patients (n:130) (n, %)**	**Controls (n:74) (n, %)**	**Model**	**OR (95% CI)**	***p*-values**
AA	125 (96.2%)	69 (93.2%)	**Additive**[a]	Reference	Reference
AC	5 (3.8%)	5 (6.8%)		0.552 (0.154-1.973)	0.355
CC	0 (0.0%)	0 (0.0%)		NA	NA
AA **AC+CC**	NA		**Dominant**	NA	
AA+AC **CC**	NA		**Recessive**	NA	
A	0.981	0.966	**Allele frequency**[b]	0.561 (0.160-1.970)	0.361
C	0.019	0.034			

[a]: AA vs AC and, [b]: A vs C (Pearson's chi-square test applied)

3.2.4.2 Male Subgroup Analysis of CYP27B1

Examination of 62 controls and 57 patients were done in male subgroup of rs4646536. Allele distribution of samples and analysis results were shown in the Table 3.16. In patient group, 53 AA (93%), 4 AC (7%), and in control group, 58 AA (93.5%), 4 AC (6.5%) individuals were observed. A allele frequencies were 0.965 and 0.968 and C allele frequencies were 0.035 and 0.032 for in patients and controls, respectively.

None of the models were applied to the samples. Only, AA and AC comparison made under the additive model by chi-square ($\chi 2(1, N=119)=0.015$, $p=0.902$). Also, allele frequencies of both group were compared with equation of $\chi 2(1, N=238)=0.015$, $p=0.904$. Both analyzes were calculated as insignificant.

Table 3. 16 Genotypes and allele frequency distribution of male subgroup of rs4646536 (CYP27B1) for patients and controls.

Genotype/Allele	**Patients (n:57) (n, %)**	**Controls (n:62) (n, %)**	**Model**	**OR (95% CI)**	***p*-values**
AA	53 (93%)	58 (93.5%)	**Additive[a]**	Reference	Reference
AC	4 (7%)	4 (6.5%)		1.094 (0.261-4.596)	0.902
CC	0 (0.0%)	0 (0.0%)		NA	NA
AA / **AC+CC**	NA		**Dominant**	NA	
AA+AC / **CC**	NA		**Recessive**	NA	
A	0.965	0.968	**Allele frequency[b]**	1.091 (0.266-4.468)	0.904
C	0.035	0.032			

[a]: AA vs AC and, [b]: A vs C (Pearson's chi-square test applied)

CHAPTER 4

DISCUSSION

Multiple sclerosis (MS) is the most common nontraumatic cause of young disability. More than one million people in Europe are MS patients. Beside labor costs, only medical costs of that patients to European countries are around 15 billion Euros (Kingwell et al., 2013; Sawcer et al., 2014). Therefore, prevention of disease has an economic importance in addition to health problem.

Disease affects central nervous system due to attacks of immune system to the myelin sheet around the nerve cells (Noseworthy et al., 2000). Women are affected more than men with the ratio of 3:1 (Dobson & Giovannoni, 2019). Leading cause to MS onset is still an unknown question but various factors are reported to be related to disease. Those factors not only genetic factors but also enviromental. (Ascherio et al., 2012). Incidence and prevalence studies showed that distance to the equatorial belt correlates with the prevalence of the disease (Goldberg, 1974; Kingwell et al., 2013). Moreover, increased vitamin D level in serum was found to reduce the severity of the disease (Simpson et al., 2010). In addition, in the winter, disease severity is found to be increasing (Harding et al., 2017). All those studies led to the emergence of the vitamin D/MS hypothesis.

Vitamin D is a secosteroid mainly produced by skin (Rochel, 2022). Previously role of vitamin D is mainly defined by its activity on phosphate and calcium homeostasis. Later studies revealed vitamin D's importance for immune system (Jones, 2022; Zorzella-Pezavento et al., 2022). Activation of vitamin D requires a series of hydroxylations that are conducted by CYPs (Agnello et al., 2018). Vitamin D shows its effects by binding to VDR and altering the gene transcription. Under the light of recent information, vitamin D’s function is better understand and effects on various

diseases attracts scientist’s attention to vitamin D-disease relations (Jones, 2022; Pereira et al., 2022; Raveendran, 2022; Zorzella-Pezavento et al., 2022).

In the present study, vitamin D related genes were the subject of investigation because of vitamin D’s immunomodulatory properties and, vitamin D-MS hypothesis. Mitochondrial CYPs and VDR polymorphisms were selected for investigation.

