INVESTIGATION OF EFFECTS OF VITAMIN D AND VITAMIN D METABOLIZING CYP450 ISOZYMES ON MULTIPLE SCLEROSIS PATHOPHYSIOLOGY IN ANIMAL MODEL BY MOLECULAR APPROACHES

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ABSTRACT

INVESTIGATION OF EFFECTS OF VITAMIN D AND VITAMIN D METABOLIZING CYP450 ISOZYMES ON MULTIPLE SCLEROSIS PATHOPHYSIOLOGY IN ANIMAL MODEL BY MOLECULAR APPROACHES

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Multiple sclerosis (MS) is a complicated, recurrent, and often progressive inflammatory demyelinating autoimmune disease of the central nervous system, yet etiopathogenesis remains unsolved. MS frequency increases with increasing latitude, leading to a hypothesis that MS is inversely correlated with the duration and intensity of sunlight and vitamin D concentrations. In this study, the relationships between vitamin D supplementation, MS, VDR, and vitamin D metabolizing CYP enzymes, including CYP2R1, CYP27A1, CYP27B1, and CYP24A1 at mRNA and protein expression levels, were investigated by qRT-PCR and Western blotting techniques in female C57BL/6 mouse autoimmune encephalomyelitis (EAE) model. Both mRNA and protein expressions of Gr 1 were set to 1.00 fold, and expressions of the other groups were calculated relatively to Gr 1. At first, mice were divided into four groups; Gr 1 (control), Gr 2 (vitamin D supplemented control), Gr 3 (EAE), and Gr 4 (vitamin D supplemented EAE). According to the results of this study, vitamin D was not a preventive but a therapeutic agent since significantly higher numbers of mice were recovered in the Gr 4 (8 mice out of 12) than the Gr 3 (2 mice out of 12) ($p=0.013<0.05$). There was no statistically significant difference in CYP27A1 mRNA and protein expression among the groups. CYP2R1 mRNA expression was

significantly low in Gr 4 (0.50 fold; $p<0.05$). However, CYP2R1 protein expression was not significantly different between the groups. CYP27B1 mRNA and protein expression were significantly high in Gr 2 and Gr 4 compared to Gr 3. CYP24A1 protein expression was higher in Gr 4 (1.70 ± 0.46; $p<0.05$), which was positively correlated with the clinical score of the EAE. Although VDR mRNA expression increases with the EAE immunization, it was reverted in protein expression levels probably because of the post-transcriptional regulations by miRNAs. In conclusion, vitamin D supplementation and CYP24A1 inhibitor therapy may help to reduce the severity of MS symptoms.

Keywords: Vitamin D, Multiple sclerosis, Vitamin D metabolizing CYPs, Experimental autoimmune encephalomyelitis, VDR

ÖZ

VİTAMİN D VE VİTAMİN D METABOLİZMASINDA ROL ALAN CYP450 İZOENZİMLERİNİN HAYVAN MODELİNDE MULTİPL SKLEROZ (MS) HASTALIĞININ PATOFİZYOLOJİSİNE ETKİLERİNİN MOLEKÜLER DÜZEYDE ARAŞTIRILMASI

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Multipl skleroz (MS), merkezi sinir sisteminin komplike, tekrarlayan ve sıklıkla ilerleyici inflamatuar demiyelinizan otoimmün bir hastalığıdır, ancak etyopatogenezi henüz çözülememiştir. Ekvatoral kuşaktan uzaklaştıkça MS hastalığının görülme sıklığı artmaktadır, bu da güneş ışığının süresi, açısı ve D vitamini konsantrasyonları ile MS görülme sıklığının ters orantılı olduğunu belirten hipotezin ortaya çıkmasına sebep olmuştur. Bu çalışmada, dişi C57BL/6 farelerde deneysel otoimmün ensefalomiyelit (DOE) modeli oluşturularak, D vitamini takviyesi, MS, VDR ve D vitamini metabolize eden CYP2R1, CYP27A1, CYP27B1 ve CYP24A1 enzimlerinin mRNA ve protein ekspresyon seviyeleri arasındaki ilişkiler qRT-PCR ve Western blot teknikleri ile araştırılmıştır. mRNA ve protein ekspresyonları Gr 1'de 1.00 kat olarak kabul edilerek diğer grupların ekspresyonları hesaplanmıştır ve Gr 1 ile kıyaslanmıştır. Başlangıçta fareler dört gruba ayrılmıştır; Gr 1 (kontrol), Gr 2 (D vitamini takviyeli kontrol), Gr 3 (DOE) ve Gr 4 (D vitamini takviyeli DOE). Çalışmanın sonuçlarına göre, D vitamini önleyici değil, tedavi edici bir ajandır çünkü Gr 4'te (8 fare) Gr 3'e (2 fare) göre önemli ölçüde daha fazla sayıda farede iyileşme gözlemlenmiştir ($p=0.013<0.05$). Gruplar arasında CYP27A1 mRNA ve protein ekspresyonunda anlamlı bir fark görülememiştir. Ayrıca, CYP2R1 mRNA

ekspresyonunun, diğer gruplara kıyasla Gr 4'te (0.50 kat; $p<0.05$) önemli ölçüde düşük olduğu belirlenmiştir. Bununla birlikte, CYP2R1 protein ekspresyonu açısından gruplar arasında önemli bir fark bulunmamaktadır. CYP27B1 mRNA ve protein ekspresyonunun, Gr 3'e göre Gr 2 ve Gr 4'te önemli ölçüde yüksek olduğu görülmüştür. CYP24A1 protein ekspresyonunun, DOE'nin klinik skoru ile pozitif korelasyon göstererek Gr 4'te (1.70 ± 0.46; $p<0.05$) daha yüksek olduğu tespit edilmiştir. VDR mRNA ekspresyonu, DOE immünizasyonu sonucunda artarken protein ekspresyonu seviyelerinin sağlıklı gruplarla eşit olduğu görülmüştür. Bu durum miRNA'ların post-transkripsiyonel düzenlemelerde aldığı rol nedeniyle gerçekleşmiş olabilir. Sonuç olarak, D vitamini takviyesi ve CYP24A1 inhibitörü tedavisi MS semptomlarının şiddetini azaltmaya yardımcı olabilir.

Anahtar Kelimeler: D vitamini, Multipl skleroz, Deneysel otoimmün ensefalomyelit, D vitaminini metabolize eden CYP'ler, VDR

This study is dedicated to my lovely wife Duygu and our big family, whose help, patience, support, and love are undeniable and unforgettable. They have selflessly given more to me than I ever could have asked for.

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LIST OF ABBREVIATIONS

ABBREVIATIONS	
AP	Alkaline phosphatase
APS	Ammonium per sulfate
BCA	Bicinchoninic acid
BCIP	5-bromo 4-chloro 3-indoyl phosphate
bp	Base pair
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CFA	Complete Freund’s adjuvant
CNS	Central nervous system
CRE	cAMP response element
Ct	Threshold cycle
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
EAE	Experimental autoimmune encephalomyelitis

EDTA	Ethylenediaminetetraacetic acid
EDSS	Expanded disability status scale
EGF	Epidermal growth factor
FGF-23	Fibroblast growth factor-23
F-9	Ferriprotoporphyrin 9
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
HS	High spin
HNF4α	Hepatocyte nuclear factor 4α
IL	Interleukin
IU	International Unit
LBD	Ligand binding domain
LS	Low spin
MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger RNA
MS	Multiple sclerosis
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NBT	Nitrotetrazolium blue chloride
NCoR	Nuclear corepressor
OD	Optical density
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PTH	Parathyroid hormone
PVDF	Polyvinylidene fluoride
PXR	Pregnane X receptor
qRT-PCR	Quantitative real time polymerase chain reaction
RDI	Recommended daily intake
rpm	Revolutions per minute
RNA	Ribonucleic acid
RRMS	Relapsing-remitting multiple sclerosis
RXR	Retinoid X receptor
SDB	Sample dilution buffer
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptors
TBS	Tris-buffered saline
TBST	Tris-buffered saline and Tween 20
TEMED	Tetramethylethylenediamine
TNFα	Tumor necrosis factor α
UVB	Ultraviolet B radiation
VDR	Vitamin D receptor
VDRE	Vitamin D response element
4PL	4 parameter logistic

LIST OF SYMBOLS

SYMBOLS

Λ_{max}	Maximum absorption wavelength
ΔC_t	Threshold cycle difference
^{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	Correlation coefficient
R^2	Coefficient of determination

CHAPTER 1

INTRODUCTION

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is one of the most common neurological autoimmune diseases that affect the central nervous system (CNS). It is an inflammatory demyelinating disease with axonal damage, which usually shows relapses and sometimes gradual progression (Noseworthy et al., 2000). The disease can involve multiple parts of the central nervous system, and clinical findings vary depending on these areas. The most common symptoms are primarily motor and sensory disorders, and vision loss, brainstem findings-double vision, balance and coordination disorders, and bladder dysfunctions (Compston et al., 2008). Although the etiopathogenesis of MS has not been fully revealed, inflammation and demyelination in the myelin sheath develop on a genetic and an immunological basis triggered by an environmental factor (Ghasemi et al., 2017).

The disease is mostly seen in young adults (20-40 years old) and is twice as common in women as in men (Simone et al., 2000). In addition, studies have found that geographical factors have an effect on the incidence of MS disease. For example, it was reported that the prevalence of MS disease increases with distance from the equatorial belt (Compston et al., 2008; Goldberg, 1974). The fact that the risk of MS increases with distance from the equatorial belt and the prevalence of MS is close to each other even in different races at the same latitudes has led to the emergence of the MS-Vitamin D hypothesis (Goldberg, 1974). According to this hypothesis, which was put forward about 40 years ago, Vitamin D deficiency suggests that it may be a risk factor for MS. In the following years, with the discovery of the immunomodulatory effects of Vitamin D, research on the MS-Vitamin D hypothesis has increased. Vitamin D is thought to be a molecule that not only prevents disease

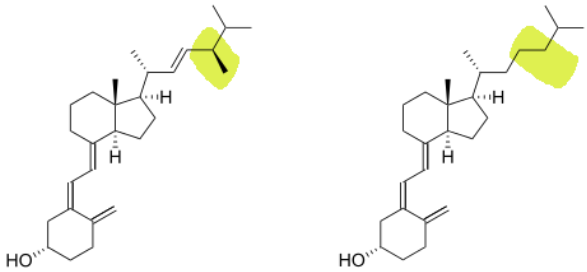
but also has the potential to reduce the symptoms of MS (Goldberg et al., 1986; Pierrot-Deseilligny et al., 2017). Furthermore, in observations made in patients with MS, it has been found that some characteristic symptoms of the disease fluctuate seasonally (Harding et al., 2017). In the first observations, it was determined that the patients had fewer relapses in the spring and summer compared to the winter season in Switzerland (Wüthrich et al., 1970). In later studies, it was shown that the Expanded Disability Status Scale (EDSS) score and serum vitamin D concentration have a negative correlation (J Smolders et al., 2008; Van Der Mei et al., 2007). In addition, it was reported that the serum vitamin D concentration of patients with relapsing-remitting MS (RRMS) is lower in the relapse period than in the remission period (Soilu-Hanninen et al., 2005). In addition to this information, in another study, it was shown that an increase of 10 nmol/L in serum vitamin D level of RRMS patients revealed a 9-12% decrease in the risk of attack frequency (Simpson et al., 2010).

Although many results supporting the MS-Vitamin D hypothesis have been reported in clinical studies, no significant success has yet been achieved in the cause and treatment of the disease. Therefore, Experimental Autoimmune Encephalomyelitis (EAE) MS animal models are used in studies to reveal the mechanism and treatment of MS disease. Thanks to the strong similarities between EAE and MS, three drugs (glatiramer acetate, mitoxantrone, and natalizumab) that are now effectively used in treating the disease were introduced by the EAE-MS research into clinical use (Karandikar et al., 2002; Rudick et al., 2008; Sivertseva et al., 2009). Research in the EAE model of MS has also provided solid support for the vitamin D-MS hypothesis. According to the study of Lemire et al., $1,25(OH)_2D_3$ injections, which were started 3 days before immunization of SJL/J mice and continued daily, were shown to reduce disease morbidity and mortality (Lemire et al., 1991). In another study, the preventive effect of vitamin D on disease development was demonstrated in the B10.PL mouse model of EAE (Cantorna et al., 1996). It was reported that vitamin D supplementation applied in the most severe period of acute EAE has a therapeutic effect on the symptoms of the disease (Mayne et al., 2011). Although there are a

limited number of publications reporting that vitamin D supplementation has preventive and mitigating effects in the EAE animal model, the molecular mechanism of these effects has not yet been clarified. There are not enough studies examining the relationship between multiple sclerosis and vitamin D metabolizing enzymes and vitamin D receptor (VDR) at the gene and protein expression level in the EAE model.

1.2 Vitamin D

Vitamin D is a secosteroid with well-known classical effects on calcium and phosphorus homeostasis and maintains bone health (Omdahl et al., 2002; Zalewski et al., 2016). It stimulates intestinal absorption of calcium and phosphorus while it stimulates the differentiation of osteoclasts in the bone. In humans, mainly vitamin D is produced in the skin *via* ultraviolet B radiation (UVB; 290-315 nm) from the sun, while minor amounts can be taken by the diet. Vitamin D is mainly found in two forms, cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2), from animal and plant sources, respectively. There is an additional methyl group on 24^{th} carbon in ergocalciferol which is the only structural difference between cholecalciferol and ergocalciferol (Figure 1.1). Synthesis of vitamin D_3 in the skin depends on the 7-dehydrocholesterol concentration in the epidermis, melanin pigment, and the angle of UVB radiation, which is determined by latitude, altitude, season, and time of day (T. C. Chen et al., 2010).



Ergocalciferol
(Vitamin D2)

Cholecalciferol
(Vitamin D3)

Figure 1.1 Two main forms of Vitamin D.

Conversion of provitamin D_3 (7-dehydrocholesterol) to previtamin D_3 in the skin starts with the absorption of UVB radiation which causes photolysis of 7-dehydrocholesterol, and then previtamin D3 is isomerized to cholecalciferol in the plasma membrane (Wacker et al., 2013). Either produced in the skin or taken by the diet, vitamin D is transported to the liver by vitamin D binding protein (DBP) in the blood (Bouillon et al., 2020). In the liver, activation of vitamin D starts with hydroxylation at 25^{th} carbon to produce 25-hydroxyvitamin D ($25(OH)D_3$) by cytochrome P450 enzymes (Sakaki et al., 2005). Although it is not biologically active, $25(OH)D_3$ is used as an index to determine vitamin D levels due to its long half-life (10-50 days) in the blood (Datta et al., 2017). In the kidney, this molecule undergoes 1α-hydroxylation by another cytochrome P450 (CYP27B1) enzyme, and biologically active vitamin D, 1,25-dihydroxyvitamin D (calcitriol), is produced (Holick et al., 1987). This active metabolite is inactivated and eliminated *via* hydroxylation at 24^{th} carbon to make 1,24,25-trihydroxyvitamin D_3 (calcitroic acid) by the CYP24A1 enzyme in the kidney. Besides hormone and other metabolites, the expression and activity of CYP27B1 and CYP24A1 are highly regulated by the active vitamin D metabolite ($1,25(OH)_2D_3$) (Jones et al., 2012; Zierold et al., 1994). This active metabolite can regulate the expression of many genes involved in cell proliferation, apoptosis, cell differentiation, calcium homeostasis, and the immune response by binding to a nuclear receptor, VDR (Fernández-Barral et al., 2020;

Martens et al., 2020; Pike et al., 2010; Zheng et al., 2017). Figure 1.2 gives the complete pathway for vitamin D metabolism (Rouphael et al., 2018).

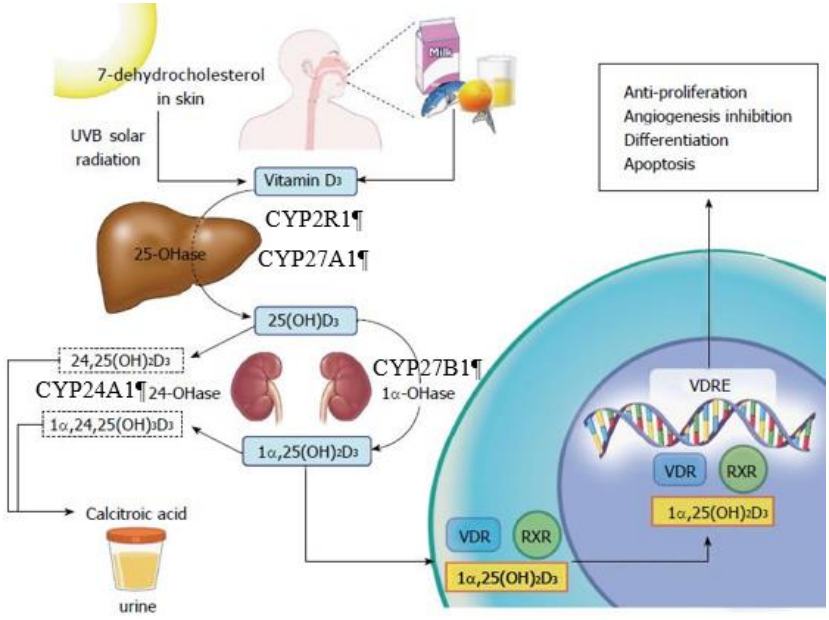


Figure 1.2 Pathway of vitamin D metabolism and effects of vitamin D (Rouphael et al., 2018).

Besides classical effects like calcium homeostasis, vitamin D has an immune regulatory function. It prevents differentiation and proliferation of B cells and immunoglobulin secretion (S. Chen et al., 2007; Lemire et al., 1984). Moreover, vitamin D suppresses T cell proliferation and causes the transition from Th1 phenotype to Th2 phenotype (Bhalla et al., 1984; Boonstra et al., 2001; Mattner et al., 2000). In addition, it increases T regulator cell number by affecting T cell maturation (Barrat et al., 2002). As a result of these immunological effects, vitamin D prevents the production and secretion of inflammatory cytokines (IL-17, IL-21) while inducing anti-inflammatory cytokines (IL-10) (Xia et al., 2019). Vitamin D is also involved in the regulation of monocytes and dendritic cells (DC) (Piemonti et al., 2000). It prevents the production of inflammatory cytokines (IL-1, IL-6, IL-8, IL-12 ve TNFα) by monocytes (Almerighi et al., 2009). Also, it prevents the differentiation and maturation of dendritic cells (Almerighi et al., 2009). Inhibition of the DC differentiation and maturation is significant in the context of autoimmunity

and self-tolerance. Typically, mature DCs promotes antigen recognition of T cells and induce an immune response against an antigen; however, immature DCs increases tolerance and prevents autoimmune response against self-antigens. Because of these immune regulatory effects of Vitamin D, its deficiency or abnormalities at the receptor level are considered a risk factor for autoimmune diseases.

1.3 Vitamin D Receptor

Vitamin D receptor (VDR) is a nuclear receptor for calcitriol [1,25$(OH)_2D_3$], the active vitamin D metabolite. In humans, its gene is located on chromosome 12q13.11 (geneID:7421). VDR protein has an amino acid length of 427 and a molecular mass of 48.3 kDa (Figure 1.3) (Itoh et al., 2020). It has a DNA-binding domain (DBD) and ligand-binding domain (LBD) in the structure similar to other nuclear receptors like the pregnane X receptor (PXR) (Bouillon et al., 2008; Rachez et al., 2000). VDR is expressed in various tissues and has highly conserved homology in structure, ligand binding, and functionality among different species (Bouillon et al., 2008; L. Yang et al., 2012).

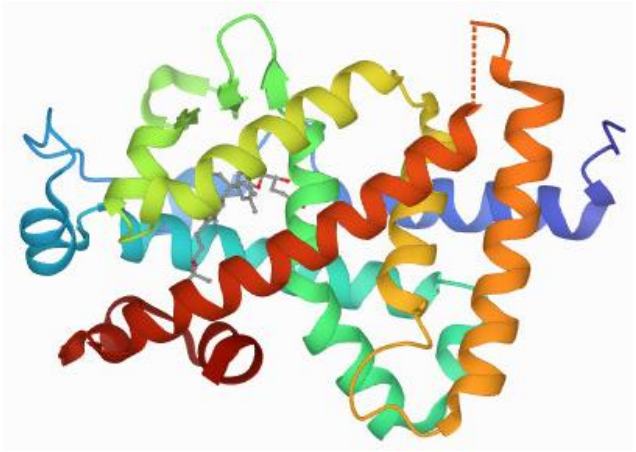


Figure 1.3 Crystal structure of vitamin D receptor (Itoh et al., 2020).

Vitamin D transcriptionally regulates several genes *via* binding to VDR. Mechanistically, first, 1,25$(OH)_2D_3$-VDR complex forms a dimer with the retinoid

X receptors (RXRα, RXRβ, RXRγ) and binds vitamin D response elements (VDRE) in the promoter region of the genes, and other nuclear proteins are recruited into the promoter region to regulate their transcription (Figure 1.4) (Bikle, 2014; von Essen et al., 2012). The VDRE may have either stimulatory or inhibitory effects on the related gene. These effects of the VDRE may depend on the type of RXR dimerizing with VDR. The steroid receptor activator complex (SRC) and the vitamin D receptor-interacting protein complex (DRIP) or mediator complex are the complexes that are responsible for the activation (Rachez et al., 2000; York et al., 2010). Nuclear corepressor (NCoR) and silencing mediator of retinoic acid and thyroid receptor (SMRT) are responsible for the inhibitory effects of VDRE; these corepressors bind to VDR when $1,25(OH)_2D_3$ is absent (L. Yang et al., 2012).

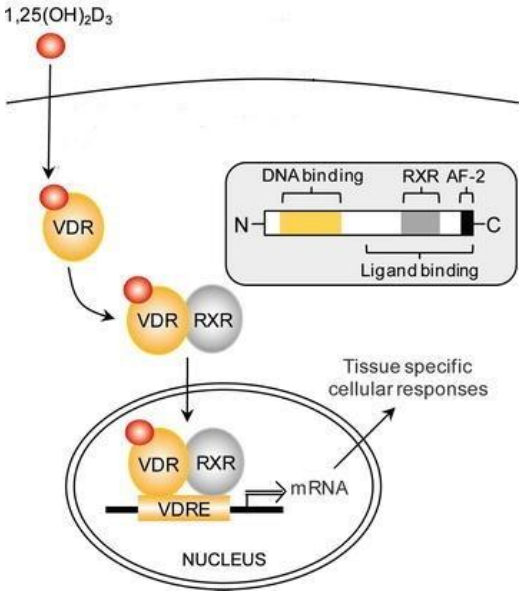


Figure 1.4 The action mechanism of VDR (von Essen et al., 2012).

