ANALYZING THE EXPRESSION PATTERNS OF VITAMIN D METABOLIZING CYP27B1 AND CYP24A1 IN BRAIN TISSUE OF VITAMIN D TREATED MICE WITH MULTIPLE SCLEROSIS (MS)

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ABSTRACT

ANALYZING THE EXPRESSION PATTERNS OF VITAMIN D METABOLIZING CYP27B1 AND CYP24A1 IN BRAIN TISSUE OF VITAMIN D TREATED MICE WITH MULTIPLE SCLEROSIS (MS)

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The etiopathogenesis of Multiple Sclerosis (MS), an inflammatory demyelinating autoimmune disease of the central nervous system, is still unknown. MS is a complex, recurring, and frequently progressing condition. There is a hypothesis that MS is adversely associated with the length and intensity of sunlight exposure and vitamin D concentrations since MS frequency rises with increasing latitude. A female C57BL/6 mouse model for autoimmune encephalomyelitis (EAE) was used in this investigation to examine the associations between vitamin D supplementation, MS, and the vitamin D metabolizing CYP enzymes CYP27B1, and CYP24A1 at the protein expression levels. Initially, mice were separated into four groups: Gr 1 (Control), Gr 2 (Vitamin D supplemented control), Gr 3 (EAE – MS Model), and Gr 4 (vitamin D supplemented EAE – MS Model). The protein expression levels of Gr 1 were adjusted to 1.00 fold, and expression levels of the other groups were determined relative to Gr 1. No statistically significant difference was observed in mice brain CYP27B1 and CYP24A1 protein expressions among the groups. However, there was a slight decrease in the mice brain CYP24A1 protein expressions

of vitamin D supplemented groups (Gr 2 and Gr 4) compared to their control groups (Gr 1 and Gr 3). In conclusion, in the future both CYP24A1 inhibitor therapy and vitamin D supplementation might be tested out to assist reducing the intensity of MS symptoms.

Keywords: Vitamin D, Multiple Sclerosis (MS), Vitamin D metabolizing CYPs, Experimental autoimmune encephalomyelitis (EAE), Brain

ÖZ

D VİTAMİNİ UYGULANMIŞ MULTİPL SKLEROZ (MS) HASTASI FARELERİN BEYİN DOKULARINDA D VİTAMİNİ METABOLİZMASINDA YER ALAN CYP27B1 VE CYP24A1 ENZİMLERİNİN İFADELERİNİN ANALİZİ

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Merkezi sinir sisteminin inflamatuar demiyelinizan otoimmün bir hastalığı olan Multipl Skleroz'un (MS) etyopatogenezi hala bilinmemektedir. MS karmaşık, yinelenen ve sık sık ilerleyen bir hastalıktır. Multipl sklerozun güneş ışığına maruz kalmanın uzunluğu ve yoğunluğu ile D vitamini konsantrasyonları ile olumsuz ilişkili olduğu teorisi vardır, çünkü multipl skleroz sıklığı enlem arttıkça artar. Bu çalışmada, D vitamini takviyesi, MS ve D vitaminini metabolize eden CYP enzimleri CYP27B1 ve CYP24A1 arasındaki ilişkileri protein ekspresyon seviyelerinde incelemek için deneysel otoimmün ensefalomiyelit (DOE) için dişi C57BL/6 fare modeli kullanılmıştır. Çalışma kapsamında Gr 1'in protein ekspresyon seviyeleri 1.00 olarak kabul edilmiş ve diğer grupların ekspresyonları Gr 1'e göre belirlenmiştir. Başlangıçta fareler dört gruba ayrılmıştır: Gr 1 (kontrol), Gr 2 (D vitamini destekli kontrol), Gr 3 (DOE) ve Gr 4. (D vitamini takviyeli DOE). Gruplar arasında CYP27B1 protein ekspresyonunda istatistiksel olarak anlamlı bir fark gözlemlenmemiştir. Ayrıca, CYP24A1 protein ekspresyonu açısından da gruplar arasında istatistiksel olarak anlamlı fark tespit edilmemiştir. Bununla birlikte, D

vitamini takviyeli grupların (Gr 2 ve Gr 4) protein ekspresyonlarında kontrol gruplarına (Gr 1 ve Gr 3) kıyasla hafif bir azalma olduğu belirlenmiştir. Sonuç olarak, CYP24A1 inhibitör tedavisi ve D vitamini takviyesinin, MS semptomlarının yoğunluğunun azaltılmasına yardımcı olabilir.