VDR is a nucleic receptor that change gene expression after binding to its ligand. Two SNPs of VDR were investigated. One of them is rs731236 (TaqI) and the other is rs7975232 (ApaI). TaqI polymorphism located on the exon 9 of the gene and presents the A to G change. This change is synonymous and therefore as a result of that no amino acids change occurs in the protein. But, this polymorphism might be important for the mRNA stability. ApaI polymorphism is on the intron 8 which is located at the 3’ end of the exon and does also not cause any amino acid change in the protein. Still it can lead to an alternative splicing or mRNA stability. ApaI and TaqI polymorphisms are also related with lower vitamin D concentrations. These two polymorphic regions generally investigated together because they are close to each other and linkage disequilibrium between them might alter the zinc finger of the nuclear response element (Ruiz-Ballesteros et al., 2020). These polymorphisms may lead to changes in the expression of many genes.

VDR polymorphisms were studied in many populations including Turkish population. Still, the obtained results are controversial (Kamisli et al., 2018; Yucel et al., 2018). In a Turkish population study, rs731236 (TaqI) and rs7975232 (ApaI) polymorphisms revealed no significant correlation with MS (Kamisli et al., 2018). Also, there are other studies reported, supporting those results in different populations (Čierny et al., 2016). However, three different studies claimed that the ApaI polymorphism increase the susceptibility to the disease and controversly an Iranian study relates same polymorphism with the decreased risk (Tizaoui et al., 2015; Zhang et al., 2018; Křenek et al., 2018; Mohammadi et al., 2020). In our study,

we also could not find any significant correlation between ApaI polymorphism and MS susceptibility parallel to Kamisli et al., 2018. Similar to ApaI, there data on TaqI polymorphism is also controversial. For instance, Yucel et al., 2018 claimed that TaqI is resulting in significant difference between patients and controls in Turkish population (Yucel et al., 2018). However, studies that did not find any correlation with that polymorphism and MS susceptibility were also reported (Tizaoui et al., 2015; Zhang et al., 2018). In our study, whole population and male/female subgroups were analyzed. Only a borderline significance (p=0.051) was observed in the dominant model of general population. According to our results, G allele decreases the susceptibility to MS (OR:0.633). An Iranian population study revealed similar results to ours (Mohammadi et al., 2020). These controvesial results might be caused by either ethnic difference or difference in sample sizes of the studies. Beside ethnicity or sample size, most of the studies do not use life style as a parameter. This factors might lead to an alteration in the results. Whatever is the cause, controversial results makes these SNPs interesting and worth further investigation.

CYP27A1 is one of the key enzymes for bile acid biosynthesis. It also hydroxylates vitamin D in the liver from its 25th carbon. It is not the only enzyme capable of 25 hydroxylation of vitamin D. Literature shows CYP2R1 but not CYP27A1 is crucial for blood vitamin D levels (Krasniqi et al., 2021). Even CYP27A1 is not a key enzyme for vitamin D, CYP27A1 is related with some other diseases like a lipid storage disorder cerebrotendinous xanthomatosis (CTX) (InanlooRahatloo et al., 2013). CYP27A1 and MS relation was investigated from a different polymorphic region and no significant association was revealed (Agnello et al., 2018). In our study, our selected region on CYP27A1 gene is rs6709815. This position is located in the intron between exon1 and exon2, and so it will not lead to any change in the protein structure of the enzyme. It might also play role on alternative transcripts of the gene or it can effect the mRNA's stability (Wang, H. F., et al., 2007). In our study, no significant relation was observed between MS and rs6709815. Both female and male subgroups were also analyzed individually and results were found to be not

significant. One interesting point about rs6709815 was the fact that no GG genotype was observed. G allele is wild type allele and observing the presence of G allele in our population was expected. It is known that, in some European subpopulations, frequency of mutant T allele is higher than wild type G allele. However, European subpopulations' frequencies are still different from ours. This might be due to the ethnicity, genetic variance between different ethnic groups or the our population size used in our study. The population in our study was relatively small and, there were no studies regarding the rs6709815 in Turkish population. In the present work, dominant and recessive models could not be applied during the analysis as a result of missing GG genotype. Only GT genotype was compared to TT genotype in additive model. Same polymorphic region (rs6709815) was investigated in different populations. In one case, caucassians subjets' blood vitamin D levels and relation of several polymorphisms including rs6709815 were investigated. In that study, rs6709815 seem not to have any effect on blood vitamin D levels (Bu et al., 2010). In another study, older Australian subjects were supplemented with vitamin D. After supplementation, blood vitamin D level changes were measured and several polymorphisms', including rs6709815's, effect on their blood vitamin D level changes were investigated. rs6709815 was again found to show no correlation with the blood vitamin D levels change of older Australian subjects (Waterhouse et al., 2014). Those results were not suprising because CYP27A1 is not the only enzyme hydroxylating vitamin D (Nelson, 2009). Our study was based on the vitamin D/MS relation and, selected polymorphic region seem to be unaffective on blood vitamin D levels according to literature information (Bikle, 2021). This support ours which revealed insignificant correlation because CYP27A1 has little effect on blood vitamin D levels compared to CYP2R1. However, this gene could be crucial if any dysfunction occur on CYP2R1. Because CYP27A1 hydroxylates vitamin D in any circumstances, independent from the blood vitamin D levels (Bikle, 2021).