Due to the gene regulatory effects of vitamin D *via* VDR, any defects or changes in the VDR gene could lead to severe problems in the activation of genes related to calcium homeostasis, immune regulation, cell proliferation and differentiation. For example, a point mutation in the human VDR gene cause resistance to $1,25(OH)_2D_3$ in rickets (Sone et al., 1990). Moreover, It was reported that VDR deficiency is related to inflammatory bowel disease in mice (Kim et al., 2013). In another study,

VDR deficiency increases tumor burden by enhancing Wnt/β-catenin signaling in colon cancer (Larriba et al., 2011). Furthermore, It was found that VDR deficiency increases the rate of aging progress in mice brains (Minasyan et al., 2007). Along with these findings, It has been reported that a VDR polymorphism (rs2228570) is associated with increased susceptibility to MS (Cancela Díez et al., 2021).

1.4 Cytochrome P450s

Cytochrome P450 (CYP) enzymes are a membrane-bound superfamily of hemoproteins involved in the metabolism of xenobiotics and endogenous compounds. The term "P450" stands for a pigment with maximum absorption at 450 nm when the reduced state of the enzyme is complexed with carbon monoxide (Omura et al., 1964). The nomenclature of a cytochrome P450 enzyme includes the acronym for cytochrome P450 "CYP" followed by a numeral denoting the CYP family (*e.g.*, CYP2, CYP24, and CYP27) followed by a capital letter for subfamily (*e.g.*, CYP2R, CYP27A, CYP27B, and CYP24A) (D. Nelson et al., 1996). Another numerical represents individual CYP enzymes (*e.g.*, CYP2R1, CYP27A1, CYP27B1, and CYP24A1) (D. Nelson et al., 1996). Amino acid sequence similarity is at least 40% within the same family, while at least 55% homology is shared in the same subfamily (Coon et al., 1992). 13,000 genes representing over 400 gene families of CYPs were identified across all the biological kingdoms (D. R. Nelson, 2009). In humans, there are 18 families, 43 subfamilies, and 57 individual CYP enzymes with different metabolic activities (Table 1.1) (D. R. Nelson, 2009). The CYPs are involved in the metabolism of xenobiotics and endogenous compounds such as vitamins, fatty acids, steroids, and hormones by oxidation and reduction reactions, including dealkylation, oxygenation, hydroxylation, deamination, and epoxidation by using NADPH as a cofactor in a variety of tissues including, the liver, kidney, brain, and many others (Adali et al., 1996; Guengerich, 2008; Hrycay et al., 2012; R. P. Meyer et al., 2002; Omiecinski et al., 1990).

Table 1.1 Human CYP genes and their functions (D. R. Nelson, 2009).

CYP Families	***Names***	***Function***
CYP1 (3 genes)	1A1, 1A2, 1B1	Xenobiotic Metabolism
CYP2 (16 genes)	2A6, 2A7, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 2R1, 2S1, 2U1, 2W1	Xenobiotic and Steroid Metabolism
CYP3 (4 genes)	3A4, 3A5, 3A7, 3A43	Xenobiotic and Steroid Metabolism
CYP4 (12 genes)	4A11, 4A22, 4B1, 4F2, 4F3, 4F8, 4F11, 4F12, 4F22, 4V2, 4X1, 4Z1	Fatty Acid Metabolism
CYP5 (1 gene)	5A1	Thromboxane A_2 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)	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 D_3 Activation
CYP39 (1 gene)	39A1	Cholesterol Biosynthesis
CYP46 (1 gene)	46A1	Cholesterol Biosynthesis
CYP51 (1 gene)	51A1	Cholesterol Biosynthesis

CYPs are the membrane-bound enzymes mainly located in the endoplasmic reticulum and mitochondria (Black, 1992). Their active sites are connected to the membrane and span the membrane to reach the cytosol, enabling them to accept substrates from both environments (High et al., 1992). CYPs have a conserved haem structure called ferriprotoporphyrin 9 (F-9) located at the center of the enzyme's active site (Figure 1.5) (Hill et al., 1970). The iron molecule in the F-9 structure is normally found in the ferrous form, Fe^{2+}; however, the substrate binds to enzyme when the iron molecule is in the ferric form (Fe^{3+}). Fe^{3+} exists in two spin states, low spin (LS) and high spin (HS). In the LS state, five 3d electrons are all paired, while in the HS state, the five 3d electrons are unpaired (Shannon et al., 1970). In substrate-free form, the LS state is stabilized by a water molecule (Poulos et al., 1986). With the binding of the substrate, the iron-water complex is dislocated from the enzyme, which changes the coordination state of the Fe^{3+} from a six-fold to a five-fold, and iron moves out of the plane of the F-9 structure (Raag et al., 1989). Inhibitors of CYP enzymes increase the stability of the LS state by binding to the Fe^{3+} and removing the water molecule (Poulos et al., 1987).

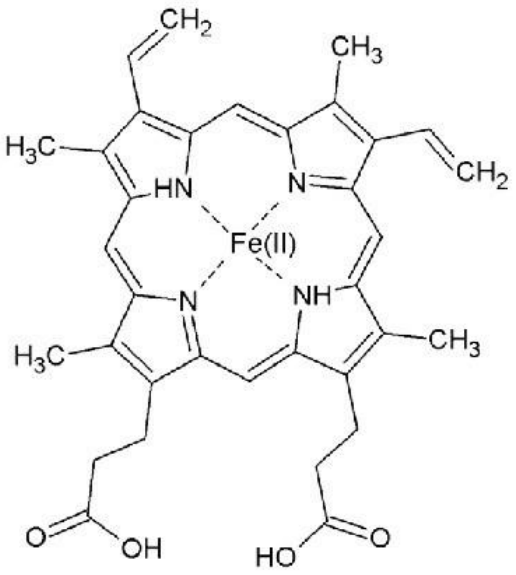


Figure 1.5 Chemical structure of ferriprotoporphyrin 9 (F-9).

Structural similarity at the active site of the CYP enzymes provides a common catalytic mechanism for the insertion of an oxygen molecule into a substrate (Figure 1.6). In the first step, the substrate binds to the Fe^{3+} form of a CYP enzyme, followed by an electron transfer from NAD(P)H, which the NADPH-P450 reductase enzyme reduces Fe^{3+} to Fe^{2+}. In the third step, the binding of O_2 molecule to reduced enzyme-substrate complex forms an unstable structure that leads to the formation of $Fe^{+3}O_2^-$ RH. This unstable complex is reduced again by the NADPH-P450 reductase enzyme to generate "activated oxygen"-CYP-substrate complex. In the fifth step, O-O bond is cleaved, and it is followed by the release of the H_2O to generate high-valent FeO^{3+}. In the seventh step, a hydrogen atom of the substrate binds to $FeO^{3+,}$ and R^*-(Fe-OH)$^{+3}$ is produced. In the next step, the activated carbon (R^*) reacts with the hydroxyl group, and the product is formed (R-OH). In the ninth, final step, the product dissociates from the enzyme's active site (Guengerich, 2001).

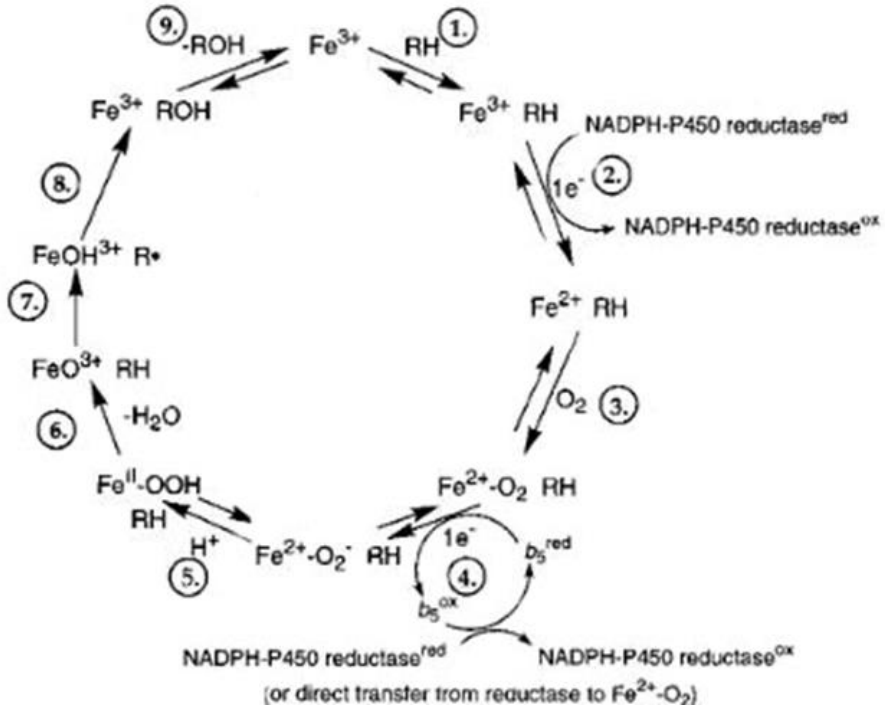


Figure 1.6 Catalytic cycle of CYPs for the oxygenation of substrates (Guengerich, 2001).

1.4.1 Vitamin D Metabolizing Cytochrome P450s

There are four major CYP isozymes responsible for the metabolism of vitamin D3 including, CYP2R1 and CYP27A1, (25-hydroxylase); CYP27B1, (1α-hydroxylase); CYP24A1, (24-hydroxylase).

1.4.1.1 CYP2R1

CYP2R1 is a microsomal cytochrome P450 monooxygenase that catalyzes the hydroxylation at C-25 of both cholecalciferol and ergocalciferol, mainly expressed in the liver (Figure 1.7) (Bergadà et al., 2014; Shinkyo et al., 2004). In humans, its gene is located on chromosome 11p15.2 (geneID:120227). CYP2R1 protein has an amino acid length of 501 and a molecular mass of 57 kDa, which is highly conserved among species; for example, 89% amino acid sequence homology exists between humans and mice (Cheng et al., 2003). Although vitamin D 25-hydroxylation is mainly carried out in the liver, CYP2R1 expression was found in many extrahepatic tissues, including kidneys, testes, and brains (Choudhary et al., 2005).

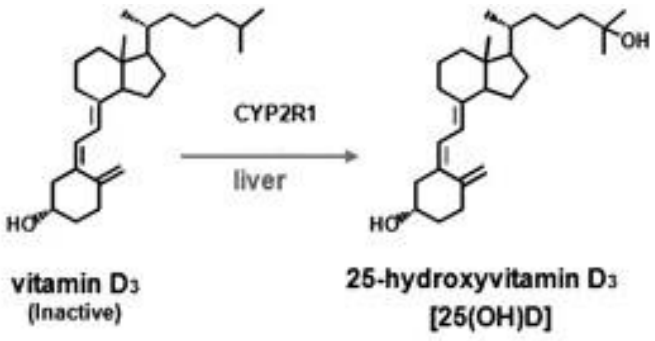


Figure 1.7 25-Hydroxylation of vitamin D catalyzed by CYP2R1.

Typically, the CYP2R1 protein consists of α-helices with β-sheets with the heme group (Figure 1.8) (Strushkevich et al., 2008). The dimer interface of the protein and the active site is formed by F and G helices (Strushkevich et al., 2008). The small G'-helix on the loop of the G helix is highly hydrophobic and associates with the ER

membrane (Williams et al., 2000). The heme group is bound to CYP2R1 at Arg109, Trp133, Arg137, His381, Ser442, and Arg446 amino acid positions by hydrogen bonding (Strushkevich et al., 2008). G and I helices forms the enrty channel for the substrate (Cojocaru et al., 2007). The enzyme's active site is covered mainly by hydrophobic amino acids, vital for accessing non-polar substrates to the active site *via* hydrophobic interactions (Roizen et al., 2017).

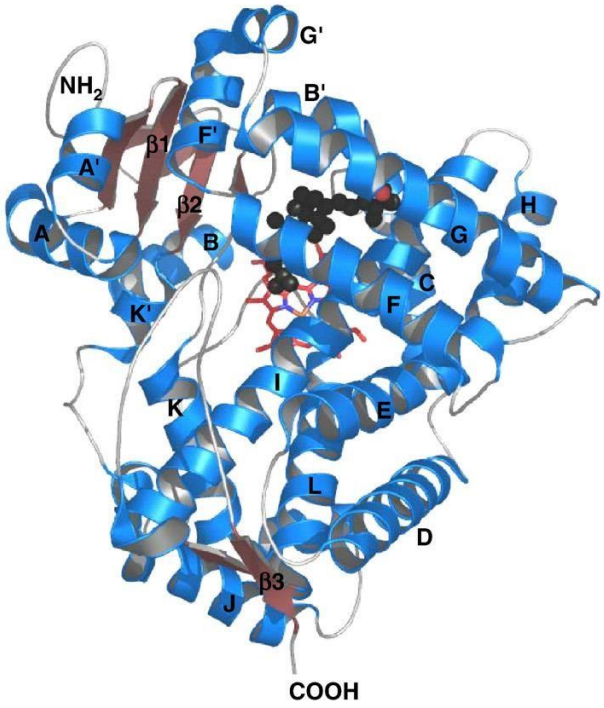


Figure 1.8 3D structure model of human CYP2R1. The heme is red, while Vitamin D3 is shown above the heme with spheres are black (Strushkevich et al., 2008).

CYP2R1 gene expression is regulated by several transcription factors either may induce or suppress gene expression of CYP2R1 depending on metabolic signals (Bouillon et al., 2019). According to a study that compares obese and control mice, CYP2R1 mRNA expression was (~40%) lower in the livers of obese mice, which correlates with lower serum 25(OH)D_3 concentration (Bassatne et al., 2019). Besides obesity, type 1 and type 2 diabetes conditions also cause a significant decrease in both CYP2R1 gene and protein expressions (Aatsinki et al., 2019). It has been reported that decrease in CYP2R1 mRNA expression is controlled by PPARγ-

coactivator-1α (PGC1α) which needs a nuclear receptor to bind the promoter region of CYP2R1 including estrogen-related receptor α (ERRα), VDR, and glucocorticoid receptor (GR) (Aatsinki et al., 2019).

According to a study by Zhu and colleagues, CYP2R1 is a major 25-hydroxylase enzyme in vitamin D metabolism. They compared the 25(OH)D_3 concentrations of wild type, Cyp2r1$^{-/-}$, Cyp27a1$^{-/-}$, and Cyp2r1$^{-/-}$/Cyp27a1$^{-/-}$ knock out mice groups; 25(OH)D_3 concentration of Cyp27a1$^{-/-}$ was similar to wild type mice while it decreased significantly in Cyp2r1$^{-/-}$ mice (Zhu et al., 2013). Thus any mutations in this gene may result in severe diseases due to the decreased circulating activated vitamin D_3 levels. A mutation that substitutes proline for leucine at position 99 of the encoded protein (L99P) in CYP2R1, which may impair protein folding, is associated with rickets and low circulating levels of 25(OH)D_3 (Cheng et al., 2004; Thacher et al., 2017). Besides mutations, polymorphisms of CYP2R1 are a risk factor for several diseases, such as rs10766197 CYP2R1 single nucleotide polymorphism (SNP) is associated with an increased risk of multiple sclerosis (Scazzone et al., 2018; J. jing Yang et al., 2019).

1.4.1.2 CYP27A1

CYP27A1 is a mitochondrial cytochrome P450 monooxygenase that catalyzes many reactions, including hydroxylation at C-25 of cholecalciferol to activate vitamin D_3 and C-27 of cholesterol to break down cholesterol to bile acid (Figure 1.9) (Bergadà et al., 2014; Sawada et al., 2000). In humans, its gene is located on chromosome 2q35 (geneID:1593) with 9 exons and is mainly expressed in the liver (Barchetta et al., 2012). CYP27A1 protein has a molecular mass of 60 kDa with an amino acid length of 531 consisting of 498-amino acid mature enzyme and a 33-amino acid mitochondrial signal sequence (Cali et al., 1991). Although vitamin D 25-hydroxylation is mainly carried out in the liver, CYP27A1 expression was found in many extrahepatic tissues and cells, including kidney, brain, and endothelial cells (S. Anderson et al., 1989; Reiss et al., 1997).

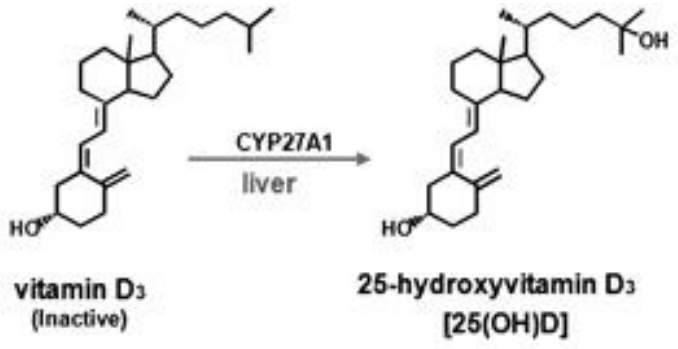


Figure 1.9 25-Hydroxylation of vitamin D catalyzed by CYP27A1.

Charvet and colleagues proposed a 3 D model for the structure of CYP27A1 protein consists of α-helices with β-sheets with the heme group where lysine at 476th position is a part of the active site of the enzyme (Figure 1.10). They showed that mutation in this position causes changes in the K_d of the enzyme towards its substrate (Charvet et al., 2013).

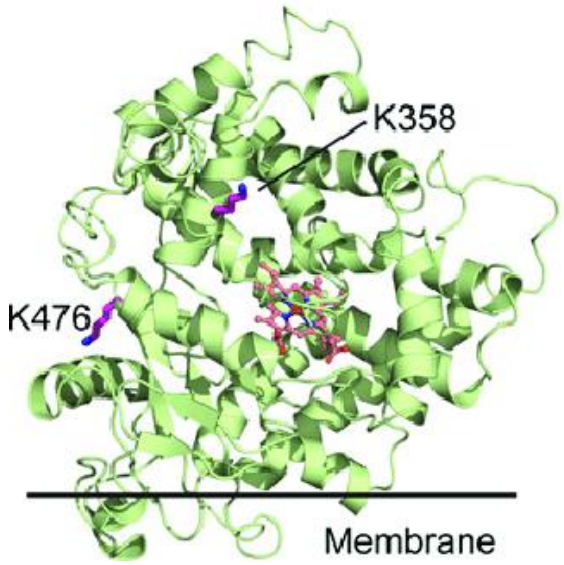


Figure 1.10 3D structure model of human CYP27A1. Green color represents α-helices, β-sheets, loops, and turns of protein while pink spheres represent ferriprotoporphyrin IX (Charvet et al., 2013).

CYP27A1 gene expression is regulated by several transcription factors either may induce or suppress gene expression of CYP27A1.In rats, mRNA expression is up-

regulated by the cholesterol while down-regulated by the bile acid (Twisk et al., 1995; Xu et al., 1999). In another study, it was reported that PPARγ and retinoid x receptor (RXR) up-regulates CYP27A1 in humans (Quinn et al., 2005). Hepatocyte nuclear factor 4α (HNF4α) also stimulates CYP27A1 mRNA expression, and any mutation that prevents binding of HNFα in the promoter region reduces the activity of the promoter (W. Chen et al., 2003).

Although CYP27A1 catalyzes the 25 hydroxylation of vitamin D_3, its primary function is considered catalysis of 27 hydroxylation of cholesterol. Moreover, the key enzyme in the 25 hydroxylation of vitamin D_3 is CYP2R1 (Rosen et al., 1998; Zhu et al., 2013). As a result, It was believed that CYP27A1 is not related to diseases rooted in vitamin D deficiency but to cholesterol metabolism abnormalities.

1.4.1.3 CYP27B1

CYP27B1 is a mitochondrial cytochrome P450 monooxygenase that catalyzes the rate-limiting reaction in the activation of vitamin D in the kidney *via* hydroxylation at 1α position of 25(OH)D_3 (Figure 1.11) (Bouillon et al., 1995; Melo et al., 2020; Sawada et al., 2001). In humans, its gene is located on chromosome 12q14.1 (geneID:1594) with 9 exons and is mainly expressed in the kidney. CYP27B1 protein has a molecular mass of 56.5 kDa with an amino acid length of 508 (Fu et al., 1997). Although 1α-hydroxylation of 25(OH)D_3 is mainly carried out in the kidney, CYP27B1 expression has been found in extra-renal tissues, including liver, pancreas, thyroid gland, prostate, colon, and bone (Bikle et al., 2018).

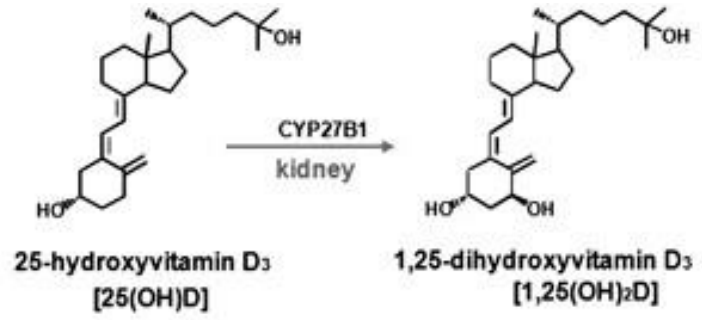


Figure 1.11 1α-Hydroxylation of vitamin D catalyzed by CYP27B1.

Yamamoto and colleagues proposed a homology 3D model for the structure of the human CYP27B1 enzyme (Figure 1.12). It comprises 17 helices and 6 β-strands where F and G loops form the substrate entry site (Yamamoto et al., 2004). Mutations at Gln^{65} and Thr^{409} positions prevent binding of substrate to CYP27B1 and are considered part of the substrate-binding site where the 25-OH group of $25(OH)D_3$ can form the hydrogen bond with Gln^{65} and Thr^{409} (Yamamoto et al., 2005). The heme group is located between the L and the I helices, where the sulfide of Cys^{455} provides the axial ligand at the fifth coordination site of the heme iron (Yamamoto et al., 2004).

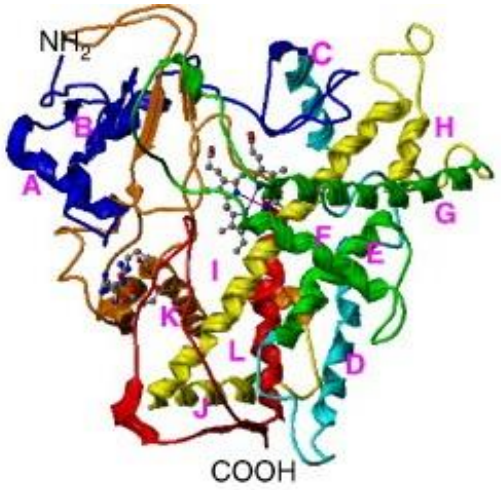


Figure 1.12 3D structure model of CYP27B1, presented in ribbon loop structure while heme is shown as ball and stick (Yamamoto et al., 2004).