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

This thesis work is dedicated to me.

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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
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
HS	High spin
IL	Interleukin
IU	International Unit
LBD	Ligand binding domain
LS	Low spin
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NBT	Nitrotetrazolium blue chloride
OD	Optical density
PBS	Phosphate buffered saline
PTH	Parathyroid hormone
PVDF	Polyvinylidene fluoride
PXR	Pregnane X receptor
RDI	Recommended daily intake

rpm	Revolutions per minute
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 elements

LIST OF SYMBOLS

SYMBOLS

Λ_{max}	Maximum absorption wavelength
ΔC_t	Threshold cycle difference
^oC	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 a chronic inflammatory disease of central nervous system characterized by inflammation and demyelination of neurons associated with varying degrees of axonal and neuronal wound that mostly result in acute physical or cognitive impairments as well as neurological problems in young adults (Gasemi et al., 2017). While it is certain that immune mechanisms and inflammation are crucial to the pathogenesis of MS, it is not clear if the inflammation is the first step in the pathophysiological cascade or it is a responsive event to an unknown infectious agent or intrinsic / primary CNS degeneration (Wootla et al., 2012). The primary cause of disease is mostly accepted as self-reactive immune cells gaining access and attacking components of the myelin sheath in the CNS (Rodriguez, 2009). However, the mechanism that activates, proliferates and leads to enter CNS is not known. Overall, MS is a complicated disease that results in activation of the adaptive and innate immune system, blood-brain barrier breakdown, central nervous system demyelination, and axonal and neuronal damage. As a result of these cascade of events, there are a variety of symptoms such as primarily motor and sensory disorders are mostly seen (Nourbakhsh and Mowry, 2019; Compston and Coles, 2008). MS disease has four different subtypes and the diagnosis is crucial for the progression of the disease and for the treatment choice. Subtypes are relapsing remitting MS (RRMS), primary progressive MS (PPMS), secondary progressive MS (SPMS), and progressive relapsing MS (PRMS). Apart from these, RRMS has the highest incidence rate (approximately 87%) among the other subtypes. It is

characterized by sudden acute attacks followed by duration of remission (Gasemi et. al., 2017).

MS etiopathogenesis is highly complex as other neurological diseases so that both genetic susceptibility and environmental exposures are studied. (Nourbakhsh and Mowry, 2019). MS disease is mostly seen in females compared to men with an approximately 3:1 (F:M) ratio (Dobson and Gjovannoni, 2018). Moreover, it is suggested that MS risk is increasing with latitude, where the latitudinal gradient is highly correlated with Ultraviolet B radiation (UVB) exposure. As UVB stimulates the synthesis of cutaneous vitamin D, it is thought that there might be a correlation between the level of serum vitamin D and MS risk. Based on that, low vitamin D levels, decreased intake of vitamin D, reduced outdoor activity, and genetic polymorphisms that interprets the vitamin D metabolism are correlated with MS susceptibility. MS-Vitamin D hypothesis that emerged in 1970s suggests vitamin D deficiency as a plausible risk factor for MS (Goldberg, 1974). Since then, several studies have emerged on the immunomodulatory properties of vitamin D and its relation to MS.

At first, vitamin D is thought to be a molecule that could not only diminish the symptoms but could also prevent the disease (Goldberg et al., 1986; Pierrot-Deseilligny et al., 2017). First hypothesis is aroused from Goldberg in 1974. He suggested that deficiency of vitamin D, calcium and magnesium could give rise to abnormal lipid composition and unstable myelin which could lead to MS development in the later adulthood of people with genetic susceptibility (Goldberg et al., 1974). In 1983, the discovery of vitamin D receptors (VDRs) on human immune cells gave rise to studies on immunomodulatory effect of vitamin D (Provyedini et al., 1983). Later, first clinical research was conducted and they found that vitamin D supplementation reduces the relapse rate by 50% comparing pre-treatment levels (Goldberg et al., 1986). In addition, in another study, it was found that serum vitamin D concentration in the relapse period was lower than in the remission period of patients with relapsing-remitting MS (RRMS) (Soilu-Hanninen et al., 2005). Moreover, it was shown that when in serum vitamin D level was

increased to 10 nmol/L, there was a 9-12% decrease in the risk of attack frequency in RRMS patients (Simpson et al., 2010). Despite of all studies, still significant result that confirms the validity of MS-Vitamin D hypothesis has not been achieved with the cause and the treatment of the disease.