CYP27B1 is mainly found in renal tissues and the only known enzyme that catalyzes the α carbon hydroxylation of 25(OH)D to produce active form of the vitamin D

(Meyer et al., 2017; Sawada et al., 2001). This property make CYP27B1 important for calcium, phosphate homeostasis and vitamin D related diseases. For instance, a promoter region polymorphism of CYP27B1 seems significant for Addison's disease, Grave's disease and Hashimoto's thyroiditis in German population (Lopez et al., 2004). Common point of these diseases is the fact that they are all autoimmune disorders which indicates the effect of CYP27B1 on immunity. This is not surprising because of the known immunomodulatory effects of vitamin D. Because of that CYP27B1 was also investigated for its relation to MS. Those studies revealed also controversial results. According to some studies, rs118204012 of CYP27B1 is insignificant for MS susceptibility (Agnello et al., 2017). rs118204012 is a synonymous polymorphism and thought to be pathogenic in databases. Yet, rs118204012's MAF is low and population size is small according to variant frequency. Because of that, Agnello et al. might not observe significant results. However, another study relate A allele of rs12368653 (G→A) of CYP27B1 to MS (Sawcer et al., 2011). In addition to that Sundqvist et al. reported the association of more than one polymorphism of CYP27B1 with MS in Swedish population (Sundqvist et al., 2010). In our study, we focused on rs4646536 (A→G). rs4646536 is located in intron 6 of the gene and cannot affect the protein structure. According to Lopez et al. rs4646536 shows linkage disequilibrium (LD) with some alleles that are related with diabetes mellitus type 1 (Lopez et al., 2004). While investigating the effect of rs4646536 on diabetes mellitus type 1, a rare variant was noticed. Common alleles for that position is A or G but in rare variant allele could be C. There is no population frequency information about C allele on databases. G allele of rs4646536 presents significant correlation with lower vitamin D levels in blood, diabetes mellitus type 1 and MS susceptibility (Lopez et al., 2004; Sundqvist et al., 2010; Yu et al., 2019). According to literature, this position seems to be important for MS susceptibility and like diabetes reason of that might be linkage disequilibrium. Therefore, we decided to investigate rare variant. Main reason was MAF of the alleles. For G allele frequencies varies from 15% to 77%. Because of those variations

among populations, it would be hard to relate polymorphism to the disease. We weren't able to observe any CC genotype in our population. This was an expected consequence of the selected variant. In addition, we could not obtain any significant result in neither complete population nor subpopulations. Moreover, dominant and recessive models could not be applied as a consequence of missing CC genotype. Even our data didn't revealed statistically significant results, this is the first study revealing the existence of C allele in a population.

CYP24A1 is a renal, mitochondrial monooxygenase and responsible for the breakdown of vitamin D (Alyaa Hussein Hammadi & Shatha Hussein Ali, 2021). It can hydroxylate vitamin D from different positions. Result of that hydroxylations are either calcitrioic acid or lactone. Calcitrioic acid is excreted from the body by urine (St-Arnaud & Jones, 2018). It's activity is affected from blood vitamin D levels and this enzyme works in a way contrary to CYP27B1. Activity of CYP24A1 is important for active vitamin D levels of the body (Bikle, 2021). Active vitamin D levels affect calcium balance of the body and bone mineralization. Because of vitamin D's importance on homeostasis, CYP24A1 was investigated for its relation to various diseases. In a Chinese population study, rs56229249 was found to decrease risk of hypertension but rs2762934 and rs1977297 of CYP24A1 were found to increase the hypertension risk (Bao et al., 2020). In another study rs34043203 and rs2762934 were found to be related to increased breast cancer risk and rs1570669 was found to reduce the risk (Fuhrman et al., 2013). rs1570669's protective effect on urinary cancers and ischemic stroke was also shown (Sun et al., 2021; Yang et al., 2020). Association CYP24A1 with MS was investigated in many studies. C allele of rs2762939 of CYP24A1 was found to be associated with the severity of the disease (Agnello et al., 2022). Orton and coworkers marked several CYP24A1 polymorphisms as a risk factor for MS including rs1570669 (Orton et al., 2011). Yet some studies could not find any significant relation with any SNP of CYP24A1 and MS (Scazzone et al., 2021). rs1570669 is the region of interest in our study. Alleles at that position is G or A. It is an intron variant and, is not expected to