The excessive amount of circulating $1,25(OH)_2D_3$ leads to granulomatous disease, which also causes hypercalcemia. These side effects occur when the 1-α-hydroxylase activity of the CYP27B1 enzyme is dysregulated. Normally, circulating $1,25(OH)_2D_3$ level is strictly controlled by regulating CYP27B1 in the kidney. At the transcriptional level, CYP27B1 expression is under the control of several transcription factors. It was reported that MAPK signaling *via* MEK/ERK1/2 plays a vital role in the transcriptional regulation of CYP27B1 in the kidney (Chanakul et al., 2013). Cyclic AMP (cAMP) is the well-known signal pathway that regulates the activation of MAPKs. There are three cAMP response-element (CRE) sites in the promoter of the CYP27B1 gene to regulate gene expression *via* cyclic AMP mediated signaling pathway (Brenza et al., 1998; Kong et al., 1999). Epidermal growth factor (EGF) and parathyroid hormone (PTH) stimulate the expression of CYP27B1 through this pathway (T. C. Chen, 2015). In another study, it was reported that CYP27B1 mRNA expression is suppressed by fibroblast growth factor-23 (FGF-23) both in renal and extra-renal tissues of normal mice (Chanakul et al., 2013). Besides regulation at the transcriptional level, the product of the enzyme, $1,25(OH)_2D_3$, inhibits the enzymatic activity of CYP27B1 (Bikle, 2014). It is also inhibited by its substrate when the substrate concentration is higher than 4 times the K_m value (Tang et al., 2010).

1.4.1.4 CYP24A1

CYP24A1 is a mitochondrial cytochrome P450 monooxygenase that has both 23- and 24-hydroxylase activity to inactivate the active vitamin D metabolite ($1,25(OH)_2D_3$) to $1,24,25(OH)_3D_3$, in the kidney (Figure 1.13) (Jones et al., 2012; Sakaki et al., 2005). In humans, its gene is located on chromosome 20q13.2 (geneID:1591) with 13 exons and is mainly expressed in the kidney. CYP24A1 protein has a molecular mass of 58.9 kDa with an amino acid length of 514. Rat CYP24A1 model contains 12 α-helices (A-L) and four β-sheets (β1-β4) (Figure 1.14) (Annalora et al., 2010). The substrate-binding cavity is composed of 2 β-sheets (β1

and β4) and 5 α-helices (E, F, G, I, and K) surrounding the heme (Annalora et al., 2010).

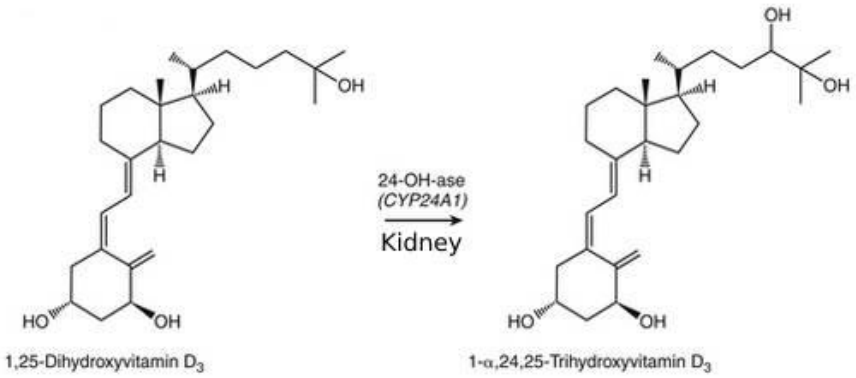


Figure 1.13 24-Hydroxylation of vitamin D catalyzed by CYP24A1.

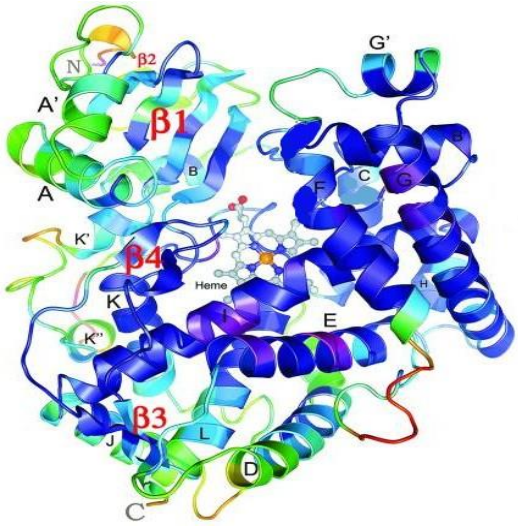


Figure 1.14 3D structure model of human CYP24A1.

Similar to CYP27B1, to control circulating $1,25(OH)_2D_3$ level, CYP24A1 is strictly regulated by PTH, FGF-23, and $1,25(OH)_2D_3$ but in a diametrical manner (Omdahl et al., 2002). For example, PTH inhibits renal CYP24A1 expression while inducing renal CYP27B1 expression (Carpenter, 2017; Melo et al., 2020). Moreover, CYP24A1 gene expression is induced by FGF-23 and $1,25(OH)_2D_3$, whereas FGF-23 inhibits CYP27B1 (Chanakul et al., 2013; Melo et al., 2020). CYP24A1 has a vitamin D response element (VDRE) in the promoter region (Zierold et al., 1994).

When active vitamin D is present, the vitamin D-VDR complex can bind to RXR and recruit other activators to bind VDRE to induce promoter activity and gene expression (Jones et al., 2012). It has also been reported that dexamethasone, a glucocorticoid drug, increases both mRNA expression and enzyme activity of CYP24A1 in the kidney (Aatsinki et al., 2019).

Increased expression and activity of CYP24A1 are related to various diseases in experimental animal models and patients, including X-linked hypophosphatemia, chronic kidney disease, and type 1 diabetes (Helvig et al., 2010; Hough et al., 1983; Tenenhouse et al., 1988). The increased degradation rate of $1,25(OH)_2D_3$ by CYP24A1 is considered the leading cause of these pathologies. Several studies are showing different cancer types have elevated basal CYP24A1 expression in which tumor cells do not respond to $1,25(OH)_2D_3$ supplementation (M. G. Anderson et al., 2006; Friedrich et al., 2003; N. King et al., 2012; Townsend et al., 2005). Because of its clinical importance, selective CYP24A1 inhibitors and vitamin D analogs, including VID-400, SDZ 89-443, CTA091, and CTA018, are the new strategies to prevent rapid degradation $1,25(OH)_2D_3$ in these patients.

1.5 Aim of the Study

Multiple sclerosis (MS) is complicated, repetitive, and frequently progressive inflammatory demyelinating autoimmune disease of the central nervous system. MS frequency increases with increasing latitude, leading to a hypothesis in which MS is inversely correlated with duration and intensity of sunlight and vitamin D concentrations. Although many studies show a strong inverse correlation between MS and vitamin D, these studies lack a biochemical and molecular point of view to investigate this relationship. The literature is limited to cell culture and mRNA expression studies that are insufficient to understand the regulation mechanism of vitamin D metabolizing enzymes since many modifications occur at post-transcriptional and translational levels. In addition, *in vitro* vitamin D metabolism study is not a suitable way to understand the relationship between multiple sclerosis and vitamin D metabolizing enzymes considering the main sites of vitamin D metabolism, liver, and kidney. Moreover, there is no study investigating the effects of vitamin D on the clinical course of MS, expression of VDR, and vitamin D metabolizing CYP450 enzymes at both mRNA and protein levels by using the Experimental Autoimmune Encephalomyelitis (EAE) mouse model.

In this study, the effects of vitamin D on the course of multiple sclerosis (MS) were investigated by molecular and biochemical approaches in the EAE mouse model. This study may come into prominence to explain the source of the relationship between MS and vitamin D by investigating MS, vitamin D, VDR, and vitamin D metabolizing enzymes as a whole. In addition, this is the first study investigating the effects of vitamin D on MS with the mRNA and protein expressions of cytochrome P450 enzymes (CYP27A1, CYP2R1, CYP27B1, and CYP24A1) and vitamin D receptor which have crucial roles in vitamin D metabolism and function. In this content, this study may be a reference for the prospective studies in this field. Moreover, the findings of this research may provide a new pathway related to vitamin D metabolizing enzymes for the development of new drugs for MS therapy.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and Materials

BCIP®/NBT liquid substrate (B1911), cholecalciferol (C1357), bovine serum albumin (BSA; A7511), 2-amino-2(hydroxymethyl)-1,3-propanediol (Tris; T1378), ammonium persulfate (APS; A-3678), bromophenol blue (B5525), glycerol (G5516), glycine (G-7126), ß-mercaptoethanol (M6250), hydrochloric acid 37% (HCl; 07101), methanol (34885), ethanol (24105), sodium dodecyl sulfate (SDS; L4390) and tween 20 (P1379) were purchased from Sigma-Aldrich Chemical Company, Saint Louis, Missouri, USA.

Ethylenediaminetetraacetic acid (EDTA; A5097) was acquired from Applichem GmbH, Germany.

Mod LabDiet® Laboratory with no added vitamin D pellet (1817422) was obtained from TestDiet, Saint Louis, Missouri, USA.

Hooke Kit™ MOG_{35-55}/CFA emulsion with pertussis toxin (EK-2110) was purchased from Hooke Laboratories, Lawrence, Missouri, USA.

Potassium dihydrogen phosphate (KH2PO4; 04871), di-potassium hydrogen phosphate (K2HPO4; 05101), sodium hydroxide (06462), sodium chloride (NaCl; 1.06400), sodium hydroxide (NaOH; 06462) were the products of E. Merck, Darmstadt, Germany.

GeneJet RNA purification kit (K0732), T-PER tissue protein extraction reagent (78510), Halt™ protease inhibitor cocktail (87786), PageRuler plus prestained protein ladder (26619), Pierce™ BCA protein assay kit (23225), Pierce™ ECL Western Blotting Substrate (32106), and RevertAid first strand cDNA synthesis kit

(K1622) were purchased from Thermo Fisher Scientific, Waltham, Massachusetts, USA.

50 bp DNA ladder (N3236S) and DNase I (M0303S) were the product of the New England Biolabs, Ipswich, Massachusetts, USA

Non-fat dry milk (170-6404), tetra methyl ethylene diamine (TEMED; 161-0801), TGX Stain-Free™ FastCast™ acrylamide kit 10% (161-0183), 10X TGS buffer (161-0732) were the products of Bio-Rad Laboratories, Richmond, California, USA.

FastStart universal SYBR® green master mix (04913850001) was purchased from Roche Diagnostics Gmbh, Germany

25(OH) vitamin D ELISA kit (ab213966), goat anti-rabbit alkaline phosphatase conjugated secondary antibody (ab6722), and recombinant anti-GAPDH antibody (ab181602) were the product of Abcam, Cambridge, United Kingdom.

The CYP2R1 (sc-20772), CYP27A1 (sc-133491), CYP27B1 (sc-367714), CYP24A1 (sc-16464), and donkey anti-goat AP (sc-2022) antibodies were purchased from Santa Cruz (Santa Cruz, CA). VDR (ab138491) antibody was the product of the Proteintech Group, Rosemont, Illinois, USA.

Primers for PCR were made by Sentebiolab Ankara, Turkey.

In this study, only analytical grade and the highest grade of pure chemicals were used.

2.2 Animal Studies

All procedures of animal studies of this thesis work were approved by Bilkent University Animal Experimentation Ethics Committee. 10-12 weeks old female C57BL/6 mice weighing 20-25 g were used. They were produced and housed at the Animal Experimental Unit in Bilkent University. Mice were randomly assorted into four groups as given in Table 2.1 and placed in individually ventilated cages.

Table 2.1 Experimental groups of the female C57BL/6 mice.

	Groups	*Number of Mice*
Control-1	Group 1 (Healthy)	6
Control-2	Group 2 (Healthy + Vitamin D)	6
Control-3	Group 3 (EAE)	12
Experimental	Group 4 (EAE + Vitamin D)	12

All mice were fed a vitamin D deficient diet. Vitamin D supplementation to Group 2 and Group 4 was done *via* the drinking water as cholecalciferol (≥98% purity). The recommended daily intake (RDI) for vitamin D in mice is 1.5 IU/g of diet (Mallya et al., 2016). According to the study of Bachmanov et al., it was reported that daily food and water intake of C57BL/6 mice were approximately 4 g and 6 mL, respectively (2002). Guided by this information, IU/mL of vitamin D in drink water of mice was calculated;

(1.5 IU/g x 4 g of the pellet)/ 6 mL of water = 1 IU/mL (RDI for vitamin D in water)

Four times higher concentration of RDI for vitamin D was supplemented to mice to observe the effects of vitamin D supplementation. For this purpose, 4 IU/mL vitamin D containing drink water was prepared;

4 IU/mL / 40 IU/μg Cholecalciferol = 0,1 μg/mL cholecalciferol in drink water.

First, cholecalciferol dissolved in absolute ethanol and then added to the drinking water of vitamin D supplemented groups. Final concentration of ethanol and cholecalciferol were 0.1 % (v/v) and 0.1 μg/mL, respectively. To eliminate the effect of the alcohol, 0.1 % of ethanol was added to the drinking water of Group 1 and Group 3 without vitamin D. Vitamin D supplementation was started on the same day of immunization and continued for 30 days.

2.2.1 Experimental Autoimmune Encephalomyelitis (EAE) Immunization

C57BL/6J (10-12 weeks old) mice were immunized with Hooke Labs' Kit (Hooke Labs Inc., #EK-2110) according to the manufacturer's instructions. 100 µL of myelin oligodendrocyte glycoprotein/complete Freund's adjuvant ($MOG_{35–55}$/CFA) emulsion, total 200 µL, was injected subcutaneously into each mouse at two sites (lower and upper back of mice). Then, pertussis toxin (80 ng in 100µL PBS/animal) was injected intraperitoneally into each mice 2 h and 24 h after the immunization. The mice of Group 1 and Group 2 were treated the same as the immunized groups, except MOG_{35-55} peptide was replaced by phosphate buffered saline (PBS). Ear tags were used to monitor mice individually and the mice were observed daily after immunization for 30 days. The clinical score of the disease is assigned according to the manufacturer's scoring chart (Table 2.2). After 30 days of the EAE immunization, under anesthesia, blood samples were taken from the aorta to determine serum vitamin D levels, and the mice were sacrificed by perfusion with PBS. The kidney, liver and brain organs were isolated and stored at -80 ºC until further analysis.

Table 2.2 Clinical observations and mouse EAE scoring.

Score	***Clinical Observation***
0	The tail has tension and is erect.
0.5	The tip of the tail is limp.
1	The tail is limp
1.5	The tail is limp, and the hind leg is inhibited.
2	The tail is, and the hind legs are weak.
2.5	The tail is limp, and the mouse is dragging hind legs.
3	The tail is limp, and the hind legs are completely paralyzed.
3.5	In addition to score 3, when the mouse is placed on its side, it cannot right itself.
4	In addition to score 3.5, there is partial front leg paralysis.
4.5	In addition to score 4, the mouse is not alert.
5	Death

2.3 Serum 25(OH) Vitamin D Measurement

Before sacrificing the mice by perfusion with PBS, heart blood samples were collected. The blood samples of the mice were centrifuged at 2500 rpm for 5 minutes at 4 ºC to obtain serum. Serum 25(OH)D_3 levels were measured by using the ELISA kit (ab213966, Abcam) according to manufacturers' protocol. All of the standards and the samples were assayed as duplicates. Briefly, 90 µL dissociation buffer was added to each well. Then 10 µL of the standards (0.5, 4.8, 24.4, 71.6, 279, 1010 ng/mL) and samples were pipetted into appropriate wells while 10 µL sample diluent solution was added into the maximum binding and non-specific binding (NSB) wells. After 5 minutes of incubation on a plate shaker at room temperature, 50µL of the 1X conjugate was pipetted into each well while 50 µL of the conjugate diluent was added into NSB wells. The plate was sealed and incubated for 1 hour on a plate shaker at room temperature. After that, the plate was washed with wash buffer 3 times, and the wash buffer was aspirated. Next, 200 µL pNpp substrate solution was added to each well, and the sealed plate was incubated for 30 minutes on a plate shaker at room temperature. Finally, 50 µL of stop solution was pipetted into each well, and optical density (OD) was read at 405 nm in the microplate spectrophotometer (Multiskan™ GO, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Serum 25(OH)D_3 concentration was calculated as follows:

Average Net OD_{405} = Average OD_{405} – Average NSB OD_{405}

Net OD versus concentration of 25(OH)D_3 for standards was plotted. A curve was fitted through the data points by using 4 parameter logistic (4PL) curve fitting. The serum 25(OH)D_3 concentration of the mice was determined by interpolating the standard curve.

2.4 Total Protein Extraction

The kidney, liver, and brain organs were removed after perfusion of mice with PBS solution. The organs were homogenized *via* the cryogenic grinding method, in which organs were homogenized in liquid nitrogen filled ceramic mortar by grinding with a pestle. Before use, the 1% Halt™ protease inhibitor cocktail was added to the T-PER™ tissue protein extraction reagent. Approximately 50 mg of the homogenate was weighed, and 500 µL of the T-PER™ reagent was added in a microcentrifuge tube. The resulting homogenate-reagent mixture was centrifuged at 10,000 x g for 5 minutes. The supernatant containing total protein was taken and stored at -80 ºC until use.

2.5 Determination of Protein Concentration

Protein concentrations of total protein extract obtained from the mice kidney, liver, and brain samples were determined by the BCA (Bicinchoninic Acid) assay. The principle of this method is that Cu^{+2} is reduced to Cu^{+1} by protein in alkaline conditions, which reacts with BCA and causes color change (Smith et al., 1985). This color change can be measured by a spectrophotometer at 562 nm, and the intensity of the color is proportional to protein concentration.

Pierce™ BCA protein assay kit was used to measure protein concentrations of the samples, and the manufacturers' manual was followed. Bovine serum albumin was used as the standard with series of different concentrations (25, 125, 250, 500, 750, 1000, 1500, 2000 µg/mL). All the measurements, including blank, standards, and the samples, were performed as duplicates. 25µL of standards and the samples were added into the wells of 96 well plate. Then, 200 µL of working reagent (reagent A: B, 50:1) was added into each well and mixed on a plate shaker for 30 seconds. The plate was sealed and incubated for 30 minutes at 37 ºC. After incubation, absorbance was read at 562 nm in the microplate spectrophotometer (Multiskan™ GO, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Net OD_{562} of standards and

samples was calculated by subtracting OD_{562} of blank from OD_{562} of the standards and the samples. Net OD_{562} versus concentration of bovine serum albumin standards was plotted, and it was used to determine the concentration of samples.

2.6 Determination of Protein Expressions by Western Blotting Technique

Effects of vitamin D and EAE immunization on protein expression of CYP27A1 and CYP2R1 in the liver, CYP27B1 and CYP24A1 in the kidney, and VDR in the brain of the mice were analyzed by the Western blot method (Towbin et al., 1979). GAPDH was used as a loading control. Before western blotting, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Then, in a discontinuous buffer system, 4% separating gel and 10% stacking gel was prepared using the TGX Stain-Free™ FastCast™ acrylamide kit as described in Table 2.3.

Table 2.3 Gel casting preparation volumes for one gel.

Components	*Stacking Gel 4%*	*Separating Gel 10 %*
Stacker A	1 mL	-
Stacker B	1 mL	-
Resolver A	-	2.5 mL
Resolver B	-	2.5 mL
TEMED	2 µL	5 µL
10% APS	10 µL	25 µL

SDS-PAGE was performed on 10% separating gel in a discontinuous buffer system. Vertical gel electrophoresis was carried out using Mini-PROTEAN tetra cell mini trans blot module (Bio-Rad, Richmond, CA). First, the 4250 µL of prepared 10% separating gel solution was dispensed into the 1.0 mm space between glass plates on the casting stand. Then, 1500 µL of 4% stacking gel solution was poured on the

separating gel, and the 15 wells comb was inserted. After the polymerization, the comb was removed.

The samples were diluted with dH_2O according to the following formula to get 2 mg/mL final protein concentration;

$$V = \frac{[\text{Conc. of protein}]}{2.67} \times 20 - 20$$

V is the volume of dH_2O to be added to dissolve 20 µL of the sample.

The samples were diluted with the 4x sample dilution buffer (pH 6.8, 0.25M Tris-HCl, 8% SDS, 40% glycerol, 20 % β-mercaptoethanol, and 0.01% bromophenol blue) and incubated 3 minutes at 100 ºC heat block. 20 µg of the samples were loaded into wells, while 3 µL of protein ladder was loaded as a marker. After loading the samples, the gel running module was placed in the main buffer tank filled with 1X TGS buffer. The tank was connected to the Bio-Rad power supply, and electrophoresis was run at 200 V.

The gel was removed from the glasses for western blotting and placed into the transfer buffer (25 mM Tris, 192 mM Glycine) for 10 minutes. The PVDF membrane was cut as equal size with the gel and immersed in 100% methanol for 20 seconds to prewet the membrane. Then the membrane was equilibrated in transfer buffer for 5 minutes. The gel and the PVDF membrane were sandwiched between blotting papers, as shown in Figure 2.1. The transfer sandwich was placed between the top and the bottom cassettes of the Trans-Blot® Turbo® semi-dry transfer system (Bio-Rad Laboratories, Richmond, CA, USA). The transfer was carried out at a constant 25 volt and up to 1 ampere for 30 minutes.

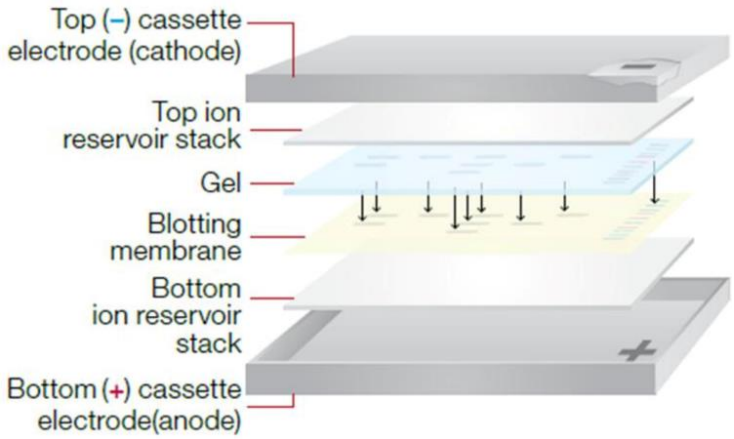


Figure 2.1. Preparation of western blot sandwich (Bio-Rad, 2021).

After the transfer was completed, the membrane was equilibrated with TBS (20 mM Tris-HCl pH 7.4, 500 mM NaCl) for 10 minutes. Then the membrane was incubated with blocking solution (5% non-fat dry milk in TBS) at room temperature for an hour on a shaker. After that, the membrane was incubated with the primary antibody of protein of interest for 2 hours at room temperature on a shaker. Next, the membrane was washed with TBST (20 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.05% Tween 20) 3 times, each of which was 5 minutes. After the washing, the membrane was incubated with secondary antibodies for an hour on a shaker. Again the membrane was washed with TBST 3 times, each of which was 5 minutes. Finally, the membrane was incubated with the BCIP®/NBT alkaline phosphatase substrate. The primary and the secondary antibodies and their dilutions are given in Table 2.4. Chemidoc XRS+ (Bio-Rad, USA) was used to take images. The band intensities were analyzed by Image J visualization software developed by NIH.

Table 2.4 Primary and secondary antibody dilutions.