Although the kidney is the main organ for the synthesis of circulating $1,25(OH)_2D$, cell types inside and outside of central nervous system (CNS) that involves T cells, B cells, monocytes, macrophages, dendritic cells, microglia, astrocytes, and neurons also carry out the hydroxylation into the active form of vitamin D (Provyedini et al., 1983; Eyles at al., 2005). As all of these cells comprise VDRs in both resting and activated forms, it is possible to study auto- and paracrine effects of vitamin D. It was shown that the inactive form of vitamin D (25(OH)D) has been encountered in cerebrospinal fluid and the upregulation of enzymatic cascade that activates vitamin D and VDRs has observed in active MS lesions (Smolders et a., 2013). Studies showed that vitamin D has anti-inflammatory effects in activated lymphocytes, myeloid cells, and glia and also homeostatic functions in neurons. Therefore, vitamin D is suggested to be a promoter for homeostatic state both in immune and CNS-resident cells (Smolders et al., 2011).

Since clinical studies have not been releasing significant success, Experimental Autoimmune Encephalomyelitis (EAE) MS animal models started to be used. As EAE model and MS share strong similarities, it has been used to study the mechanism and find possible treatments for MS. The study conducted by Cantorna et al., revealed that vitamin D has preventative effect on the development of MS disease in B10.PL mouse model of EAE (Cantorna et al., 1996). Also, another study showed that vitamin D has therapeutic effects on the symptoms of the disease even on the most severe period of acute EAE (Mayne et al., 2011). Even though, there are publications on the vitamin D supplementation in EAE model, the mechanism which involves vitamin D metabolizing enzymes in the brain that would reveal the relationship with MS is scarce.

1.2 Vitamin D

Vitamin D is a lipid-soluble vitamin that also has a mode of action like other steroid hormones. It can be synthesized in the body differing from other vitamins which should be taken from the outside. Importantly, it has impact on phosphorus and calcium metabolism and maintains bone health (Sintzel et al., 2018). There are two existing forms of vitamin D (Figure 1.1). The first one is called vitamin D_3 or cholecalciferol which can be produced in the skin upon sunlight or ultraviolet light exposure or could be taken by diet. The second one is ergocalciferol or vitamin D_2 and it is taken from the irradiation of plants or plant materials or foods. There is only one structural difference between vitamin D_3 and vitamin D_2 which is the fact that vitamin D_2 has an additional methyl group on 24th carbon. Vitamin D is synthesized from 7-dehydrocholesterol in the skin by ultraviolet B radiation (UVB; 290-315 nm), which photolysis the 7-dehydrocholesterol to form pre-vitamin D_3 (Lips., 2006). Therefore, synthesis of vitamin D depends on the concentration of 7-dehydrocholesterol, intensity and the angle of UV irradiation which all vary with season and latitude (Christakos et al., 2012).

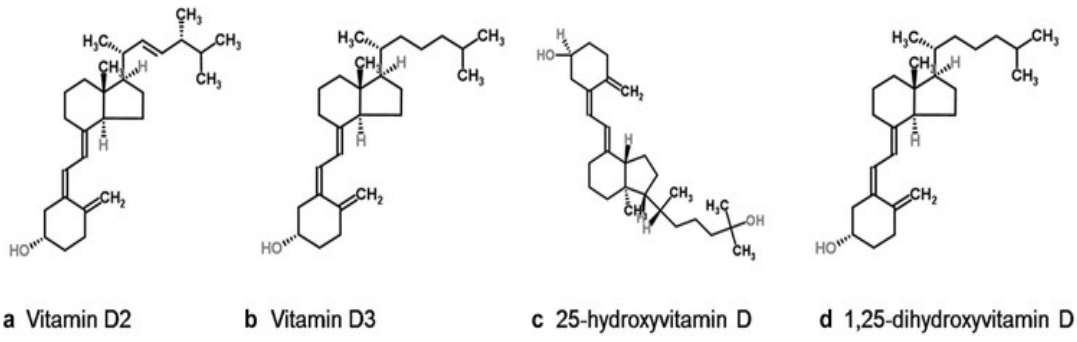

Figure 1.1 Chemical structures of the two main forms of vitamin D a) vitamin D_2 and b) vitamin D_3 and c) bioactive vitamin D_3 metabolite and d) calcitriol (Chemspider., 2017).