result in any change in the protein structure. It is located in the 9th intron. Our results revealed the G allele as the risky allele and the results obtained in the dominant model were found to be significant. Morever, in the additive model, heterozygot AG genotype also result in another statistically significant result. The reasons of that result might be the linkage disequilibrium or mRNA stability. As a result of the changes caused by that polymorphism, activity of CYP24A1 might be increased which might lead to a decrease in the amouts of circulating active vitamin D. Literature contains controversial results also about rs1570669. Several studies defined the protective effect of rs1570669 on diseases like, urinary cances or ischemic stroke (Yang et al., 2020). Some others related G allele of rs1570669 with the increased risk of MS (Orton et al., 2011). Vitamin D shows protective effect on ischemic stroke and MS according to literature (Yang et al., 2020; Zorzella-Pezavento et al., 2022). Yet, according to literature the G allele of rs1570669, decrease ischemic stroke risk and increase MS risk. From the vitamin D perspective, ischemic stroke and MS risks should be affected from rs1570669's polymorphism in the same direction but the results revealed the opposite. Overall, our results were controversial to Scazzzone et al.,2021 but support Orton et al., 2011 study (Orton et al., 2011; Scazzone et al., 2021). Accordingly, rs1570669 should be investigated further to understand its role and shed light onto the controversial results about its effect on different diseases.

In our study, we had several limitations, one of them being the population size. We have studied with 187 patients. This number is good for investigating a rare disease like MS but many exonic rare variants became out of scope for us. The study carries the risk of undetection of a rare allele. On the other hand, selecting variants with higher MAFs has its own risk. In a rare disease, it is hard to associate a variant with higher MAF to the prevalence or the progression of the disease.

Another limitation of our study might be the use of RFLP. In RFLP studies, several properties of the selected region becomes important. For instance, polymorphic point

should be amplified with unique primers and amplified DNA must carry a restriction site specific to selected polymorphism. In addition to that, DNA fragments after restriction must be seperately detectable on gel. All these expectations and limitations about experimental design lead us to select certain SNPs in this study.

Still, beside those limitations, we have discovered several significant associations in our study. We believe, our results might shed light onto the future studies. For instance, samples of CYP27B1 which were detected as C allele carriers should be sequenced to confirm our results. Moreover, VDBP, CYP3A4 and CYP2R1 can be included in another study to investigate all vitamin D related proteins in the body. In addition, several other polymorphic regions of the genes investigated in this study were already present in the literature, yet, many of them were controversial. Those polymorphisms are needed to be investigated in detail in the future.

CHAPTER 5

CONCLUSION

Multiple Sclerosis is the most common reason of nontraumatic young disability. It attracts interest of many researchers around the world. Yet, its etiology is still a mystery. After the recognition of incidence correlation with lattitude, researches started to focus on vitamin D and MS relation. Untill now, no clear evidence for the underlaying causes of the onset of MS was provided. However each study contributes to our understanding of the MS. According to literature and our findings, the disease not only have a single genetic cause. Certain environmental triggers are also found to contribute to the onset and progression of the MS.

In our study, for the first time we investigated the association of certain polymorphism with MS in the Turkish population. We have followed the path of vitamin D through body. We selected the mitochondrial monooxygenases' genes that are playing a role on vitamin D activation or inactivation (CYP27A1, CYP27B1, CYP24A1) and VDR. For VDR, rs731236 (restriction enzyme; TaqI, base change; A to G) and rs7975232 (restriction enzyme; ApaI, base change; C to A), for CYP27A1, rs6709815 (restriction enzyme; BfuA1, base change; G to T), for CYP27B1, rs4646536 (restriction enzyme; BsmI, base change; A to C) and for CYP24A1, rs1570669 (restriction enzyme; HphI, base change; A to G) were examined in the present study. In the study, 187 MS patients and 140 healthy control subjects were involved. For each polymorphic region, region of interest was amplified with specific primers and the amplified PCR products were cut with restriction enzymes. Those restriction enzymes which were selected for each region, were sensitive to single base change at selected positions on the genes. According to gel patterns, samples were genotyped. After genotyping, results were analyzed and

p<0.05 was accepted as significant. Three different models and allele frequencies were used in statistical analyzes. Those models were additive, dominant and recessive. Samples were also grouped according to gender (female and male subgroups) and all statistical analyzes were also applied to gender subgroups.