Protein	*1st Antibody*	*2nd Antibody*
GAPDH	1/2000	1/2000
CYP2R1	1/200	1/2000
CYP27A1	1/200	1/2000
CYP27B1	1/1000	1/2000
CYP24A1	1/1000	1/2000
VDR	1/500	1/1000

2.7 Determination of mRNA Expression

2.7.1 Isolation of Total RNA From Tissue Homogenates

The kidney, liver, and brain organs were removed after perfusion of mice with PBS solution. The organs were homogenized *via* the cryogenic grinding method, in which organs were homogenized in liquid nitrogen filled ceramic mortar by grinding with a pestle. GeneJet RNA purification kit was used to isolate total RNA, and the manufacturer's protocol was followed. Approximately 30 mg of each sample was taken into a 2 mL centrifuge tube. 300 µL of lysis buffer was added, and the lysate was passed through a 20-gauge sterile syringe. 600µL of proteinase K was added and vortexed thoroughly. After 10 minutes of incubation at room temperature, the lysate was centrifuged for 10 minutes at 12,000 x g. The supernatant was transferred into a new 2 mL centrifuge tube, and 450 µL absolute ethanol was added and mixed by pipetting. The lysate was transferred into the purification column and centrifuged for 1 minute at 12,000 x g. The purification column was placed into a new 2 mL collection tube. 700 µL of Wash Buffer 1 was added and centrifuged for 1 minute at 12,000 x g. The flow-through was discarded, and the purification column was placed back into the collection tube. 600 µL of Wash Buffer 2 was added and centrifuged for 1 minute at 12,000 x g. After discarding the flow-through, the purification column was placed back into the collection tube, and 250 µL of Wash

Buffer 2 was added and centrifuged for 1 minute at 12,000 x g. The purification column was placed into a new sterile 1.5 mL centrifuge tube, 100 µL of nuclease-free water was added and centrifuged for 1 minute at 12,000 x g to elute RNA. The purification column was discarded and stored at -80°C until use.

Isolated RNA concentration was quantified by measuring the absorbance at 260 nm and the purity was assessed by the 260/280 nm ratio by BioDrop µLite+ (Biochrom, United Kingdom) spectrophotometer.

2.7.2 Genomic DNA (gDNA) Removal from RNA

RNase-free DNase I was used to removing gDNA and prevent PCR amplification of the gDNA template. 10 µg of the isolated RNA, 10 µL of 1X DNase I reaction buffer, and 1 µL Dnase I was mixed. The total volume of the mixture was completed to 100 µL with nuclease-free water. It was incubated at 37 ºC for 10 minutes. Before cDNA synthesis, DNase I was inactivated by adding 1 µl of 0.5 M EDTA and incubating at 75 ºC for 10 minutes.

2.7.3 cDNA Synthesis

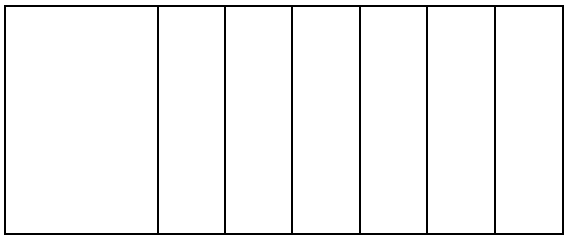
Reverse transcription of RNA to cDNA was performed with RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). 1 µg of total isolated RNA and 1 µL of oligo dT primer in a PCR tube. The final volume of the mixture was completed to 10 µL with nuclease-free distilled water. The solution was mixed gently and spun down by microcentrifuge. The mixture was incubated at 70°C for 5 minutes. After that, 5 µL of 5X reaction buffer, 0.5 µL Ribolock, 0.5 µL of reverse transcriptase, 2.5 µL of 10 mM dNTP, and 6.5 µL nuclease-free distilled water were added. It was mixed gently and spun down by microcentrifuge. It was incubated at 42 °C for 1 hour. Finally, the reaction was stopped by keeping at 70°C for 10 minutes and chilled on ice. cDNA was stored at -20°C for further use.

2.7.4 Quantitative Real-Time PCR

The expressions of CYP2R1, CYP27A1, CYP27B1, CYP24A1, and VDR genes in organs were analyzed by quantitative Real-Time PCR (qRT-PCR) using Corbett Rotor-Gene 6000 (Corbett life Science, PO Box 435, Concorde, NSW 2137). The 25 μL of final reaction mixture containing 100 ng cDNA, 0.5 mM reverse and forward primers, and FastStart Universal SYBR Green Master Mix (Roche, Basel, Switzerland) and RNase free distilled water. To detect any contamination, no template control (NTC) was used. As an internal standard, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene was used. The DNA amplification carried out in a reaction mixture containing a specific nucleotide sequence for related genes was given in Table 2.5. The qRT-PCR program consisted of the following cycling profile; initial melting at 95 °C for 5 minutes, amplification and quantification program repeated 45 times containing melting at 95 °C for 20 seconds, annealing at 60-63 °C (depending on the gene) for 30 seconds and extension at 72 °C for 20 seconds with a single fluorescent measurement. The melting curve was used to confirm expected product amplification without any non-specific products. Quantities of specific mRNAs in the sample were measured according to the corresponding gene and relative standard curve method. The results obtained using Corbett Rotor-Gene 600 quantitation software were normalized with internal standard GAPDH, and the Livak method was used to determine relative mRNA expression in different tissues by using C_t values (Livak et al., 2001).

Table 2.5 Primer sequence, temperature and product size of the genes.

GENE	Forward Primer (5'→ 3')	Reverse Primer (5' → 3')	Annealing Temperature (°C)	Product Size (bp)
GAPDH	GTGATGGGTGTGAACCACGAG	CATGAGCCCTTCCACAATGC	60	132
CYP2R1	ATTTGTCGGCAACATCTGCTC	CATGAACAAAGGAAGGCATGG	60	208
CYP27A1	CCTTGCCTTGGAAGCCATCAC	ATCCCAGCCATTCAGGTATCG	60	196
CYP27B1	GATTGCTAACGGCGGATGGT	TTAGTCGTCGCACAAGGTCAC	63	135
CYP24A1	AAATCCAGAGCGTGCTGCCTGAC	TAAGACTGTTCCTTTGGGTAGCGTG	63	182
VDR	CTGGCTTCAGGGACCTCACCTC	TTCGATCAGCTCCAGGGTGTGC	63	188



2.8 Statistical Analysis

Statistical analyses were performed by using the GraphPad Prism version 6 statistical software package for Windows. One-way ANOVA (Tukey's test) was used for multiple comparisons. The student's t-test was used for the comparison of two groups. The chi-square test was chosen to test the recovery of mice. All results were expressed as means with their Standard Deviation (mean ± SD), and $p<0.05$ was chosen as the level for significance.

CHAPTER 3

RESULTS

3.1 Animal Studies

3.1.1 Clinical Scoring and Observation

In this study, there were 4 groups of mice, as given previously in Table 2.1. Mice in Gr 3 and Gr 4 were immunized with the emulsion of MOG_{35-55} peptide, and daily clinical scoring was carried out after the day of immunization as described before in Table 2.2. The results of clinical observation are given in Figure 3.1 and Table 3.1. Disease development was achieved in all of the immunized mice. The mean maximum score in Gr 3 is 3.18 ± 0.56 and 3.13 ± 0.77 in Gr 4. The mean day of disease onset is 14.0 ± 3.6 and 13.3 ± 2.1 in Gr 3 (EAE) and 4 (EAE + Vit D), respectively.

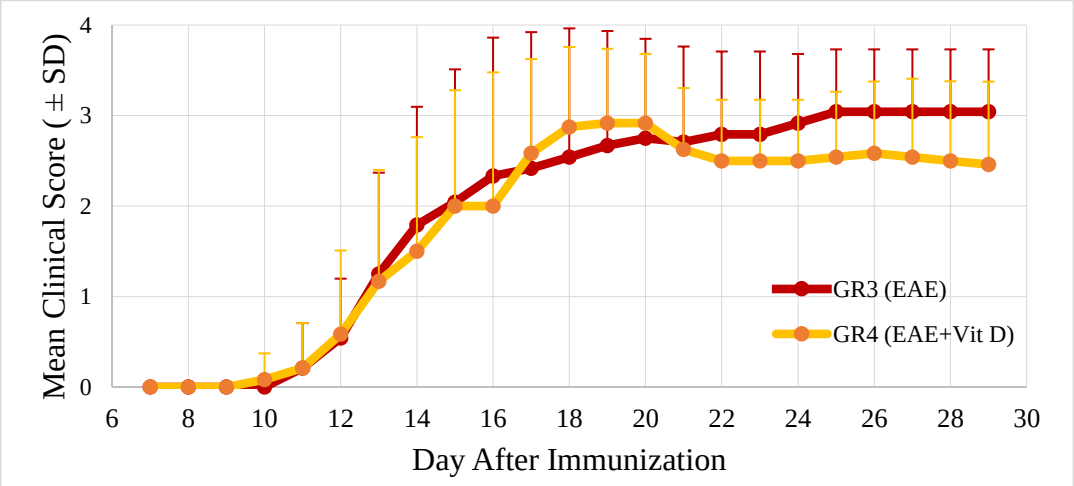


Figure 3.1 Daily clinical observation of immunized mice.

Table 3.1 Results of clinical scoring and observation of EAE immunized mice.

Group	*Number of Mice*	*Mean Day of Onset ± SD*	*Mean Maximum Score ± SD*	*Disease Incidence*
Gr 3 (EAE)	12	14.0 ± 3.6	3.18 ± 0.56	100 %
Gr 4 (EAE + Vit D)	12	13.3 ± 2.1	3.13 ± 0.77	100 %

Figure 3.2 shows the effect of vitamin D supplementation on the mean maximum score and mean day of onset of the EAE development in mice. There was no statistically significant difference in the mean maximum score (p= 0.8397) and mean day of onset (p= 0.9764) between Gr 3 and Gr 4.

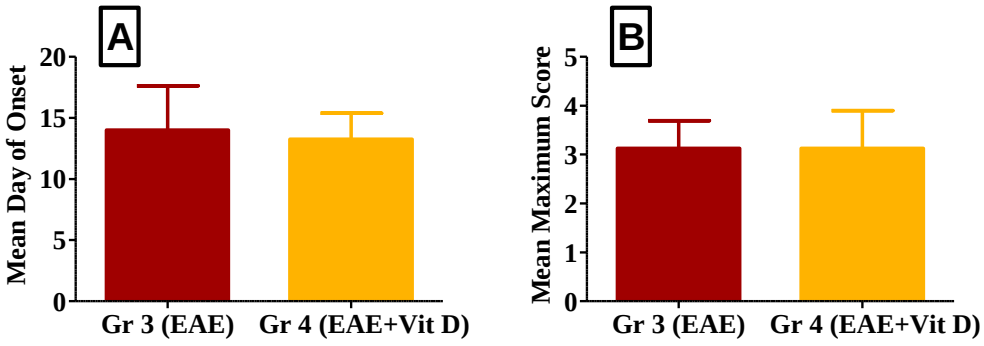


Figure 3.2 Comparison of the mean day of onset (A) and mean maximum score (B) between EAE (Gr 3) and EAE + Vitamin D (Gr 4) groups. Values are mean ± SD for 12 mice per group.

3.1.2 Serum Vitamin D Concentration

Serum 25(OH)D_3 levels were measured by using the ELISA kit (ab213966, Abcam) according to manufacturers' protocol. All of the standards and the samples were assayed as duplicates. The standard curve was constructed using 4 parameter logistic (4PL) curve fitting with 0.5, 4.8, 24.4, 71.6, 279, 1010 ng/mL of vitamin D standards

(Figure 3.3). The serum 25(OH)D_3 concentration of the mice was determined by interpolating the standard curve (R^2 = 0.9973).

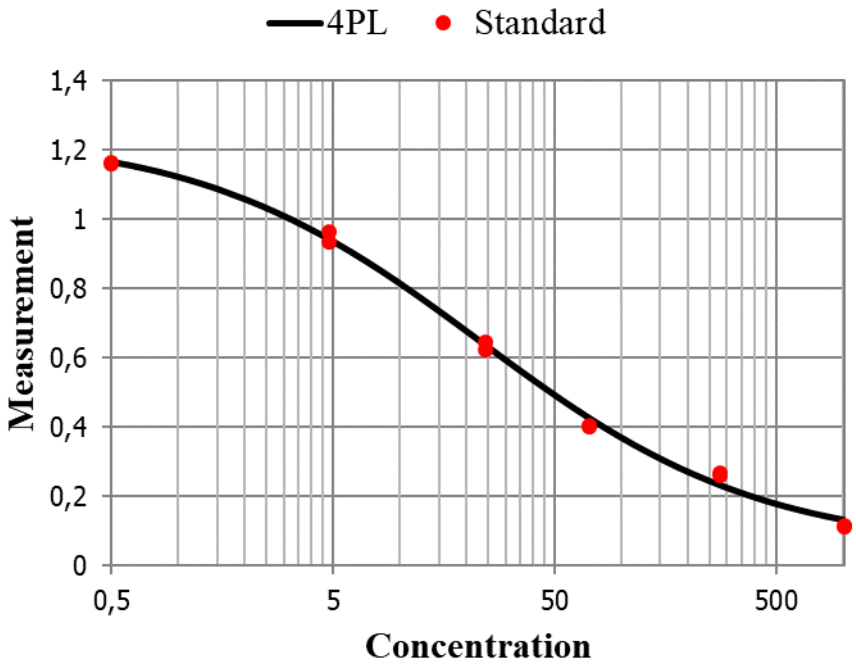


Figure 3.3 25 (OH) Vitamin D standard curve (0.5, 4.8, 24.4, 71.6, 279, 1010 ng/mL standards) using 4 parameter logistic (4PL) curve fitting (R^2= 0.9973). All measurements were done as duplicates.

As given in Figure 3.4, 25 (OH) Vitamin D concentrations were significantly higher in the Vitamin D supplemented groups of Gr 2 and Gr 4 ($p<0.0001$). Serum 25 (OH) Vitamin D concentrations of Gr 1, Gr 2, Gr 3, and Gr 4 were 24.3 ± 4.3, 111 ± 13.0, 30 ± 7.4, and 109.0± 11.9, respectively.

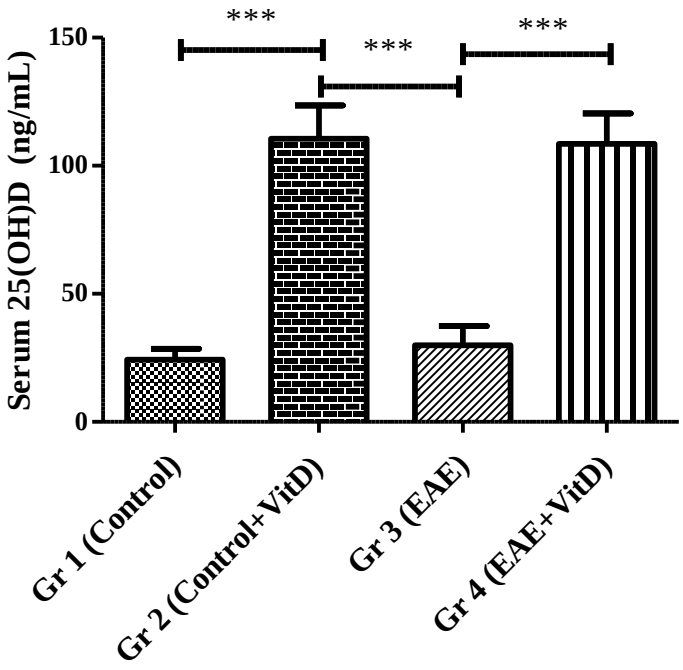


Figure 3.4 25 (OH) vitamin D concentrations in blood serum of Gr 1 (6 mice), Gr 2 (6 mice), Gr 3 (12 mice), and Gr 4 (12 mice). All measurements were done as duplicates. ***Significantly different ($p \leq 0.001$).

3.1.3 Effect of Vitamin D Supplementation on Recovery of EAE Immunized Mice

Clinical scores of each mouse were followed for 30 days starting from the EAE immunization day. Two groups (Gr 3; EAE and Gr 4; EAE + Vitamin D) were compared to determine the therapeutic effect of Vitamin D supplementation. The chi-square test was used to analyze the results. Two mice out of 12 were recovered in Gr 3 (EAE), while there were eight mice out of 12 recovered in Gr 4 (EAE + Vitamin D), and it was statistically significant ($p=0.013<0.05$).

3.2 Effect of EAE Immunization and Vitamin D Supplementation on mRNA Expressions

Effects of EAE immunization and vitamin D supplementation on mRNA expression of vitamin D metabolizing CYP450s; CYP2R1 and CYP27A1 in the liver, CYP27B1 and CYP24A1 in the kidney, and VDR gene in the brain were determined by qRT-PCR using gene-specific primers. GAPDH gene was used as an internal standard. To verify PCR amplification and the specificity amplification curve and melt curve were plotted, respectively. Relative mRNA expressions were determined by the following formula:

ΔCt (Control) = Ct (Target Gene) - Ct (GAPDH)

ΔCt (Treatment) = Ct (Target Gene) - Ct (GAPDH)

$\Delta\Delta$Ct = ΔCt (Treatment) - ΔCt (Control)

$2^{-\Delta\Delta Ct}$ = Relative mRNA expression

For this analysis, Gr 1 was the control group, while Gr 2, Gr 3, and Gr 4 were considered as the treatment groups. Statistical analysis was done by one-way ANOVA method, and multiple comparisons were performed with Tukey's test.

3.2.1 Effect of EAE Immunization and Vitamin D Supplementation on CYP2R1 mRNA Expression in Liver

The mRNA expression of the CYP2R1 gene in the liver was determined by quantitative real-time PCR (qRT-PCR). As an internal standard GAPDH gene was used. Specific primers and annealing temperatures for CYP2R1 and GAPDH genes were used as described before in Table 2.5. Relative mRNA expression of CYP2R1 was analyzed by the $2^{-\Delta\Delta Ct}$ method as described previously. To verify PCR amplification and the specificity amplification curve (Figure 3.5) and melt curve

(Figure 3.6) were plotted, respectively. As shown in Figure 3.6, there was only one peak which means one amplicon without primer dimer or non-specific PCR product.

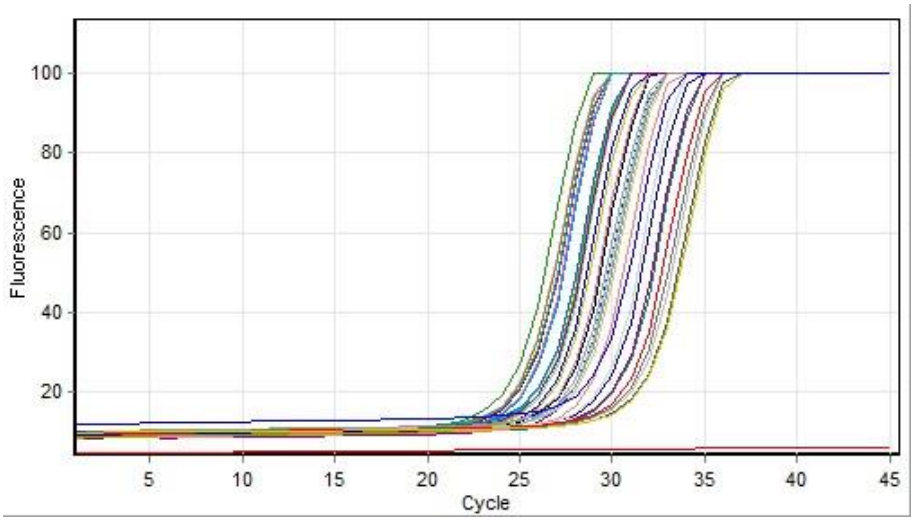


Figure 3.5 Amplification curve of qRT-PCR of CYP2R1 gene showing fluorescence accumulation at each cycle.

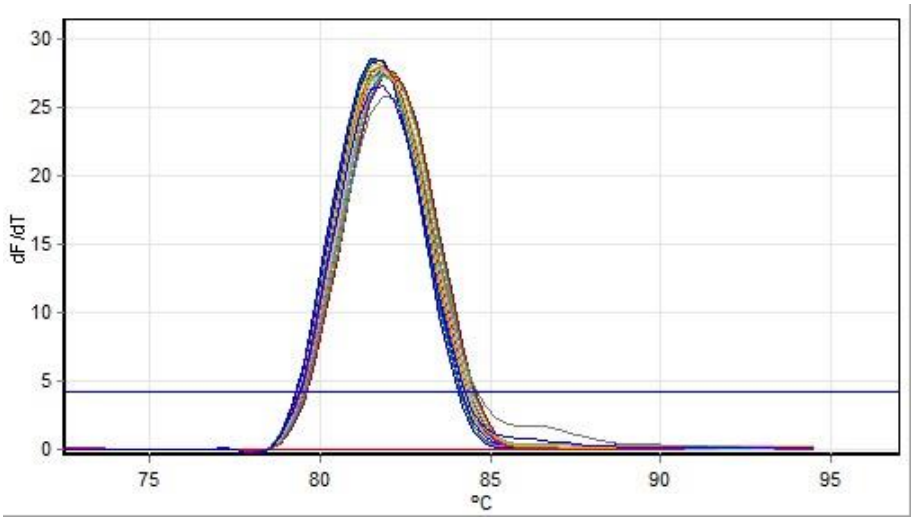


Figure 3.6 Melting curve of qRT-PCR of CYP2R1 gene showing fluorescence emission change versus temperature.

In Figure 3.7, relative mRNA expression of CYP2R1 in the liver of the 4 groups of mice was shown. There was no significant difference between groups 1, 2, and 3 in

CYP2R1 expressions. However, CYP2R1 mRNA expresion was significantly decreased in EAE immunized and vitamin D supplemented group (Gr 4; 0.50±0.24 fold) compared to the control group (Gr 1; 1.00±0.16 fold), vitamin D supplemented (Gr 2; 0.91±0.36 fold), and EAE immunized (Gr 3; 0.88±0.29 fold) groups ($p \leq 0.05$).

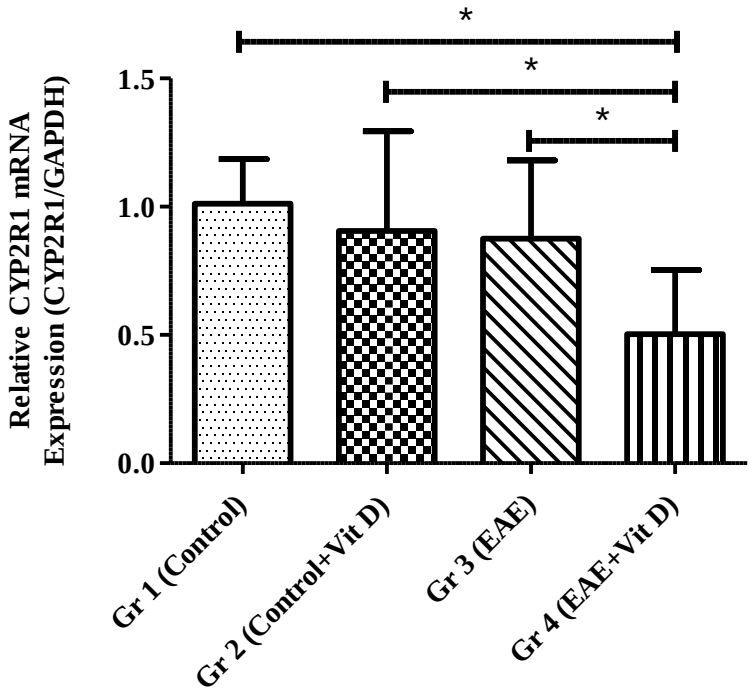


Figure 3.7 Effect of EAE immunization and vitamin D supplementation on CYP2R1 mRNA expression in the liver of mice. mRNA expression changes were determined by qRT-PCR. The quantifications were expressed as the mean ± SD of the relative expression from three independent experiments. * Significantly different, $p \leq 0.05$.