In VDR-ApaI (rs7975232), three model and allele frequency analyzes were insignificant (p>0.05) for general and subgroups. In VDR-TaqI (rs731236), dominant model analysis (AA vs AG+GG) was borderline significant (p=0.051), other models and frequency analyzes were insignificant (p>0.05). Odds ratio for that dominant model analysis of VDR-TaqI is 0.631 and this implies being a G allele carrier reduces the MS risk. For subgroups of VDR-TaqI none of the analyzes were statistically significant.

CYP27A1 (rs6709815) was investigated for the first time in Turkish population. GG genotype was not observed. As a result of missing genotype in CYP27A1, additive, dominant and recessive models could not be used. GT genotype vs TT genotype and allele frequencies were analyzed and results were insignificant (p>0.05) for general and subgroups.

In CYP27B1 (rs4646536), CC genotype was not observed. Model analyzes (additive, dominant and recessive) for CYP27B1 were not applied due to missing genotype. AA vs AC genotype and allele frequencies were analyzed and results were insignificant (p>0.05) for general and subgroups. Even though the results were insignificant, population frequency data of C allele, which is rare variant of rs4646536 was calculated for the first time in this study.

In CYP24A1 (rs1570669), additive model (AA vs AG) (p <0.001, OR:5.413) and dominant model (AA vs AG+GG) (p<0.001, OR:4.429) results of general population were significant. For male subgroup, additive model (AA vs AG) (p<0.001, OR:4.387) and dominant model (AA vs AG+GG), (p<0.001, OR:4.277) results and for female subgroup, additive model (AA vs AG), (p<0.001, OR:6.693) and

dominant model (AA vs AG+GG) ($p<0.001$, OR:4.685) results were significant similar to general population. According to additive and dominant model results of general population and subgroup analyzes, G allele increase susceptibility to the disease. Additive model (AA vs GG), recessive model and allele frequency analyzes were statistically insignificant for general population and male subgroup.In female subgroup, recessive model (AA+AG vs GG) was also statistically significant ($p=0.041$, OR:0.957) and being A allele carrier seem slightly reduce MS risk for females. Additive model (AA vs GG) and allele frequency analyzes of female subgroup was not statistically significant.

Except CYP24A1 (rs1570669) and TaqI polymorphism of VDR (rs731236), our selected polymorphisms show no association with the disease. Because of multifactorial nature of the disease all of them might contribute to disease onset but their effect cannot detectable in our samples.

In conclusion, vitamin D has protective and relieving effect on the disease. According to our CYP24A1 (rs1570669) results, effect of inactivation reaction of vitamin D could be more critical for disease onset. In Turkish population, effect of mutant allele of rs1570669 seems important. For better understanding of importance of that position, research should be repeated with different and larger population samples. Moreover rs1570669 should be investigated further in different populations for more exact results. Additionally, investigation of VDBP and CYP2R1 polymorphisms might be important for better understanding of genetic factors behind the MS. Until complete mechanism of the disease will be solved, vitamin D supplementation seems to be the best way of protection against MS onset. Furthermore, focusing on CYP24A1 blockers could be beneficial for MS treatments.

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APPENDICES

A. INFORMED CONSENTS

A.1. INFORMED CONSENT FOR PATIENTS

Multiple Skleroz (MS) hastalığı ile ilişkili olabileceğini düşündüğümüz genler üzerine araştırma yapmaktayız. Çalışmamızın adı "Multipl Skleroz Riski ile D Vitamini Metabolizmasında Rol Alan CYP Enzimlerinin ve D Vitamini Reseptörünün Genetik Polimorfizmleri Arasındaki İlişkinin Türk Popülasyonunda İncelenmesi" 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, beş gen bölgesinin, MS hastalığı üzerinde risk faktörü olarak etki edip etmediğinin 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 katılmamkonusunda 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

"Multipl Skleroz Riski ile D Vitamini Metabolizmasında Rol Alan CYP Enzimlerinin ve D Vitamini Reseptörünün Genetik Polimorfizmleri Arasındaki İlişkinin Türk Popülasyonunda İncelenmesi" isimli çalışmamızda beş gen bölgesinin, MS üzerinde risk faktörü olarak etki edip etmediğinin belirlenmesi amacıyla, 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 Yapan Araştırmacının;

Adı-Soyadı:

Adres:

Tel:

İmza:

Açıklamaya Başından Sonuna Kadar Tanıklık Eden Kişinin;

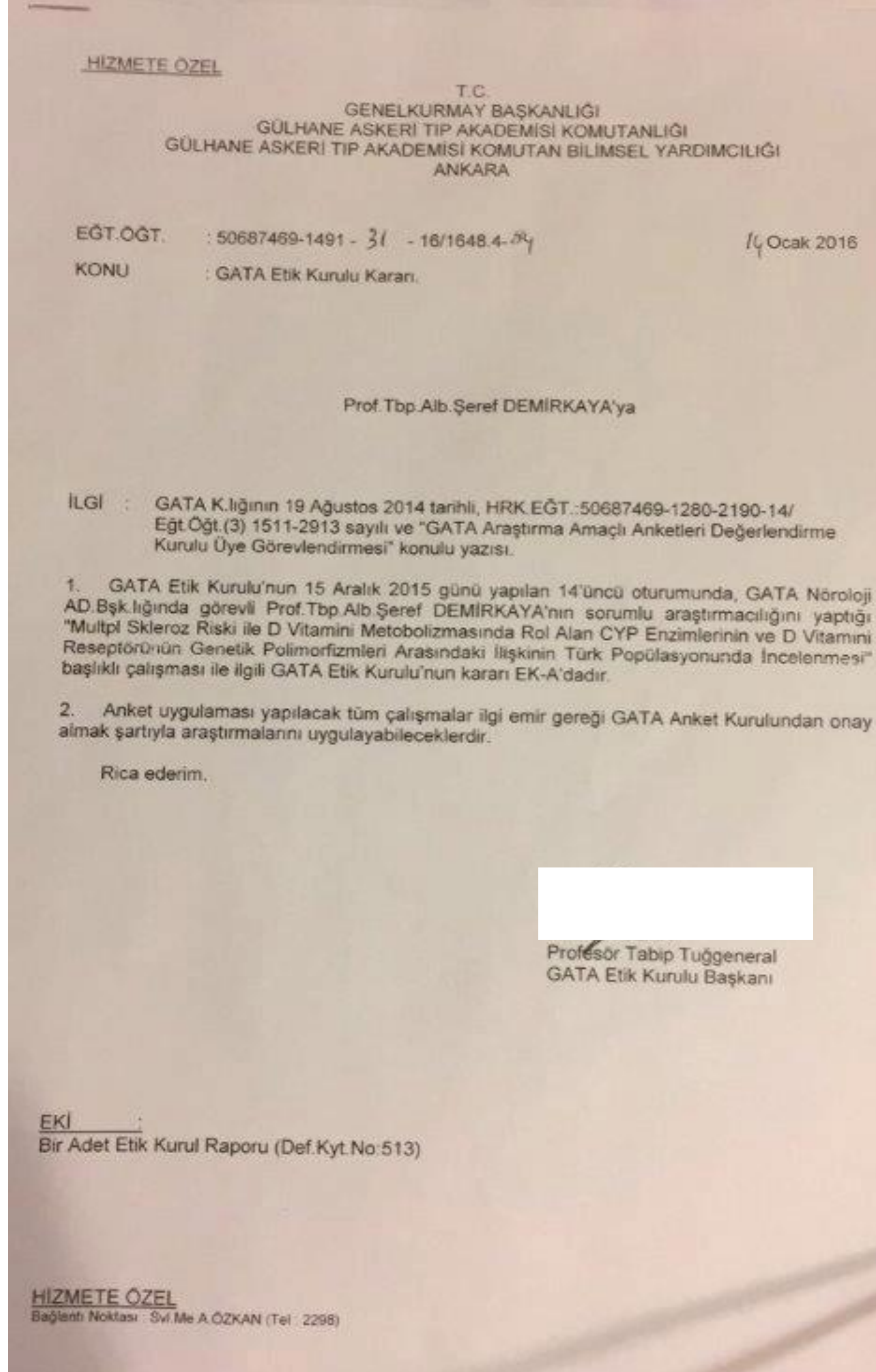
Adı-Soyadı:

Adres:

Tel:

İmza:

B. ETHICAL COMITEE APPROVAL



HİZMETE ÖZEL

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

EĞT.ÖĞT. : 50687469-1491 - 31 - 16/1648.4-04 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 Reseptörünün 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.

Profesör Tabip Tuğgeneral
GATA Etik Kurulu Başkanı

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

HİZMETE ÖZEL
Bağlantı Noktası : Svl.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.
OTURUM SEKRETERİ : Prof. Tbp. Tuğg.

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 — Prof.Hv.Tbp.Tuğg.

ÜYE — Prof.Tbp.Tuğg.

ÜYE — Prof.Diş.Tbp.Alb.

ÜYE — Prof.Tbp.Alb.

ÜYE — Prof.Tbp.Alb.

ÜYE — Prof.Hv.Tbp.Alb.