3.2.2 Effect of EAE Immunization and Vitamin D Supplementation on CYP27A1 mRNA Expression in Liver

The mRNA expression of the CYP27A1 gene in the liver was determined by quantitative real-time PCR (qRT-PCR). As an internal standard GAPDH gene was used. Specific primers and annealing temperatures for CYP27A1 and GAPDH genes were used as described before in Table 2.5. Relative mRNA expression of CYP27A1

was analyzed by the $2^{-\Delta\Delta Ct}$ method as described previously. To verify PCR amplification and specificity, the amplification curve (Figure 3.8) and melt curve (Figure 3.9) were plotted, respectively. As shown in Figure 3.9, there was only one peak which means one amplicon without primer dimer or non-specific PCR product.

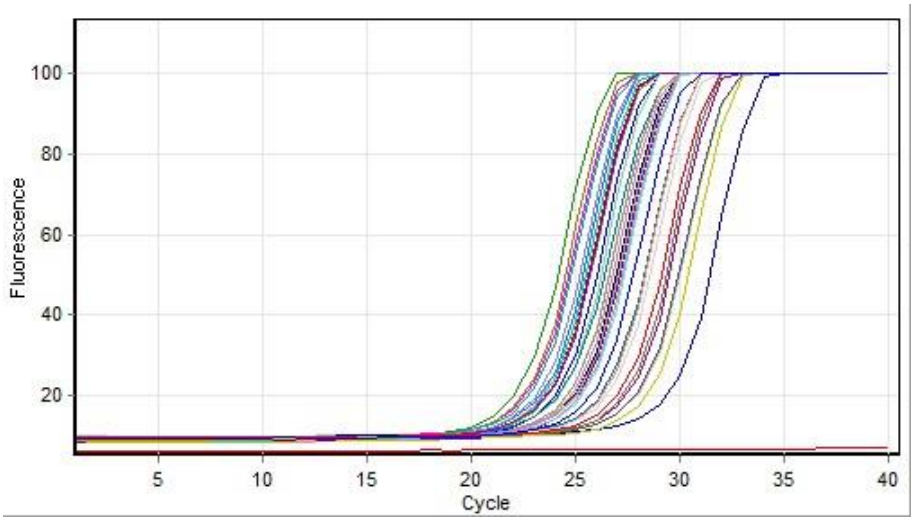


Figure 3.8 Amplification curve of qRT-PCR of CYP27A1 gene showing fluorescence accumulation at each cycle.

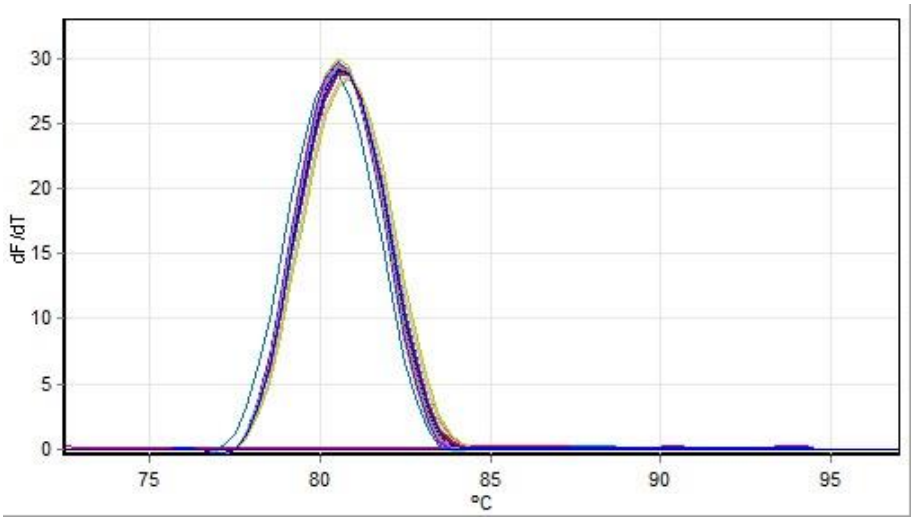


Figure 3.9 Melting curve of qRT-PCR of CYP27A1 gene showing fluorescence emission change versus temperature.

In Figure 3.10, relative mRNA expression of CYP27A1 in the liver of the 4 groups of mice was shown. There was no significant difference between 4 groups in CYP27A1 expressions, Gr 1 (1.00±0.20 fold), Gr 2 (1.05±0.21 fold), Gr 3 (0.93±0.25 fold), Gr 4 (1.01±0.10 fold).

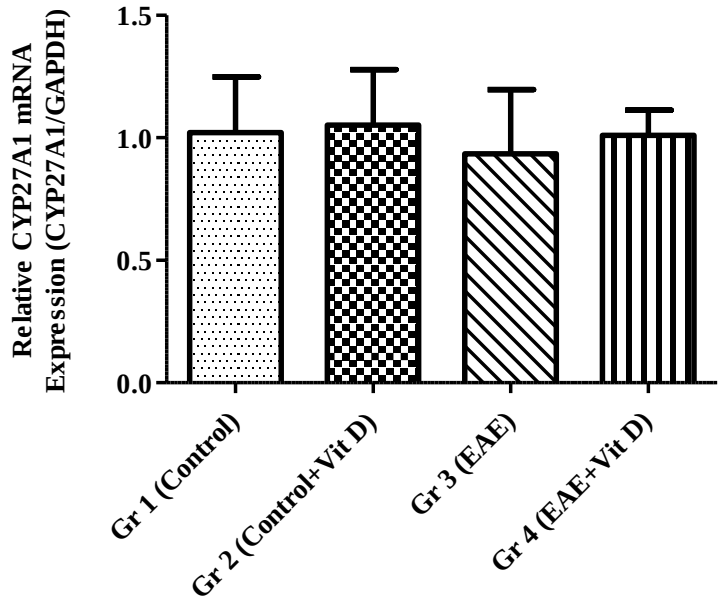


Figure 3.10 Effect of EAE immunization and vitamin D supplementation on CYP27A1 mRNA expression in the liver of mice. mRNA expression changes were determined by qRT-PCR. The quantifications were expressed as the mean ± SD of the relative expression from three independent experiments.

3.2.3 Effect of EAE Immunization and Vitamin D Supplementation on CYP27B1 mRNA Expression in Kidney

The mRNA expression of the CYP27B1 gene in the kidney was determined by quantitative real-time PCR (qRT-PCR). As an internal standard GAPDH gene was used. Specific primers and annealing temperatures for CYP27B1 and GAPDH genes were used as described before in Table 2.5. Relative mRNA expression of CYP27B1

was analyzed by the $2^{-\Delta\Delta Ct}$ method as described previously. To verify PCR amplification and specificity, the amplification curve (Figure 3.11) and melt curve (Figure 3.12) were plotted, respectively. As shown in Figure 3.12, there was only one peak which means one amplicon without primer dimer or non-specific PCR product.

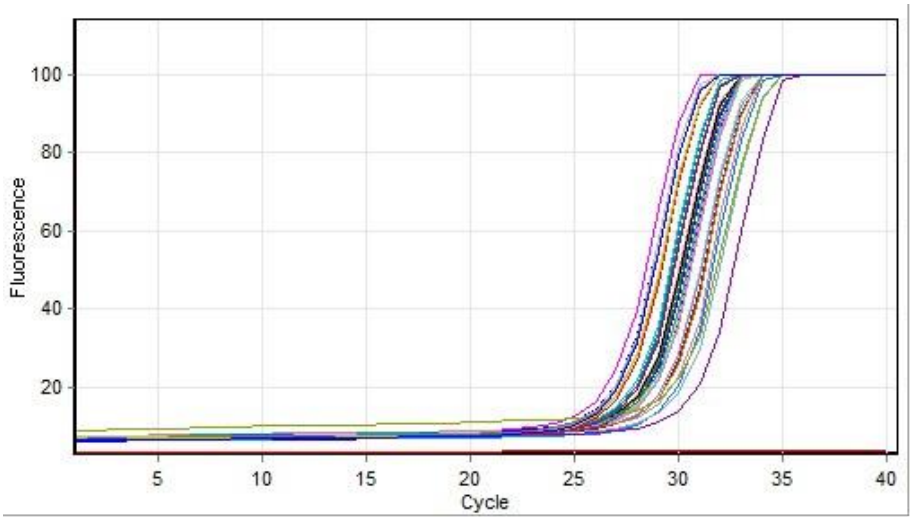


Figure 3.11 Amplification curve of qRT-PCR of CYP27B1 gene showing fluorescence accumulation at each cycle.

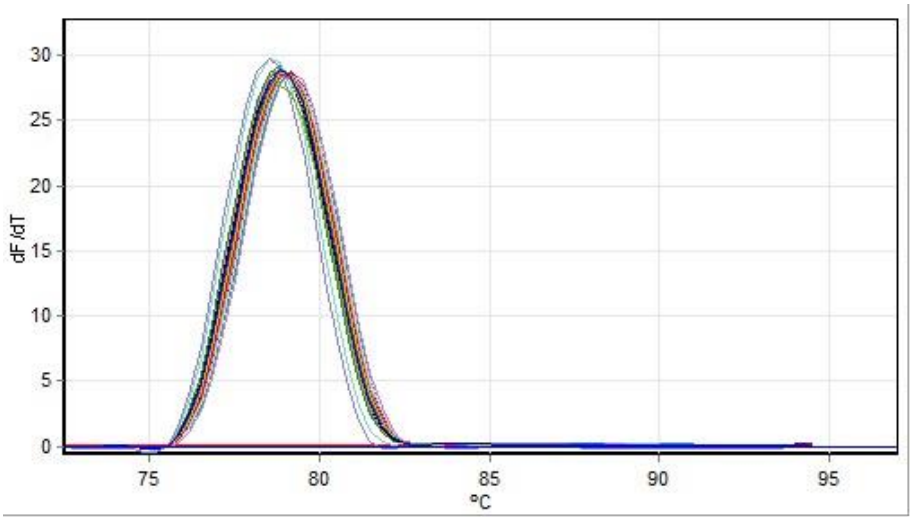


Figure 3.12 Melting curve of qRT-PCR of CYP27B1 gene showing fluorescence emission change versus temperature.

In Figure 3.13, relative mRNA expression of CYP27B1 in the kidney of the 4 groups of mice was shown. There was no significant difference between the control (Gr 1; 1.00±0.22 fold) and EAE immunized (Gr 3; 0.76 ±0.16 fold) groups. Also, there was no significant difference between vitamin D supplemented control (Gr 2; 1.65±0.77 fold) and vitamin D supplemented EAE immunized (Gr 4; 1.50±0.57 fold) groups. However, CYP27B1 mRNA expression was significantly high in Gr 2 (1.65±0.77 fold) and Gr 4 (1.50±0.57 fold) compared to Gr 3 (0.76±0.16 fold) (** $p \leq 0.01$).

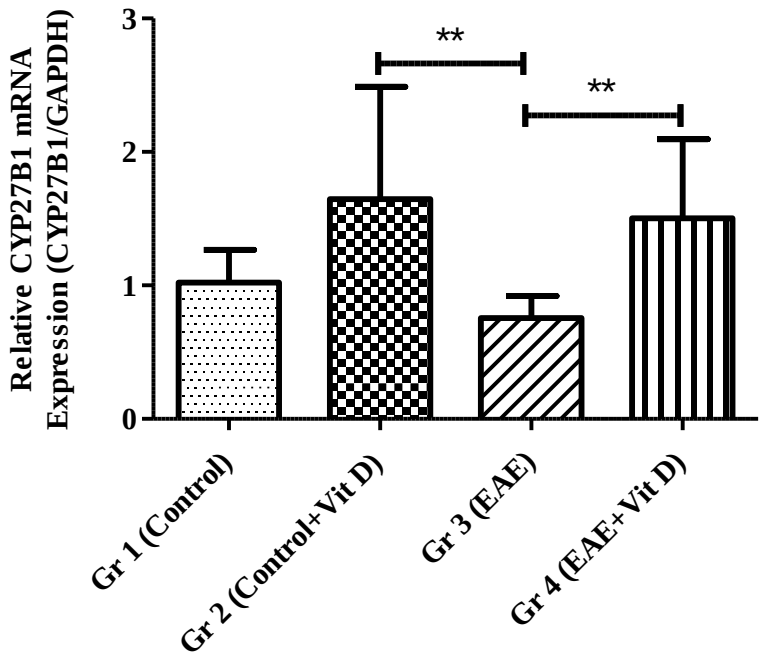


Figure 3.13 Effect of EAE immunization and vitamin D supplementation on CYP27B1 mRNA expression in the kidney of mice. mRNA expression changes were determined by qRT-PCR. The quantifications were expressed as the mean ± SD of the relative expression from three independent experiments. ** $p \leq 0.01$

3.2.4 Effect of EAE Immunization and Vitamin D Supplementation on CYP24A1 mRNA Expression in Kidney

The mRNA expression of the CYP24A1 gene in the kidney was determined by quantitative real-time PCR (qRT-PCR). As an internal standard GAPDH gene was used. Specific primers and annealing temperatures for CYP24A1 and GAPDH genes were used as described before in Table 2.5. Relative mRNA expression of CYP24A1 was analyzed by the $2^{-\Delta\Delta Ct}$ method as described previously. To verify PCR amplification and specificity, the amplification curve (Figure 3.14) and melt curve (Figure 3.15) were plotted, respectively. As shown in Figure 3.15, there was only one peak which means one amplicon without primer dimer or non-specific PCR product.

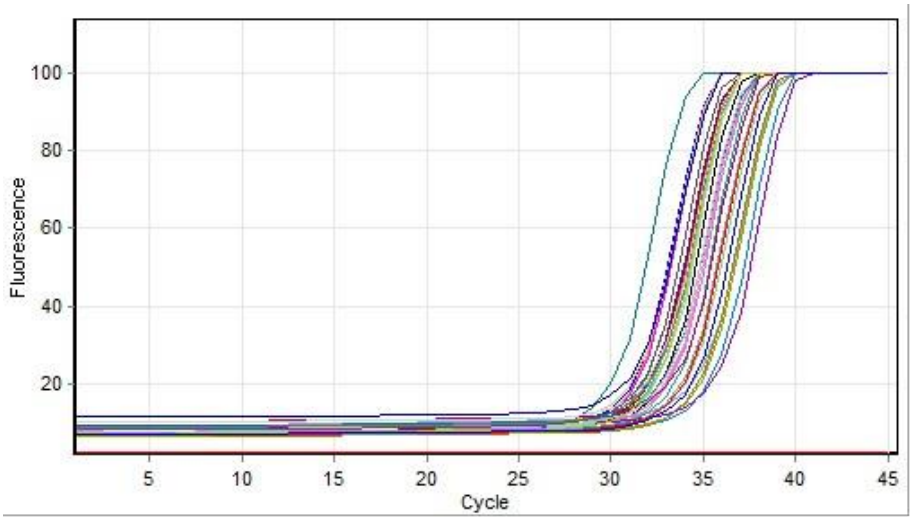


Figure 3.14 Amplification curve of qRT-PCR of CYP24A1 gene showing fluorescence accumulation at each cycle.

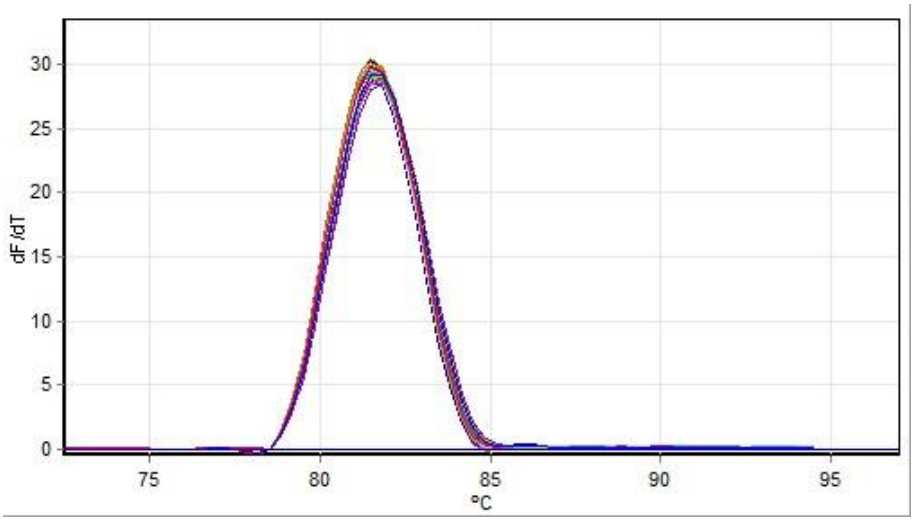


Figure 3.15 Melting curve of qRT-PCR of CYP24A1 gene showing fluorescence emission change versus temperature.

In Figure 3.16, relative mRNA expression of CYP24A1 in the kidney of the 4 groups of mice was shown. Although there was an increase in CYP24A1 mRNA expression in vitamin D supplemented healthy mice, it was not significant compared to control (Gr 1; 1.00±0.50 fold), and EAE immunized (Gr 3; 0.78±0.36 fold) groups. However, CYP24A1 mRNA expression was significantly higher in Gr 2 (1.38 ±0.10 fold) than Gr 4 (0.58±0.28 fold), **$p \leq 0.01$.

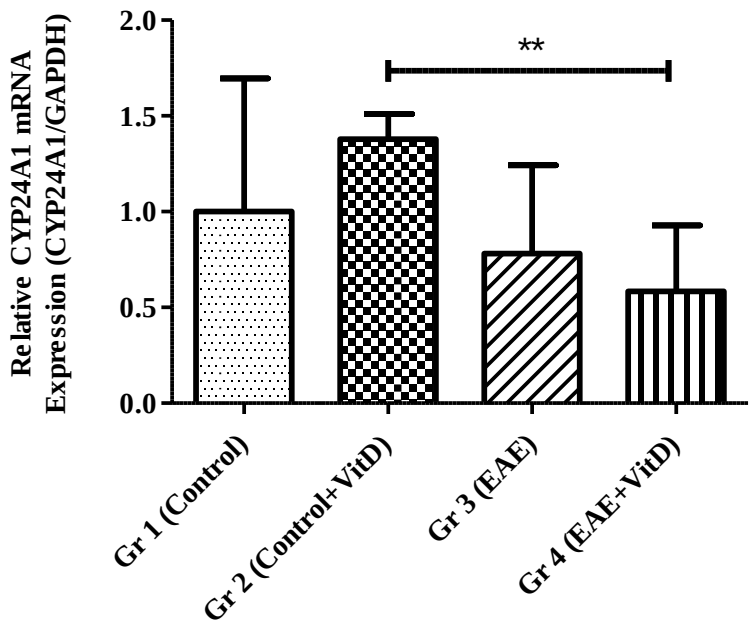


Figure 3.16 Effect of EAE immunization and vitamin D supplementation on CYP24A1 mRNA expression in the kidney of mice. mRNA expression changes were determined by qRT-PCR. The quantifications were expressed as the mean ± SD of the relative expression from three independent experiments. ** $p \leq 0.01$

3.2.5 Effect of EAE Immunization and Vitamin D Supplementation on VDR mRNA Expression in Brain

The mRNA expression of the VDR gene in the brain was determined by quantitative real-time PCR (qRT-PCR). As an internal standard GAPDH gene was used. Specific primers and annealing temperatures for VDR and GAPDH genes were used as described before in Table 2.5. Relative mRNA expression of VDR was analyzed by the $2^{-\Delta\Delta Ct}$ method as described previously. To verify PCR amplification and specificity, the amplification curve (Figure 3.17) and melt curve (Figure 3.18) were plotted, respectively. As shown in Figure 3.18, there was only one peak which means one amplicon without primer dimer or non-specific PCR product.

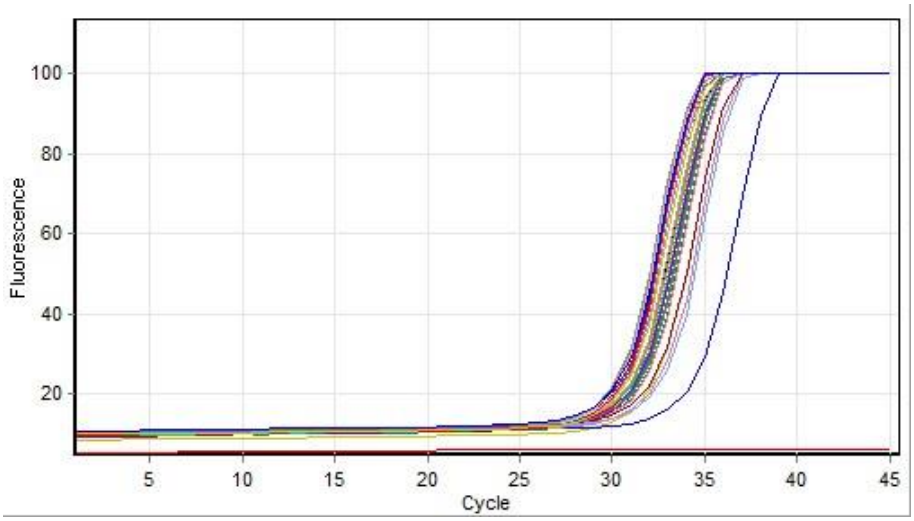


Figure 3.17 Amplification curve of qRT-PCR of VDR gene showing fluorescence accumulation at each cycle.

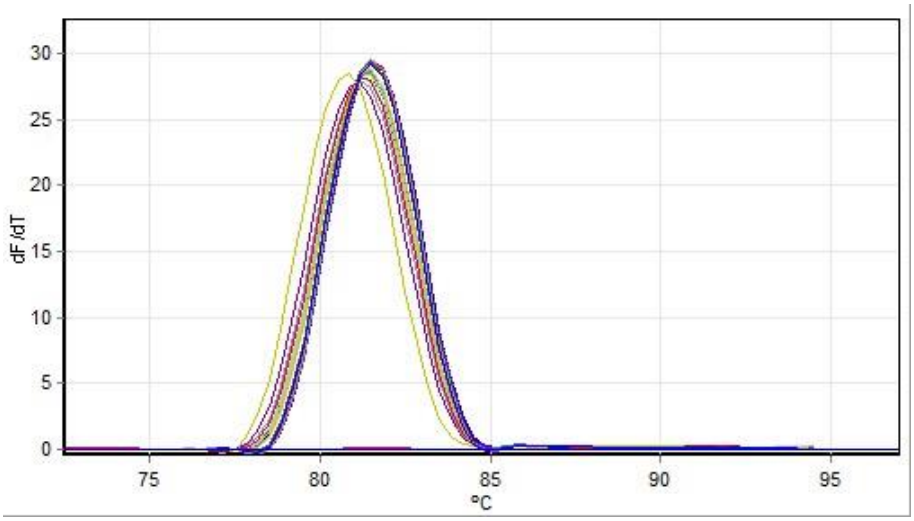


Figure 3.18 Melting curve of qRT-PCR of VDR gene showing fluorescence emission change versus temperature.