ÜYE — Prof.Tbp.Alb.

ÜYE — Prof.Tbp.Alb.

ÜYE — Prof.Tbp.Alb.

ÜYE — Prof.Hv.Tbp.Alb.

ÜYE — Prof.Tbp.Alb.

ÜYE — Doç.Dr.J.Tbp.Alb.

ÜYE — Doç.Dr.Hv.Sağ.

HİZMETE ÖZEL

C. PCR and Restriction Gel Images

C.1. Representative Gel Image of TaqI (VDR) Restriction (Patients)

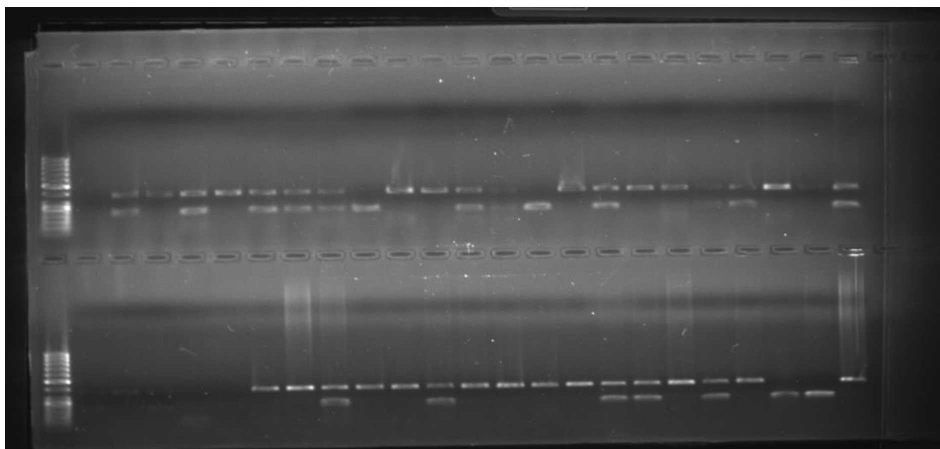


Figure C.1 Representative gel image of TaqI (VDR) restriction endonuclease digestion of patients:
First lanes in the upper and lower part of the gel represent the DNA ladder. Remaining lanes are the genotyping results of patients.

C.2. Representative Gel Image of ApaI (VDR) Restriction (Patients)

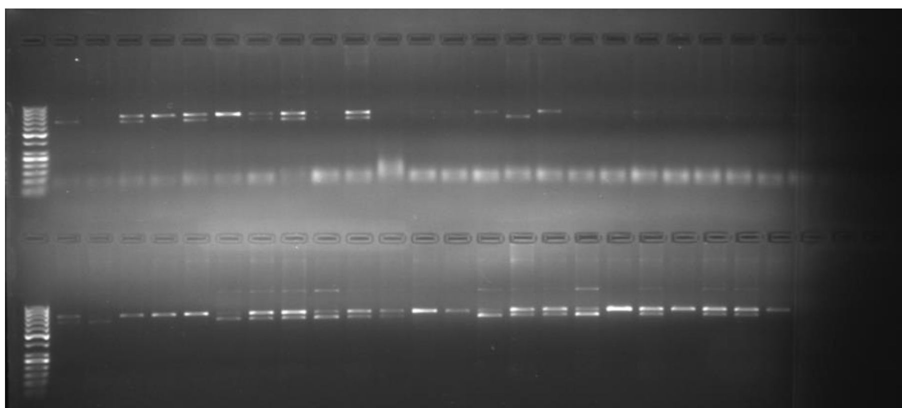


Figure C.2 Representative gel image of ApaI (VDR) restriction endonuclease digestion of patients:
First lanes in the upper and lower part of the gel represent the DNA ladder. Remaining lanes are the genotyping results of patients.

C.3. Representative Gel Image of HphI (CYP24A1) Restriction (Patients)