In Figure 3.19, relative mRNA expression of VDR in the brain of the 4 groups of mice was shown. There was no significant difference between the control (Gr 1; 1.00±0.42 fold) and vitamin D supplemented control (Gr 2; 1.10±0.14 fold) groups. However, VDR mRNA expression was significantly higher in EAE immunized groups (Gr 3; 1.52±0.33 fold and Gr 4; 1.66±0.21 fold) than healthy control groups (Gr 1; 1.00±0.42 fold and Gr 2; 1.10±0.14 fold).

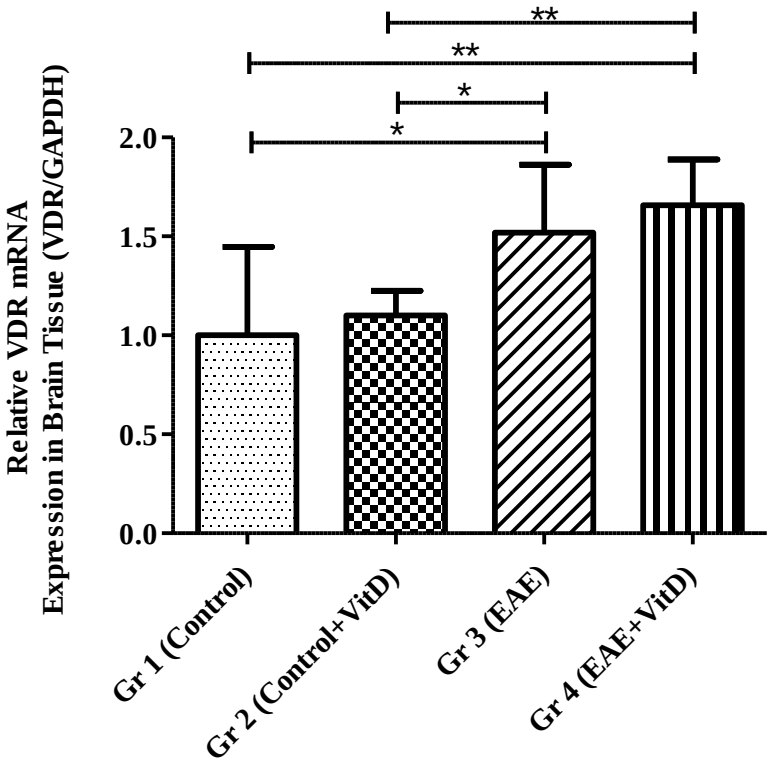


Figure 3.19 Effect of EAE immunization and vitamin D supplementation on VDR mRNA expression in the brain of mice. mRNA expression changes were determined by qRT-PCR. The quantifications were expressed as the mean ± SD of the relative expression from three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$

3.3 Protein Concentration of Mouse Liver, Kidney, and Brain

Pierce™ BCA protein assay kit was used to measure protein concentrations of the samples by following the manufacturers' manual. Bovine serum albumin was used as the standard with different concentrations (25, 125, 250, 500, 750, 1000, 1500, 2000 μg/mL). All the measurements, including blank, standards, and the samples, were performed as duplicates. Total protein concentrations in mouse kidney, liver, and brain tissue homogenates were given in Figure 3.20.

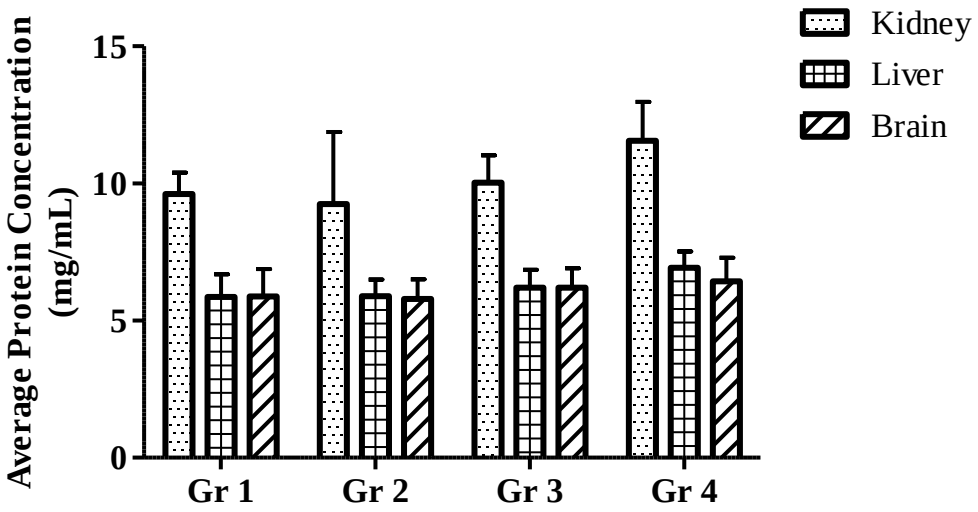


Figure 3.20 Protein concentrations in mouse kidney, liver, and brain homogenate.

3.4 Effects of EAE Immunization and Vitamin D Supplementation on Protein Expressions

Effects of EAE immunization and vitamin D supplementation on vitamin D metabolizing CYPs (CYP2R1, CYP27A1, CYP27B1, and CYP24A1) and VDR protein expressions of female C57BL/6 mice in different organs were determined by Western blotting *via* specific antibodies. GAPDH was used as the protein loading control. Primary rabbit monoclonal anti-GAPDH (1/2000 dilution) and monoclonal anti-rabbit alkaline phosphatase conjugated secondary (1/2000 dilution) antibodies were used for immunochemical detection of GAPDH protein. The intensity of each band was quantified as an arbitrary unit, relative peak area (RPA) by Image J software. This RPA was relatively set to 1.00 for Gr 1 (control), and the protein expression of the other groups was calculated relatively to Gr 1. Multiple comparisons of protein expression of each group were performed by one-way ANOVA. The quantifications were expressed as the mean ± SD of the relative protein expression from three independent experiments and the level of significance was chosen as $p<0.05$.

3.4.1 Effects of EAE Immunization and Vitamin D Supplementation on CYP2R1 Protein Expression in Liver

CYP2R1 (57 kDa) protein expression was determined by the Western blotting technique. 40 μg protein was loaded into each well. Primary goat polyclonal anti-CYP2R1 antibody (1/200 dilution) and alkaline phosphatase (AP) conjugated secondary donkey anti-goat antibody (1/2000 dilution) were used for immunochemical detection of CYP2R1 protein (Figure 3.21/A). Band intensities were quantified by using Image J visualization software. Figure 3.21/B shows the relative protein expression of CYP2R1 where there was no significant difference among each group, Gr 1 (1.00 ± 0.05), Gr 2 (0.98 ± 0.09), Gr 3 (0.96 ± 0.1), Gr 4 (0.94 ± 0.07).

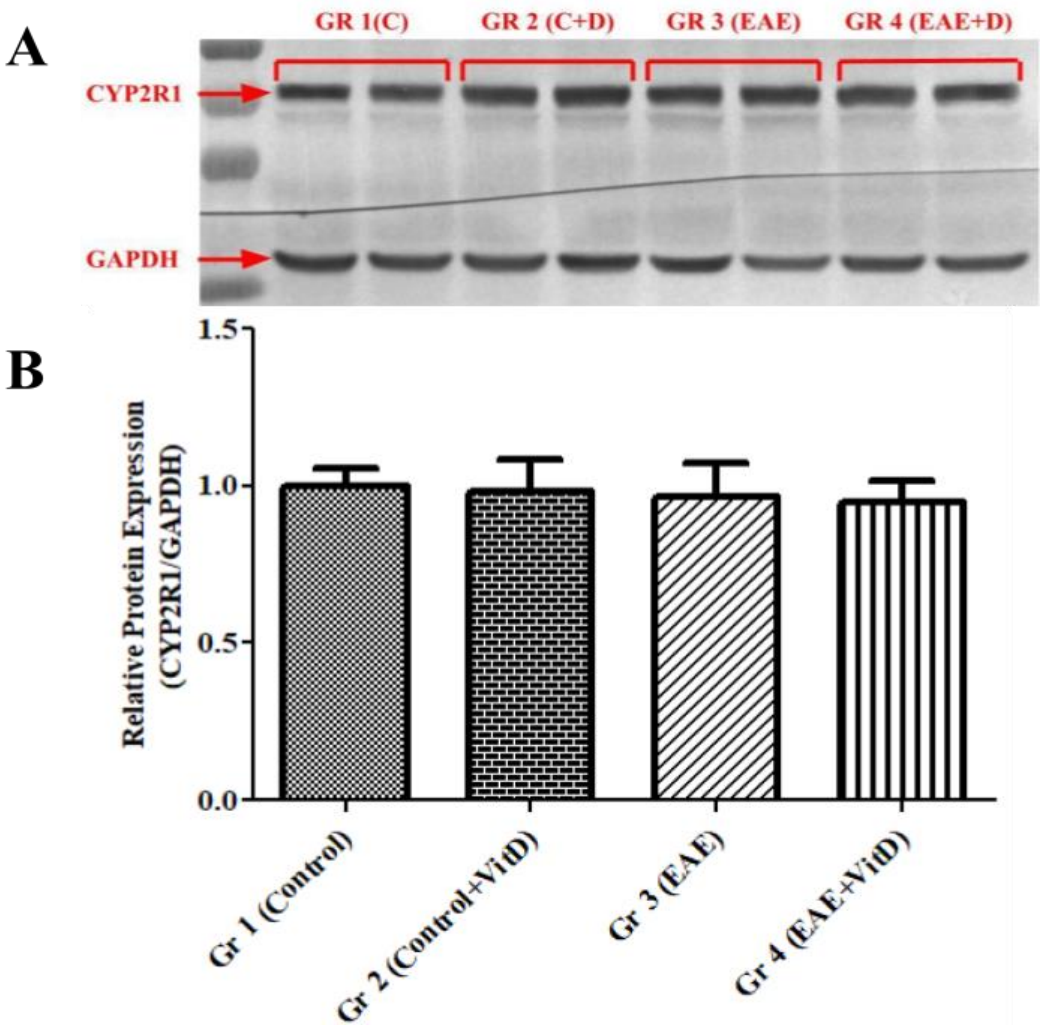


Figure 3.21 Effects of EAE immunization and vitamin D supplementation on CYP2R1 protein expression in mouse liver. A) Representative immunoblot of liver CYP2R1 protein in Gr 1 (Control), Gr 2 (Control + Vitamin D), Gr 3 (EAE), and Gr 4 (EAE + Vitamin D). B)Comparision of CYP2R1 protein expression in the four groups. Experiments were repeated at least 3 times.

3.4.2 Effects of EAE Immunization and Vitamin D Supplementation on CYP27A1 Protein Expression in Liver

CYP27A1 (60 kDa) protein expression was determined by the Western blotting technique. 40 μg protein was loaded into each well. Primary goat polyclonal anti-CYP27A1 antibody (1/200 dilution) and alkaline phosphatase (AP) conjugated secondary donkey anti-goat antibody (1/2000 dilution) were used for immunochemical detection of CYP27A1 protein (Figure 3.22/A). Band intensities were quantified by using Image J visualization software. Figure 3.22/B shows the relative protein expression of CYP27A1 where there was no significant difference among each group, Gr 1 (1.00 ± 0.18), Gr 2 (1.06 ± 0.18), Gr 3 (1.05 ± 0.20), Gr 4 (1.17 ± 0.23).

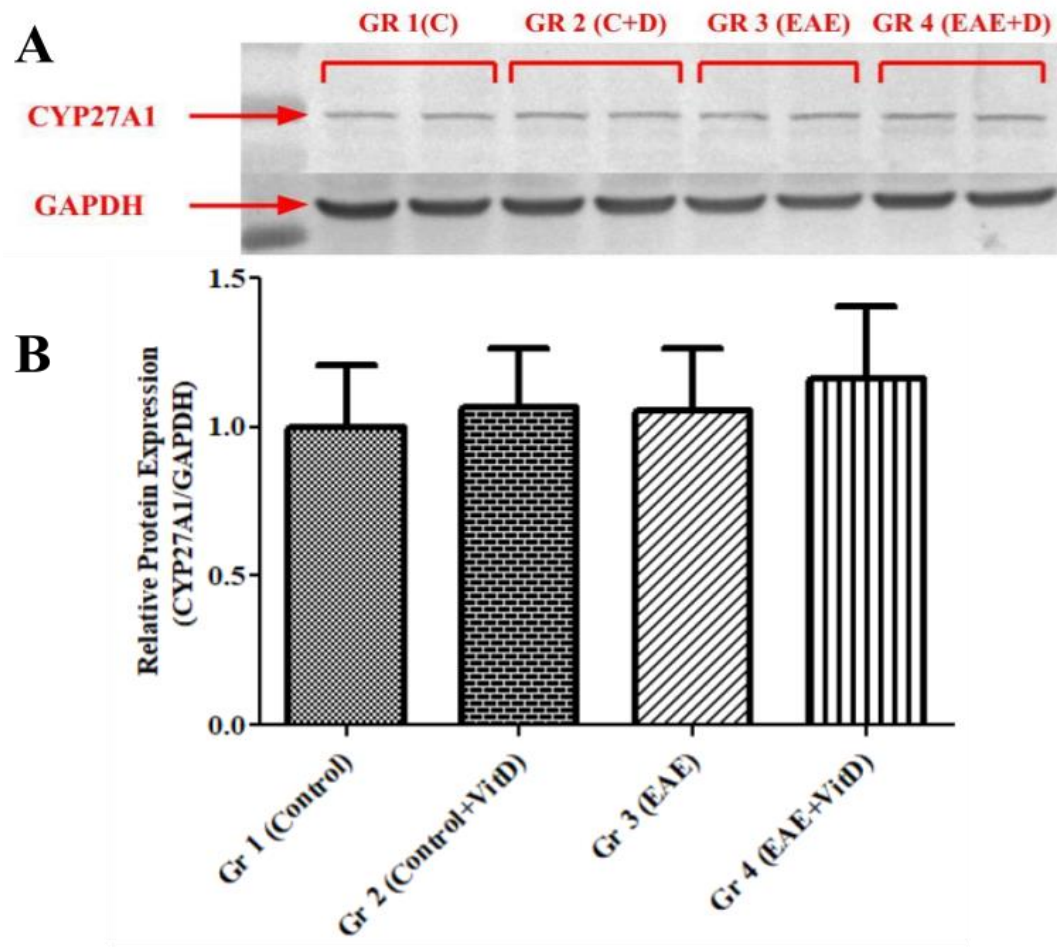


Figure 3.22 Effects of EAE immunization and vitamin D supplementation on CYP27A1 protein expression in mouse liver. A) Representative immunoblot of liver CYP27A1 protein in Gr 1 (Control), Gr 2 (Control + Vitamin D), Gr 3 (EAE), and Gr 4 (EAE + Vitamin D). B) Comparision of CYP27A1 protein expression in the four groups. Experiments were repeated at least 3 times.

3.4.3 Effects of EAE Immunization and Vitamin D Supplementation on CYP27B1 Protein Expression in Kidney

CYP27B1 (56.5 kDa) protein expression was determined by the Western blotting technique. 20 μg protein was loaded into each well. Primary rabbit polyclonal anti-CYP27B1 antibody (1/1000 dilution) and alkaline phosphatase (AP) conjugated secondary anti-rabbit antibody (1/2000 dilution) were used for immunochemical detection of CYP27B1 protein (Figure 3.23/A). Band intensities were quantified by using Image J visualization software. Figure 3.23/B shows the relative protein expressions where EAE immunized, and vitamin D supplemented group, Gr 4 (2.06 ± 0.63), has statistically significantly higher CYP27B1 protein expression than the Gr 1 (1.0 ± 0.16) and Gr 3 (1.04 ± 0.30), $p<0.001$. Although Gr 2 (1.71 ± 0.26) has higher CYP27B1 expression than Gr 1, it was not statistically significant.

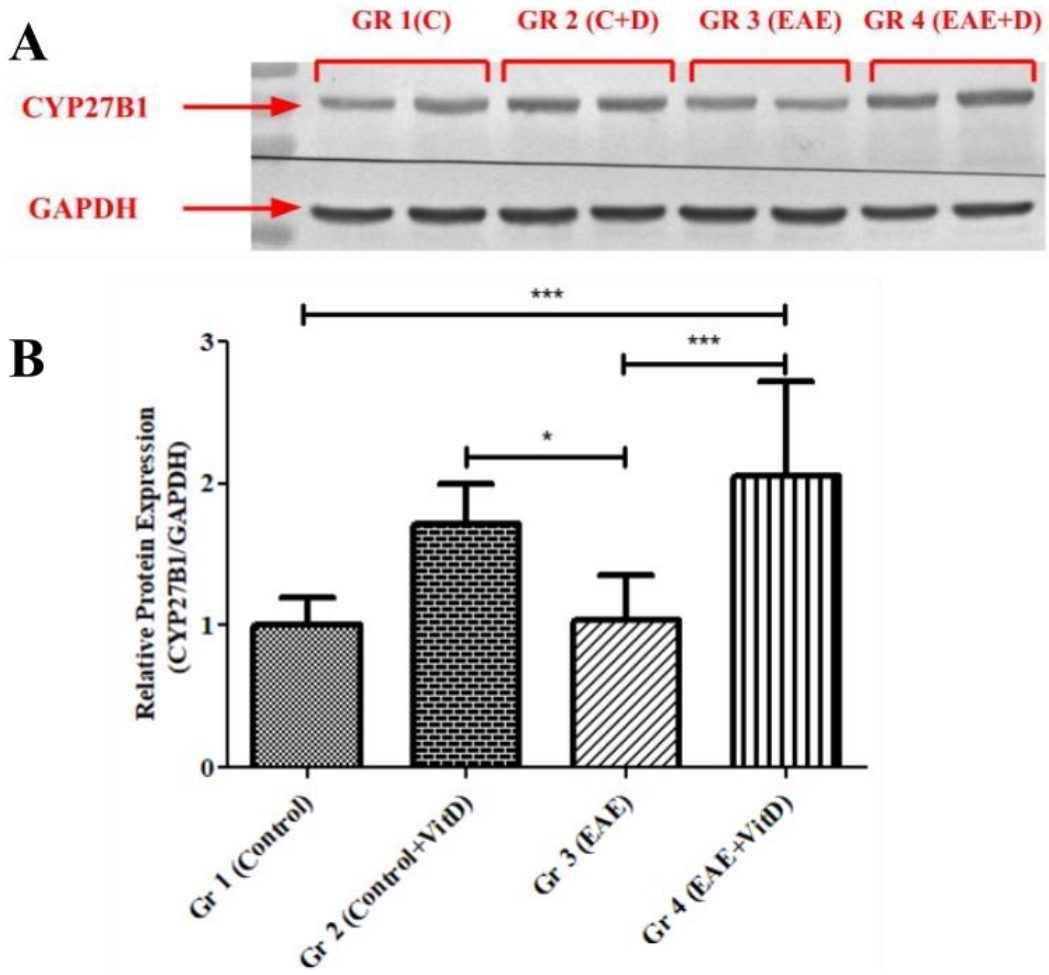


Figure 3.23 Effects of EAE immunization and vitamin D supplementation on CYP27B1 protein expression in mouse kidney. A) Representative immunoblot of kidney CYP27B1 protein in Gr 1 (Control), Gr 2 (Control + Vitamin D), Gr 3 (EAE), and Gr 4 (EAE + Vitamin D). B) Comparision of CYP27B1 protein expression in the four groups. Experiments were repeated at least 3 times. * $p<0.05$, *** $p<0.001$

3.4.4 Effects of EAE Immunization and Vitamin D Supplementation on CYP24A1 Protein Expression in Kidney

CYP24A1 (58.9 kDa) protein expression was determined by the Western blotting technique. 20 μg protein was loaded into each well. Primary rabbit polyclonal anti-CYP24A1 antibody (1/1000 dilution) and alkaline phosphatase (AP) conjugated secondary anti-rabbit antibody (1/2000 dilution) were used for immunochemical detection of CYP24A1 protein (Figure 3.24/A). Band intensities were quantified by using Image J visualization software. Figure 3.24/B shows the relative protein expressions. Although both vitamin D supplementation (Gr 2; 1.47 ± 0.45) and EAE immunization (Gr 3; 1.45 ± 0.28) caused an increase in CYP24A1 protein expression compared to the control group (Gr 1; 1.00 ± 0.30), the results were not statistically significant. However, their combined effect in Gr 4 (1.70 ± 0.46) caused a statistically significant increase in CYP24A1 protein expression compared to the control group ($p<0.05$).

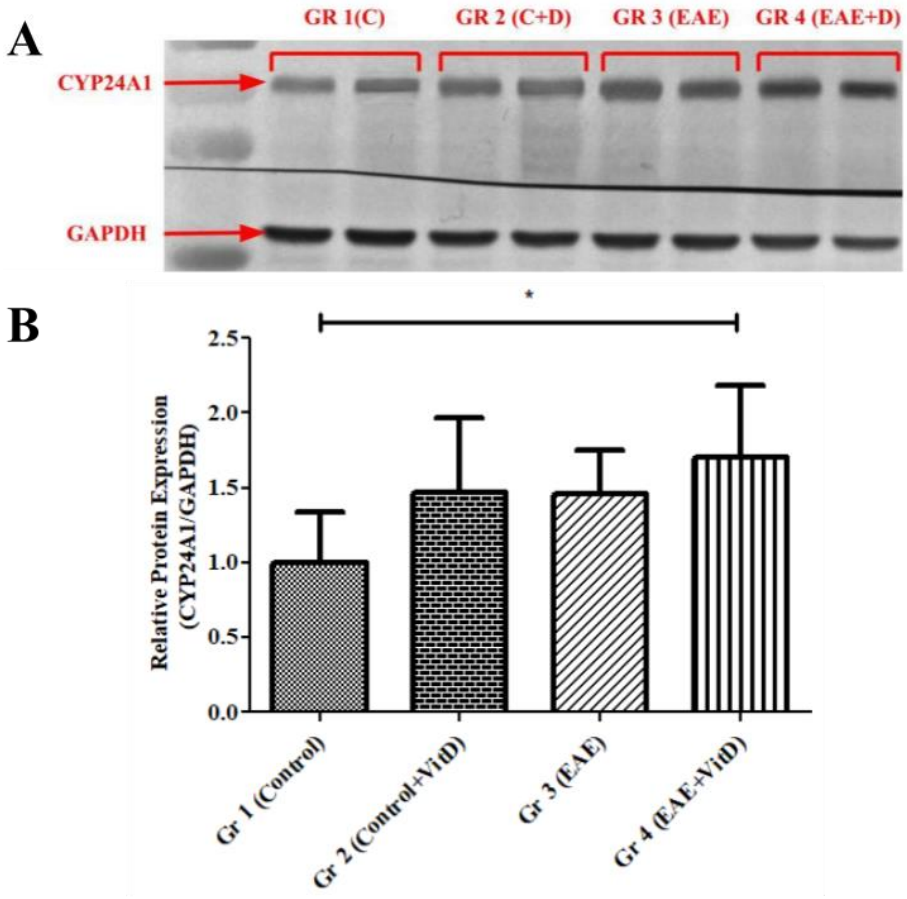


Figure 3.24 Effects of EAE immunization and vitamin D supplementation on CYP24A1 protein expression in mouse kidney. A) Representative immunoblot of kidney CYP24A1 protein in Gr 1 (Control), Gr 2 (Control + Vitamin D), Gr 3 (EAE), and Gr 4 (EAE + Vitamin D). B) Comparision of CYP24A1 protein expression in the four groups. Experiments were repeated at least 3 times. * $p<0.05$

3.4.5 Effects of EAE Immunization and Vitamin D Supplementation on VDR Protein Expression in Brain

VDR (48.3 kDa) protein expression was determined by the Western blotting technique. 40 μg protein was loaded into each well. Primary rabbit polyclonal anti-VDR antibody (1/500 dilution) and alkaline phosphatase (AP) conjugated secondary monoclonal anti-rabbit antibody (1/1000 dilution) were used for immunochemical detection of VDR protein (Figure 3.25/A). Band intensities were quantified by using Image J visualization software. Figure 3.25/B shows the relative protein expression of VDR where there was no significant difference among the 4 groups of mice, Gr 1 (1.00 ± 0.37), Gr 2 (0.91 ± 0.31), Gr 3 (0.95 ± 0.21), Gr 4 (0.81 ± 0.21).

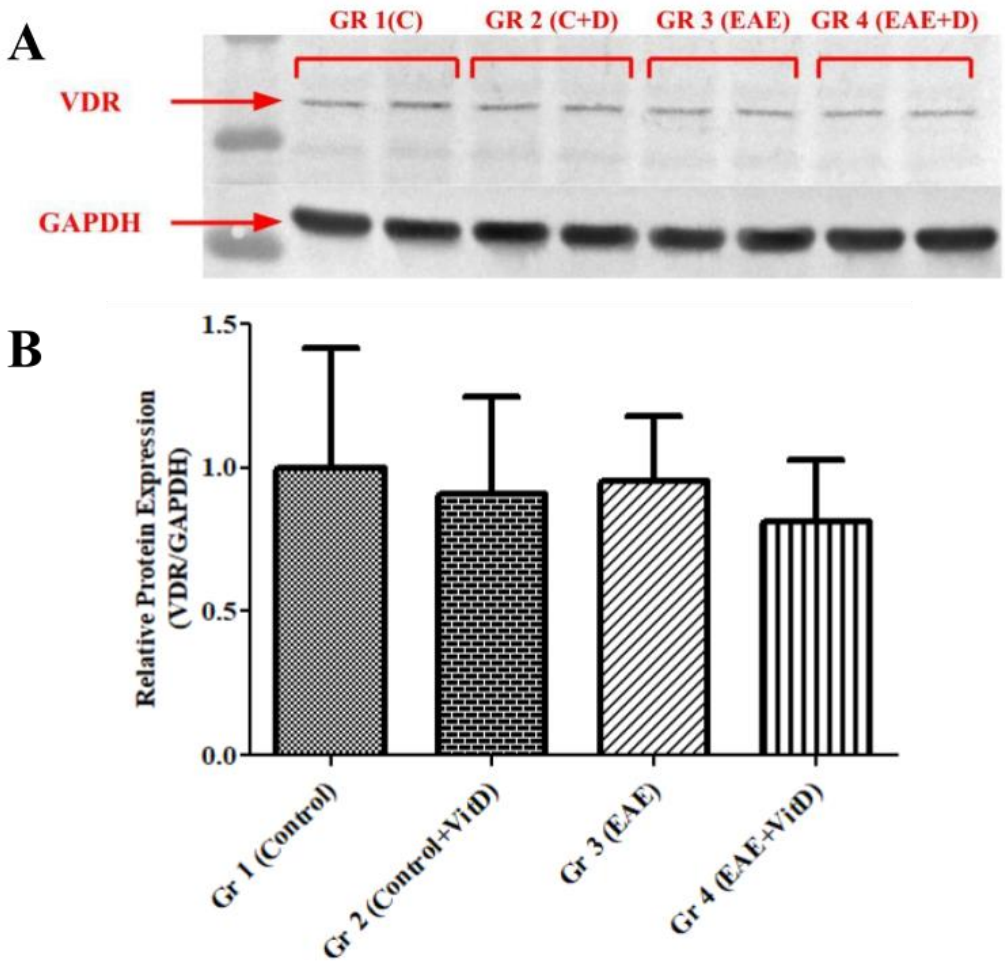


Figure 3.25 Effects of EAE immunization and vitamin D supplementation on VDR protein expression in mouse brain. A) Representative immunoblot of brain VDR protein in Gr 1 (Control), Gr 2 (Control + Vitamin D), Gr 3 (EAE), and Gr 4 (EAE + Vitamin D). B) Comparision of VDR protein expression in the four groups. Experiments were repeated at least 3 times.

CHAPTER 4

DISCUSSION

MS is a complicated, repetitive, and frequently progressive inflammatory demyelinating autoimmune disease of the central nervous system (Noseworthy et al., 2000). Although the etiopathogenesis of MS has not been fully revealed, inflammation and demyelination in the myelin sheath develop on a genetic and an immunological basis triggered by an environmental factor (Ghasemi et al., 2017). Furthermore, it was reported that the prevalence of MS disease increases with distance from the equatorial belt leading to a hypothesis in which MS is inversely correlated with the duration and intensity of sunlight and vitamin D concentrations (Compston et al., 2008; Goldberg, 1974). In the following years, with the discovery of the immunomodulatory effects of Vitamin D, research on the MS-Vitamin D hypothesis has increased. However, although many studies show a strong inverse correlation between MS and vitamin D, these studies lack a biochemical and molecular perspective to investigate this relationship. Furthermore, the literature is limited to cell culture and mRNA expression studies that are insufficient to understand the regulation mechanism of vitamin D metabolizing enzymes since many modifications occur at post-transcriptional and translational levels. In addition, *in vitro*, vitamin D metabolism study is not a suitable way to understand the relationship between multiple sclerosis and vitamin D metabolizing enzymes considering the main sites of vitamin D metabolism, liver, and kidney. Moreover, there is no study investigating the effects of vitamin D on the clinical course of MS, expression of VDR, and vitamin D metabolizing CYP450 enzymes at both mRNA and protein levels by using the EAE mouse model. Therefore, this study may fill a void in the literature by investigating the relationship between MS and vitamin D by studying MS, vitamin D, VDR, and vitamin D metabolizing enzymes as a whole *via* molecular approaches in the mouse EAE model. To understand and explain the

relationship between vitamin D and MS, it is essential to study the mRNA and protein expressions of vitamin D metabolizing cytochrome P450 enzymes (CYP27A1, CYP2R1, CYP27B1, and CYP24A1) and vitamin D receptor.

Although EAE in mice has limitations as an MS model, it is the most common animal model which is generated using several myelin antigens depending on animal and type of MS. C57BL/6 mouse strain is mainly used to study progressive MS, and the commonly used mouse myelin antigen is the MOG_{35-55} peptide. There are 300 publications in PubMed using the EAE model induced by MOG_{35-55} peptide until June 2021. Furthermore, using the EAE model, several drugs approved by Food and Drug Administration (FDA) were developed, such as fingolimod, mitoxantrone, and glatiramer acetate (Constantinescu et al., 2011). Although it is a widely used model to investigate MS, several factors should be considered while generating this model, including the age of the mouse, stress levels, handling, housing sterility to prevent involvement of any other antigen, emulsion preparation, and pertussis toxin dosing (Constantinescu et al., 2011; Linker et al., 2009; Procaccini et al., 2015). Therefore, generating this model is only possible by minimizing the effects of these factors by strictly controlling the environment in the animal house. Also, the experimental setup should be planned considering these factors, one of which is stress levels.

In this study, to prevent extra stress on mice, vitamin D supplementation was performed in drink water instead of oral gavage. EAE induction was performed on 24 mice in two groups (Gr 3 and Gr 4) as described in the methodology part, and all of the mice have developed EAE symptoms. On the day of immunization, vitamin D supplementation has been started in two groups (Gr 2 and Gr 4). After sacrificing the mice, heart blood was collected, and serum $25(OH)D_3$ concentrations of each mouse were measured by ELISA assay to confirm the success of the vitamin D supplementation. Serum $25(OH)D_3$ concentrations of Gr 1, Gr 2, Gr 3, and Gr 4 were 24.3 ± 4.3, 111 ± 13.0, 30 ± 7.4, and 109.0 ± 11.9 ng/mL, respectively. According to these results, it can be safely said that vitamin D supplementation was performed successfully. Mice in Gr 1 and Gr 3 were suffering from vitamin D insufficiency since serum $25(OH)D_3$ levels between 10 ng/mL and 30 ng/mL are considered

insufficient (Seldeen et al., 2017). The mean day of disease onset and mean maximum scores in the EAE group (Gr 3) and vitamin D supplemented EAE group (Gr 4) were compared; there was no significant difference, implying that vitamin D has no protective effect against mice EAE. As opposed to these results, in a study, EAE was developed by myelin basic protein (MBP) peptide in 8 weeks old B10.PL mice showed a significant reduction in the severity of EAE symptoms in the vitamin D supplemented group (Spach et al., 2005). Although the EAE model in this study was generated in different mouse strain and by different peptide, the most important difference may be the supplementation date of the vitamin D four weeks before the induction. Controversial to this study, it was shown that the mean maximum score of EAE in the vitamin D deficient group was significantly lower (Wang et al., 2012). Actually, these contradictions in the literature were one of the main motivation of this study. According to our results, vitamin D is neither protective nor harmful effects on EAE development and symptoms. To observe the protective effects of vitamin D, maybe it should have been started to be administered long enough before the EAE induction with any myelin antigen. Due to vitamin D supplementation on the day of immunization, vitamin D might not have enough time to program the immune system, specifically T regulatory cells, to increase tolerance to self-antigens. In a meta-analysis study, it has been reported that vitamin D supplementation causes an increase in the number of regulatory T cells (Fisher et al., 2019). Still, this effect can be observed after at least three months of vitamin D supplementation (Fisher et al., 2019).

Besides protective effects, the therapeutic effect of vitamin D was investigated. If the score of a mouse was decreased at least for 0.5, it has been considered recovered. Although there was no significant difference in the day of onset and the maximum score of EAE in Gr 3 and Gr 4, eight mice out of 12 recovered in the vitamin D supplemented EAE group (Gr 4), while only two mice out of 12 were recovered in the EAE group (Gr 3) which was statistically significant. Similar to our results, it was reported that vitamin D supplementation applied in the most severe acute EAE has a therapeutic effect on the symptoms of the disease (Mayne et al., 2011). In

addition to these results, in a study, it was shown that an increase of 10 nmol/L in serum vitamin D level of RRMS patients revealed a 9-12% decrease in the risk of attack frequency (Simpson et al., 2010).

The immunomodulatory effects of vitamin D are well-known. High doses of vitamin D reduces the proportion of inflammatory cytokine (IL-17) producing immune cells, including $CD4^+$ T-cells (Pierrot-Deseilligny et al., 2017). In addition, vitamin D supplementation reduces the release of inflammatory cytokines, such as IL-12 and IL-23, from Th1 and Th17 cells-whereas increases the production of anti-inflammatory cytokine IL-10 (Bhargava et al., 2017; Häusler et al., 2019). Besides the immunomodulatory effects, vitamin D supplementation also increases remyelination in rats by inducing oligodendrocyte lineage differentiation (Gomez-Pinedo et al., 2020). These pathways may explain the underlying mechanisms that resulted in the recovery of vitamin D-supplemented mice. Still, this study aimed to investigate the possible effects of vitamin D metabolizing enzymes and VDR on the course of MS.

Vitamin D is a secosteroid with well-known classical effects on calcium and phosphorus homeostasis and maintains bone health (Omdahl et al., 2002; Zalewski et al., 2016). In humans, mainly vitamin D is produced in the skin *via* ultraviolet B radiation (UVB; 290-315 nm) from the sun, while minor amounts can be taken by the diet. Vitamin D is mainly found in two forms, cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2), from animal and plant sources, respectively. Either produced in the skin or taken by the diet, vitamin D is transported to the liver by vitamin D binding protein (DBP) in the blood (Bouillon et al., 2020). In the liver, activation of vitamin D starts with hydroxylation at 25th carbon to produce 25-hydroxyvitamin D ($25(OH)D_3$) by cytochrome P450 enzymes (Sakaki et al., 2005). Both CYP27A1 and CYP2R1 enzymes have 25-hydroxylation activity.

In this study, mRNA and protein expressions of CYP27A1 and CYP2R1 enzymes were investigated by qRT-PCR and Western blotting, respectively. CYP2R1 mRNA expresion was significantly decreased in vitamin D supplemented EAE group (Gr 4;

0.50 fold) compared to the control (Gr 1; 1.0 fold), control and vitamin D supplemented (Gr 2 - 0.91 fold), and EAE immunized (Gr 3 - 0.88 fold) groups. In Gr 4, mRNA expression of CYP2R1 was suppressed due to the combined effect of vitamin D and EAE immunization. It is known that $1,25(OH)_2D_3$ causes suppression of the CYP2R1 gene by the JNK pathway (Sundaram et al., 2014). Moreover, fasting causes repression of CYP2R1 mRNA expression by estrogen-related receptor α (ERRα)–dependent manner (Aatsinki et al., 2019). Due to disability in mice resulting from EAE development may result in decreased food intake, which may cause fasting. Therefore this condition and the vitamin D supplementation were combined in Gr 4, in which mRNA expression of CYP2R1 has been reduced more than the vitamin D supplemented (Gr 2) and EAE (Gr 3) groups itself. However, there was no significant difference between the four groups in relative CYP2R1 protein expressions. In Gr 4, suppressed mRNA expression was restored in protein expression due to modifications at the translational level; translation efficiency may be increased. However, with the methodology of this study, it was impossible to address the mechanism of these modifications. Also, there was no significant difference between the four groups in CYP27A1 mRNA and protein expressions. Since protein has enzymatic functions, protein expression levels should be taken into account. Considering relative protein expressions of CYP2R1 and CYP27A1, 25-hydroxylation may not be a critical step for the course of MS.

In the kidney, $25(OH)D_3$ undergoes 1α-hydroxylation by another cytochrome P450 (CYP27B1) enzyme and biologically active vitamin D, $1,25(OH)_2D_3$ (calcitriol), is produced (Holick et al., 1987). Because of the crucial role of CYP27B1 in vitamin D activation, regulation of CYP27B1 mRNA and protein expression by vitamin D supplementation and EAE immunization was investigated to explain whether it has a role in the course of MS. Although there was a slight decrease of both mRNA and protein expression in the EAE immunized group (Gr 3) compared to the control group (Gr 1), it was not statistically significant. Similarly, there was no significant difference in mRNA and protein expressions between vitamin D supplemented control (Gr 2) and vitamin D supplemented EAE immunized (Gr 4) groups.

However, CYP27B1 mRNA expression was significantly high in Gr 2 (1.65 fold) and Gr 4 (1.50 fold) compared to Gr 3 (0.76 fold). Similarly, Gr 4 (2.06 ± 0.63) and Gr 2 (1.71 ± 0.26) have statistically significantly higher CYP27B1 protein expression than Gr 3 (1.04 ± 0.30). Although Gr 2 (1.71 ± 0.26) has higher CYP27B1 expression than Gr 1 (1.0 ± 0.16), it was not statistically significant. Also, there was no significant difference between Gr 1 (control) and Gr 3 (EAE). These results showed that vitamin D supplementation caused an increase in CYP27B1 mRNA and protein expression. Reasonably, an increase in protein expression may be due to the increase in mRNA expression, where a higher number of CYP27B1 transcripts may result in an increased number of translations. Thus, regulation of CYP27B1 may occur at the transcriptional level with vitamin D supplementation. Before explaining the possible regulatory pathway of vitamin D on CYP27B1, it is better to remember that in this study, vitamin D was supplemented as cholecalciferol, which underwent 25-hydroxylation and $25(OH)D_3$ produced which is the substrate of CYP27B1 enzyme. An increase in CYP27B1 mRNA expression might result from increased substrate concentration which may somehow trigger a process that causes demethylation at the promoter region of CYP27B1. However, there is no such evidence in the literature. Opposed to this result, due to increased protein expression of CYP27B1, the production of $1,25(OH)_2D_3$ should have been increased, which might cause suppression of CYP27B1 expression by calcitriol and VDR through a negative feedback mechanism (Brenza et al., 2000). However, because of the involvement of CYP24A1 in the degradation and inactivation of $1,25(OH)_2D_3$ molecules, it can not be said that active vitamin D levels were high in vitamin D supplemented groups at the end of the experiment. Also, upregulating effects of high substrate concentration might be overcome the suppressive effects of the product. Besides the effects of vitamin D supplementation, since EAE immunization itself did not cause a significant change in both mRNA and protein expression of CYP27B1 in the kidney, it may be said that CYP27B1 might not be a pivotal factor to explain the relationship between MS and vitamin D. However, in the literature, it was stated that in tolerogenic dendritic cells (DC2), decrease in CYP27B1 expression is

associated with MS risk (Shahijanian et al., 2014). Besides MS, there are many studies in the literature reporting association of the CYP27B1 enzyme with several diseases. For example, in a study, it was reported that CYP27B1 expression decreases during melanoma progression (Brożyna et al., 2013). In another study, an increase in CYP27B1 expression has been reported in schizophrenia, and it has been hypothesized that it was a defense mechanism to tolerate harmful effects of the disease condition (Manjili et al., 2018).

The active vitamin D metabolite ($1,25(OH)_2D_3$) produced by the CYP27B1 enzyme is inactivated and eliminated *via* hydroxylation at 24th carbon to make $1,24,25(OH)_3D_3$ (calcitroic acid) by the CYP24A1 enzyme in the kidney. Together with the hormone and other metabolites, the expression and activity of CYP24A1 are highly regulated by the active vitamin D metabolite ($1,25(OH)_2D_3$) (Jones et al., 2012; Zierold et al., 1994). CYP24A1 gene expression induced by $1,25(OH)_2D_3$, binding of this metabolite to VDR enables this receptor-ligand complex to interact with the VDRE in the promoter region of the CYP24A1 gene to recruit transcription factors (Meyer et al., 2020). Guided by this information, it was expected to observe increased mRNA expression of the CYP24A1 gene in vitamin D supplemented groups (Gr 2 and Gr 4). Although there was an increase in CYP24A1 mRNA expression in vitamin D supplemented healthy mice, it was not significant compared to control (Gr 1; 1.00±0.50 fold), and EAE immunized (Gr 3; 0.78±0.36 fold) groups. On the contrary, the vitamin D supplemented EAE group (Gr 4; 0.58±0.28 fold) has the lowest levels of CYP24A1 mRNA expression among the groups. Also, CYP24A1 mRNA expression was significantly higher in Gr 2 (1.38±0.10 fold) than Gr 4 (0.58±0.28 fold), $p \leq 0.01$. Although it was not statistically significant due to great standard deviation within groups, when healthy controls of EAE groups compared, it could have been said that EAE immunization might suppress the CYP24A1 gene expression. However, considering the mRNA expression and western blotting results together, this assumption was not logical, but this regulation at the mRNA level should still be studied in detail. Although separately, vitamin D supplementation in Gr 2 (1.47 ± 0.45) and EAE immunization in Gr 3 (1.45 ± 0.28)

caused an increase in CYP24A1 protein expression compared to the control group (Gr 1; 1.00 ± 0.30), the results were not statistically significant. However, their combined effect in Gr 4 (1.70 ± 0.46) caused a statistically significant increase in CYP24A1 protein expression against the control group ($p<0.05$). Besides, kidney CYP24A1 protein expression of mice in Gr 4 showed a strong positive correlation with the clinical EAE scores of the mice ($p = 0.034$, $r = 0.613$; Figure 4.1) in Gr 4. However, this correlation could not be observed in Gr 3, which may be due to the vitamin D supplementation. At the beginning of this study, it was aimed to find a possible target in the pathway of vitamin D metabolism to be able to increase its therapeutic effects. The correlation of CYP24A1 protein expression and the clinical score of EAE makes this enzyme a potential target for treating MS disease. Moreover, in Gr 3, CYP27B1 and CYP24A1 protein expression were positively correlated ($p = 0.019$, $r = 0.690$; Figure 4.2). However, for healthy groups, similar data could not be taken. When excessive vitamin D supplementation is not in the picture, CYP27B1 and CYP24A1 protein expressions positively correlate in EAE mice implying that active vitamin D producing and degrading enzymes work together under disease conditions. Therefore, low doses of vitamin D supplementations may not be effective for the treatment of MS.

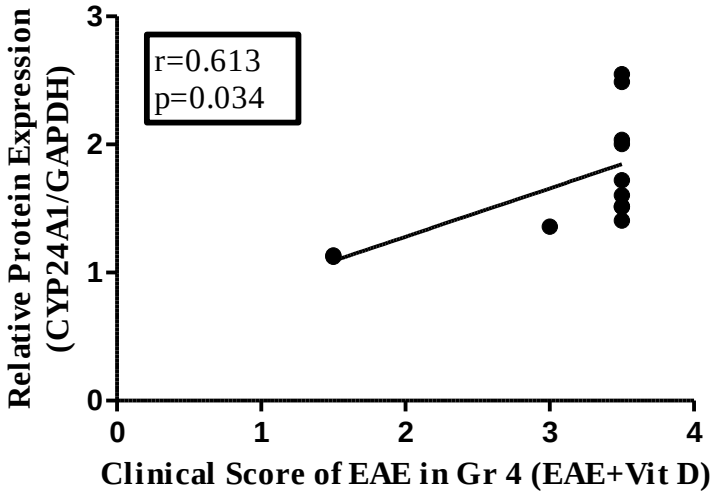


Figure 4.1 Correlation between clinical scores of EAE and CYP24A1 relative protein expression in Gr 4. The correlation coefficient (r=0.613, p=0.034<0.05) was calculated by the linear regression method.