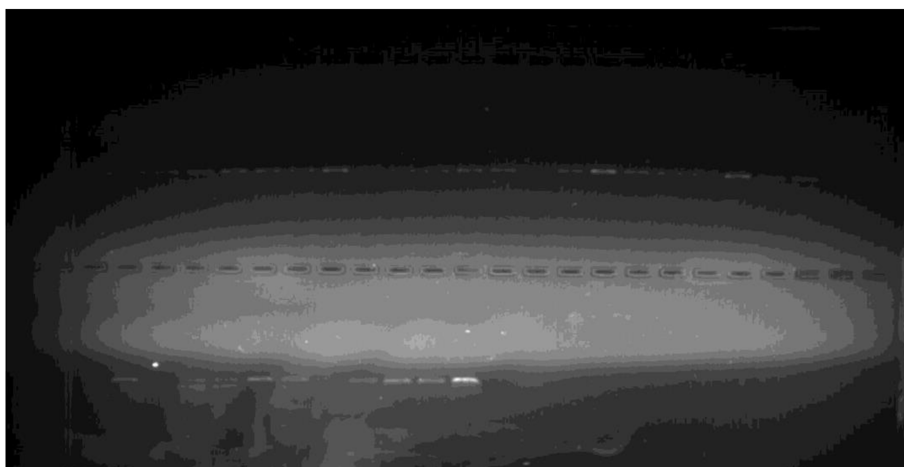


Figure C.3 Representative gel image of HphI (CYP24A1) restriction endonuclease digestion of patients:
Each lane in the upper and lower part of the gel represent the genotyping results of patients.

C.4. Representative Gel Image of BfuAI (CYP27A1) Restriction (Patients)

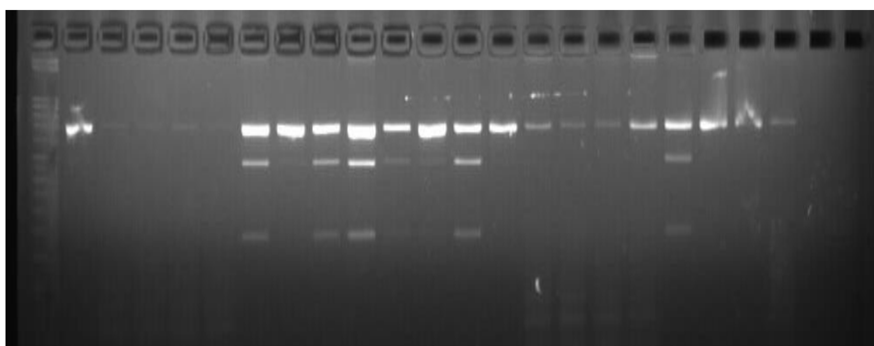


Figure C.4 Representative gel image of BfuAI (CYP27A1) restriction endonuclease digestion of patients:
First lane represents the DNA ladder. Remaining lanes of the gel represent the genotyping results of patients.

C.5. Representative Gel Image of BsmI (CYP27B1) Restriction (Patients)

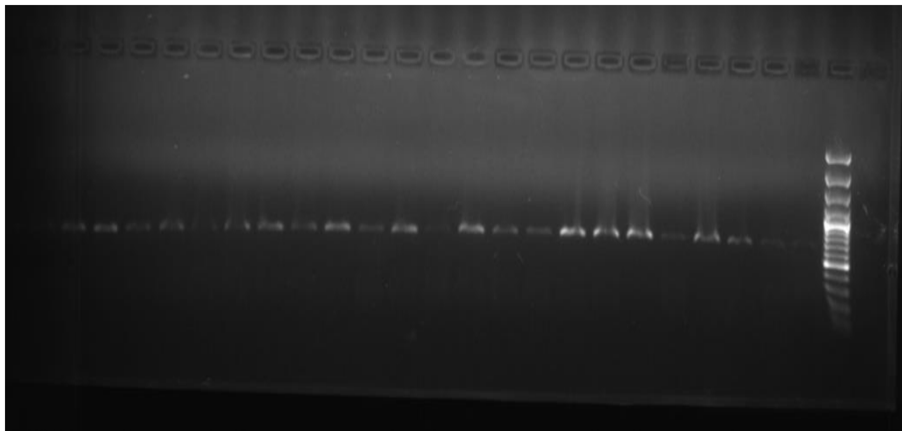


Figure C.5 Representative gel image of BfuAI (CYP27A1) restriction endonuclease digestion of patients:
Last lane represents the DNA ladder. Remaining lanes of the gel represent the genotyping results of patients.

CURRICULUM VITAE

PERSONAL INFORMATION

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EDUCATION

Degree	Institution	Year of Graduation
MS	METU Biology	2014
BS	METU Biology	2010
High School	Milli Piyango Anatolian High School	2005

WORK EXPERIENCE

Year	Place	Enrollment
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FOREIGN LANGUAGES

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PUBLICATIONS

1. Sezer, E., Can Demirdöğen, B., Demirkaya, Ş., Bulut, G., Akkulak, M., Evin, E., & Adalı, O. (2022). Association of cholesterol 7α-hydroxylase (CYP7A1) promoter polymorphism (rs3808607) and cholesterol 24S-hydroxylase (CYP46A1) intron 2 polymorphism (rs754203) with serum lipids, vitamin D levels, and multiple sclerosis risk in the Turkish population. *Neurological Sciences*, *43*(4), 2611-2620.