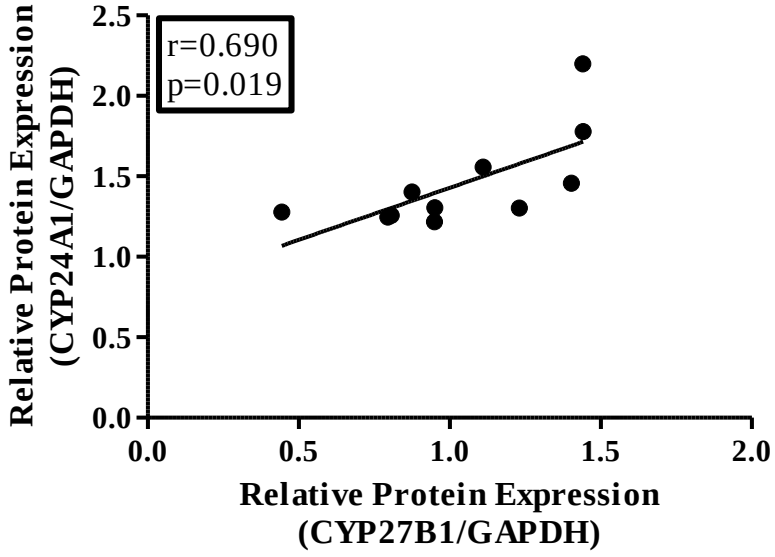


Figure 4.2 Correlation between CYP27B1 and CYP24A1 relative protein expression in Gr 3. The correlation coefficient (r=0.690, p=0.019<0.05) was calculated by the linear regression method.

Many studies in the literature report association with increased expression and activity of CYP24A1 to various diseases, such as X-linked hypophosphatemia, chronic kidney disease, and type 1 diabetes (Helvig et al., 2010; Hough et al., 1983; Tenenhouse et al., 1988). The increased degradation rate of $1,25(OH)_2D_3$ by CYP24A1 results in decreased active vitamin D concentrations, which may cause these pathologies. Also, CYP24A1 expression is elevated in tumor cells that do not respond to $1,25(OH)_2D_3$ supplementation (M. G. Anderson et al., 2006; Friedrich et al., 2003; N. King et al., 2012; Townsend et al., 2005). Apart from this thesis study, because of its clinical importance, selective CYP24A1 inhibitors and vitamin D analogs, including VID-400, SDZ 89-443, CTA091, and CTA018, are already the new strategies to prevent rapid degradation of $1,25(OH)_2D_3$. In the future, these agents may be used as a part of MS therapy with vitamin D.

Active vitamin D transcriptionally regulates several genes *via* binding to VDR (Bikle, 2014; von Essen et al., 2012). Due to the gene regulatory effects of vitamin D *via* VDR, any defects or changes in the VDR gene could lead to severe problems in the activation of genes related to calcium homeostasis, immune regulation, cell proliferation and differentiation, etc. For example, a point mutation in the human VDR gene cause resistance to 1,25(OH)2D3 in rickets (Sone et al., 1990). Moreover, it was reported that VDR deficiency is related to inflammatory bowel disease in mice (Kim et al., 2013). In another study, VDR deficiency increases tumor burden by enhancing Wnt/β-catenin signaling in colon cancer (Larriba et al., 2011). Furthermore, it was found that VDR deficiency increases the rate of aging progress in mice brains (Minasyan et al., 2007). According to the findings of this thesis work, there was no significant difference in mRNA expression of VDR gene between control (Gr 1; 1.00±0.42) and vitamin D supplemented control (Gr 2; 1.10±0.14) groups in mice brains. Similarly, there was no significant difference in mRNA expression of VDR gene between EAE (Gr 3; 1.52±0.33) and vitamin D supplemented EAE (Gr 4; 1.66±0.21) groups in mice brains. These results suggested that mRNA expression of VDR was not affected by vitamin D supplementation. However, VDR expression was significantly higher in EAE immunized groups (Gr

3 and Gr 4) than in control groups (Gr 1 and Gr 2). Similar to these findings, it was reported that VDR mRNA expression was upregulated in active MS lesions of 39 patients (Joost Smolders et al., 2013). Besides mRNA expression, there was no significant difference in protein expression of VDR among each group [Gr 1 (1.00 ± 0.37), Gr 2 (0.91 ± 0.31), Gr 3 (0.95 ± 0.21), Gr 4 (0.81 ± 0.21)]. Lack of correlation between mRNA and protein expression of VDR in mouse brain may be due to post-transcriptional regulations in EAE conditions. Although it was impossible to determine this regulatory pathway by the methodology of this study, it can be hypothesized that the translational efficiency of VDR transcripts may be affected by miRNAs expressed due to EAE conditions. For example, in a study, the effects of miR-125b on VDR expression were investigated, which downregulates the protein expression of VDR (Mohri et al., 2009). Another study reported that the contribution of miR-125b to pro-inflammatory activation of monocyte-derived macrophages was reported (Duroux-Richard et al., 2016). Similarly, in a recent study, miR-125b was associated with inflammation by the NFκB pathway (Valmiki et al., 2020). Considering the importance of VDR for the therapeutic effects of vitamin D, suppression of VDR translation by micro RNAs may be targeted in treating MS.

CONCLUSION

Multiple sclerosis (MS) is one of the most common autoimmune diseases of the central nervous system, yet etiopathogenesis remains unsolved. MS frequency increases with increasing latitude, leading to a hypothesis in which MS is inversely correlated with duration and intensity of sunlight and vitamin D concentrations. To explain the etiopathogenesis of MS and enlighten the effect of vitamin D on MS, investigators work around the globe with increased interest. However, these studies lack a holistic approach considering vitamin D metabolizing CYP enzymes and vitamin D receptor (VDR) together with vitamin D supplementation in the EAE model. In our study, we have investigated the relationship between vitamin D supplementation, MS, VDR, and vitamin D metabolizing CYP enzymes, including CYP2R1, CYP27A1, CYP27B1, and CYP24A1 at mRNA and protein expression levels. Since active vitamin D formation, degradation and function require these enzymes and VDR, determining their role in MS etiopathogenesis may help us find a potential target for treating the disease. Considering our results, although vitamin D could not prevent disease formation, it significantly helps the recovery of the mice. Moreover, since CYP24A1 protein expression correlates with the clinical score of the EAE, the CYP24A1 enzyme may be the target to increase the efficacy of vitamin D in MS. Therefore, CYP24A1 inhibitor to prevent degradation of active vitamin D and keep high vitamin D concentration in circulation might be helpful to treat MS. Fortunately, there are continuing researches to find specific inhibitors for the CYP24A1 enzyme. Although VDR mRNA expression increases with the EAE immunization, it was reverted in protein expression levels probably because of the post-transcriptional regulations. The underlying pathway for this regulation should be investigated, and new targets in that pathway should be determined. Increasing circulating vitamin D concentration or VDR expression in the cells may seem a good strategy. However, the adverse effects of long-term high concentrations of circulating vitamin D should be taken into account.

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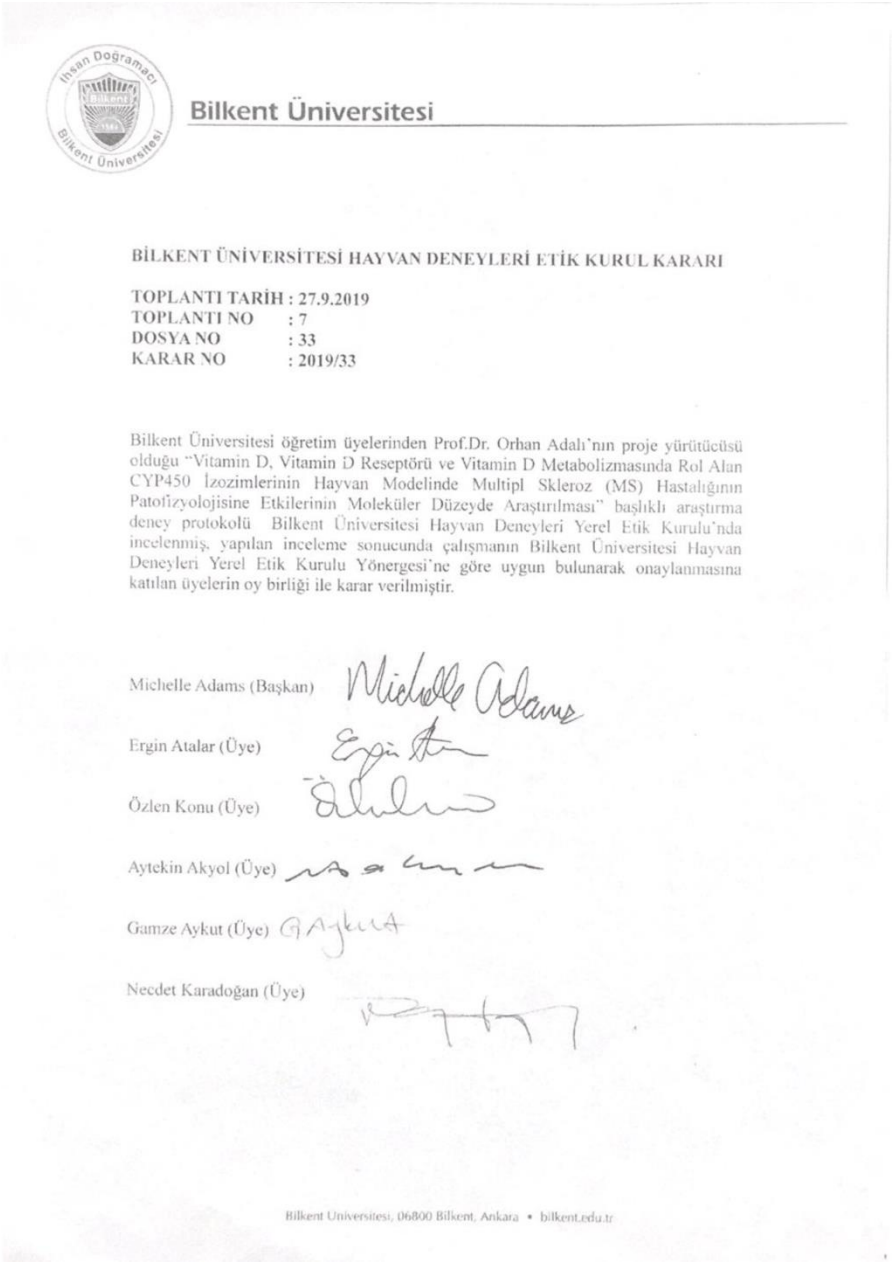
APPENDICES

A. Animal Experimentation Ethics Committee Approval Document



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DOSYA NO : 33
KARAR NO : 2019/33

Bilkent Üniversitesi öğretim üyelerinden Prof.Dr. Orhan Adalı'nın proje yürütücüsü olduğu "Vitamin D, Vitamin D Reseptörü ve Vitamin D Metabolizmasında Rol Alan CYP450 İzozimlerinin Hayvan Modelinde Multipl Skleroz (MS) Hastalığının Patofizyolojisine Etkilerinin Moleküler Düzeyde Araştırılması" başlıklı araştırma deney protokolü Bilkent Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu'nda incelenmiş, yapılan inceleme sonucunda çalışmanın Bilkent Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu Yönergesi'ne göre uygun bulunarak onaylanmasına katılan üyelerin oy birliği ile karar verilmiştir.

Michelle Adams (Başkan)
Ergin Atalar (Üye)
Özlen Konu (Üye)
Aytekin Akyol (Üye)
Gamze Aykut (Üye)
Necdet Karadoğan (Üye)

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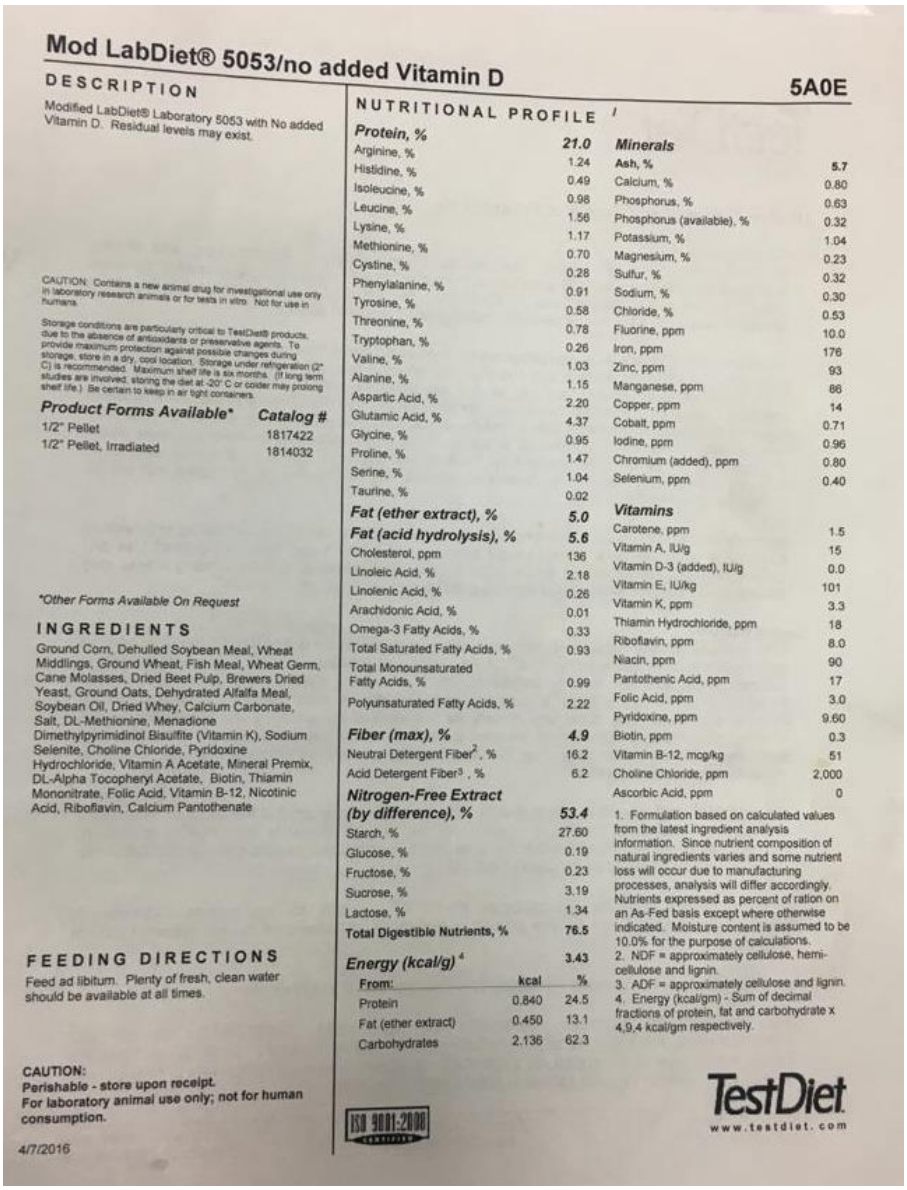
B. Vitamin D Deficient Diet Ingredients



Mod LabDiet® 5053/no added Vitamin D

5A0E

DESCRIPTION

Modified LabDiet® Laboratory 5053 with No added Vitamin D. Residual levels may exist.

CAUTION: Contains a new animal drug for investigational use only in laboratory research animals or for tests in vitro. Not for use in humans.

Storage conditions are particularly critical to TestDiet® products due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

Product Forms Available*	***Catalog #***
1/2" Pellet	1817422
1/2" Pellet, Irradiated	1814032

**Other Forms Available On Request*

INGREDIENTS

Ground Corn, Dehulled Soybean Meal, Wheat Middlings, Ground Wheat, Fish Meal, Wheat Germ, Cane Molasses, Dried Beet Pulp, Brewers Dried Yeast, Ground Oats, Dehydrated Alfalfa Meal, Soybean Oil, Dried Whey, Calcium Carbonate, Salt, DL-Methionine, Menadione Dimethylpyrimidinol Bisulfite (Vitamin K), Sodium Selenite, Choline Chloride, Pyridoxine Hydrochloride, Vitamin A Acetate, Mineral Premix, DL-Alpha Tocopheryl Acetate, Biotin, Thiamin Mononitrate, Folic Acid, Vitamin B-12, Nicotinic Acid, Riboflavin, Calcium Pantothenate

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable - store upon receipt.
For laboratory animal use only; not for human consumption.

4/7/2016

NUTRITIONAL PROFILE [1]

Protein, %	***21.0***
Arginine, %	1.24
Histidine, %	0.49
Isoleucine, %	0.98
Leucine, %	1.56
Lysine, %	1.17
Methionine, %	0.70
Cystine, %	0.28
Phenylalanine, %	0.91
Tyrosine, %	0.58
Threonine, %	0.78
Tryptophan, %	0.26
Valine, %	1.03
Alanine, %	1.15
Aspartic Acid, %	2.20
Glutamic Acid, %	4.37
Glycine, %	0.95
Proline, %	1.47
Serine, %	1.04
Taurine, %	0.02
Fat (ether extract), %	***5.0***
Fat (acid hydrolysis), %	***5.6***
Cholesterol, ppm	136
Linoleic Acid, %	2.18
Linolenic Acid, %	0.26
Arachidonic Acid, %	0.01
Omega-3 Fatty Acids, %	0.33
Total Saturated Fatty Acids, %	0.93
Total Monounsaturated Fatty Acids, %	0.99
Polyunsaturated Fatty Acids, %	2.22
Fiber (max), %	***4.9***
Neutral Detergent Fiber[2], %	16.2
Acid Detergent Fiber[3], %	6.2
Nitrogen-Free Extract (by difference), %	***53.4***
Starch, %	27.60
Glucose, %	0.19
Fructose, %	0.23
Sucrose, %	3.19
Lactose, %	1.34
Total Digestible Nutrients, %	**76.5**
Energy (kcal/g) [4]	**3.43**

From:	kcal	%
Protein	0.840	24.5
Fat (ether extract)	0.450	13.1
Carbohydrates	2.136	62.3

Minerals	
Ash, %	**5.7**
Calcium, %	0.80
Phosphorus, %	0.63
Phosphorus (available), %	0.32
Potassium, %	1.04
Magnesium, %	0.23
Sulfur, %	0.32
Sodium, %	0.30
Chloride, %	0.53
Fluorine, ppm	10.0
Iron, ppm	176
Zinc, ppm	93
Manganese, ppm	86
Copper, ppm	14
Cobalt, ppm	0.71
Iodine, ppm	0.96
Chromium (added), ppm	0.80
Selenium, ppm	0.40
Vitamins	
Carotene, ppm	1.5
Vitamin A, IU/g	15
Vitamin D-3 (added), IU/g	0.0
Vitamin E, IU/kg	101
Vitamin K, ppm	3.3
Thiamin Hydrochloride, ppm	18
Riboflavin, ppm	8.0
Niacin, ppm	90
Pantothenic Acid, ppm	17
Folic Acid, ppm	3.0
Pyridoxine, ppm	9.60
Biotin, ppm	0.3
Vitamin B-12, mcg/kg	51
Choline Chloride, ppm	2,000
Ascorbic Acid, ppm	0

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly. Nutrients expressed as percent of ration on an As-Fed basis except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.
2. NDF = approximately cellulose, hemi-cellulose and lignin.
3. ADF = approximately cellulose and lignin.
4. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

ISO 9001:2008 CERTIFIED

TestDiet
www.testdiet.com

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Evin, Emre
Nationality: Turkish (TC)
Date and Place of Birth: 16 August 1988, Kırşehir
Marital Status: Married
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EDUCATION

Degree	Institution	Year of Graduation
MS	METU Biochemistry	2014
BS	METU Molecular Biology and Genetics	2012
High School	Tokat Science High School, Tokat	2006

WORK EXPERIENCE

Year	Place	Enrollment
2015-Present	METU Dept. of Biology	Research Assistant
2011-2011	Technical University of Denmark	Internship

FOREIGN LANGUAGES

Advanced English

PUBLICATIONS

1. Çelebioğlu, H. U., Evin, E., Erden, Y., Adalı, O., & Çelebi, B., (2020). Cytotoxic Effects of Probiotic Bacteria Lactobacillus acidophilus and Lactobacillus rhamnosus Grown in the Presence of Oleuropein on Human Prostate Cancer Cells. Celal Bayar University Journal of Science, vol.16, 55-59.

2. Evin, E., Durukan, O., Akkulak, M., & Adalı, O., (2018). Effect of cisplatin treatment on protein expression of vitamin D metabolizing CYP24A1 in PC3 cell line. FEBS OPEN BIO , vol.8, 326.
3. Durukan, Ö., Akkulak, M., Evin, E., Arslan, Ş., & Adalı, O., (2018). Alteration of protein and gene expressions of Hexokinase II in PC3 cell lines by cisplatin metformin combination treatment. FEBS OPEN BIO, vol.8, 352.
4. Akkulak, M., Evin, E., Durukan, Ö., Özhan, G., & Adalı, O., (2018). Effect of quercetin on mRNA and protein expressions of vitamin D metabolizing CYP27B1 and CYP24A1 in human embryonic kidney cell line (HEK-293). FEBS OPEN BIO, vol.8, 352.
5. Evin, E., Karakurt, S., Akkulak, M., & Adalı, O., (2017). Analysis of Protein and mRNA Expressions of CYP1A1 and CYP2E1 Enzymes In Liver, Colon, and Prostate Cancer Cell Lines to Study Drug and Carcinogen Metabolism. International Congress on Advances in BioScience and Biotechnology (pp.59). Sarajevo, Bosnia & Herzegovina
6. Durukan, Ö., Evin, E., Arslan, Ş., & Adalı, O., (2017). Metformin – Cisplatin Combination Treatment Alters mRNA Expression of Hexokinase II Gene in LNCaP and PC3 Prostate Cancer Cell Lines. 3rd International Symposium on Euroasian Biodiversity (pp.323). Minsk, Belarus
7. Arıtuluk, Z. C. , Evin, E., Karakurt, S., Ezer, N., Adalı, O., & Gençler Özkan, A. M. , (2017). Investigation of Three Tanacetum L. Taxa From Turkey on The Proliferation of Human Prostate Cancer Cells and Expression of Detoxification Enzyme Activity. The 21th International Congress Phytopharm (pp.10). Graz, Austria
8. Akkulak, M., Evin, E., Durukan, Ö., Özhan, H. G. , & Adalı, O., (2017). Effect of resveratrol on mRNA and protein expressions of Vitamin D metabolizing CYP24A1 in human embryonic kidney cell line (HEK-293). 10th International Symposium on Health Informatics and Bioinformatics (HIBIT 2017)

9. Evin E., Karakurt S., Adali O. 2016. Analysis of Protein and mRNA Expressions of NQO1 and GST-Pi Enzymes in Liver, Colon, and Prostate Cancer Cell Lines to Study Drug and Carcinogen Metabolism FEBS Journal, 283:140. doi: 10.1111/febs.13808
10. Karakurt S., Akkulak M., Evin E., Adali O. 2014. Investigation of Antioxidant Potential of Tannic Acid on Human Prostate Cell Line. XXII. National Biology meeting, Eskişehir, TURKEY
11. Karakurt S., Akkulak M., Evin E., Adali O. 2014. Effects of Plant Phenolic Compounds Tannic Acid and Resveratrol on Prostate Cancer Metastasis. FEBS Journal, 281: 460. doi: 10.1111/febs.12919
12. Karakurt S., Evin E., Akkulak M., Adali O. 2014. Antiproliferative and Protective Effect of Tannic Acid on Human Prostate Cancer. EACR Sponsored 2nd Anticancer Agents Congress: Targeting Cancer Stem Cell. Bodrum-TURKEY