Vitamin D synthesis starts with photolysis of 7-dehydrocholesterol in the epidermis of the skin with UVB radiation to produce pre-vitamin D_3. Then pre-vitamin D3 isomerizes to vitamin D_3 so called cholecalciferol (Bikle, 2009). Then, vitamin D3, either produced by sunlight or taken by diet, is transported in the blood by vitamin D binding protein (DBP) to the liver. In the liver, biologically inactive cholecalciferol undergoes hydroxylation at 25th carbon to produce 25-hydroxyvitamin D ($25(OH)D_3$) so called calcidiol by one or more cytochrome P450 vitamin D 25-hydroxylases such as CYP2R1. Calcidiol which is the main circulating form of vitamin D, is then transported to kidney. In kidney, $25(OH)D_3$ undergoes 1α-hydroxylation to form 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) so called calcitriol which is the biologically active form of vitamin D. This second hydroxylation reaction is carried out by one of the cytochrome P450 enzymes called CYP27B1. In addition, in kidney calcitriol is also deactivated and eliminated via hydroxylation at 24th carbon to make 1,24,25-trihydroxyvitamin D_3 so called calcitroic acid by another cytochrome P450 enzyme (CYP24A1) in the kidney (Christakos et al., 2012). In addition, active vitamin D metabolite ($1,25(OH)_2D_3$) have a role in the regulation of CYP27B1 and CYP24A1 enzymes except other metabolites and hormones. It negatively regulates the CYP27B1 and also reciprocally regulate the CYP24A1 enzyme (Jones et al., 2012; Zierold et al., 1994). Figure 1.2 illustrates the main steps of the pathway for vitamin D metabolism in the brain (Gáll et al., 2021).

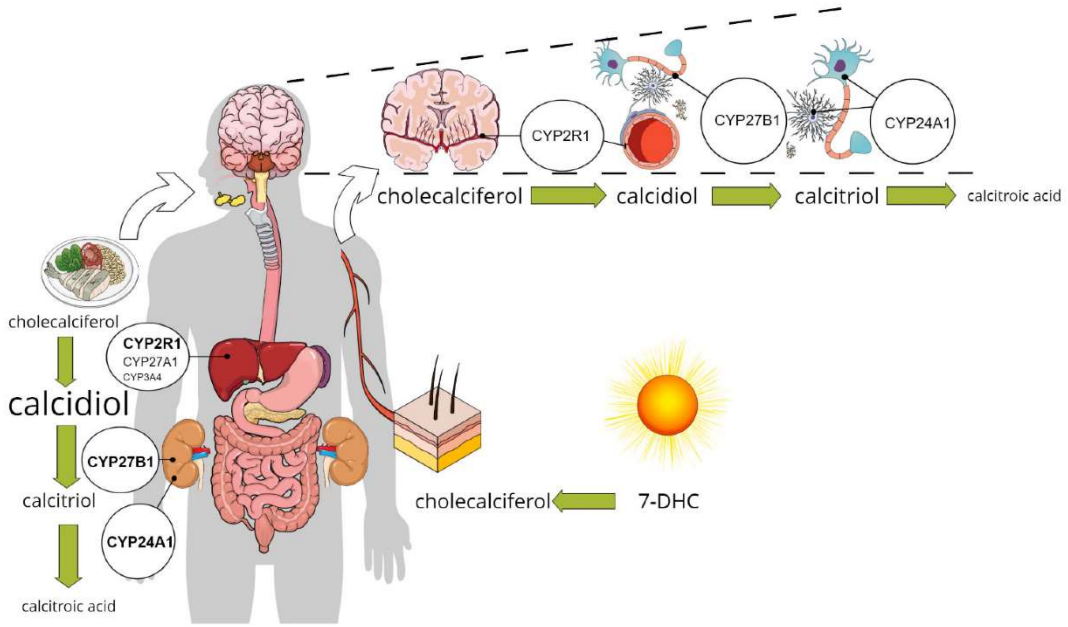

Figure 1.2 Vitamin D metabolism in the brain (Gáll et al., 2021).

Later, this active vitamin D metabolite ($1,25(OH)_2D_3$) released into the bloodstream and by binding to vitamin D binding protein it is transported to target tissues. Moreover, it binds to intracellular vitamin D receptor (VDR) to exert its biological effects (Yang et al., 2012). This binding then gather cofactors in order to form transcriptional complex which will bind to vitamin D response elements. This interaction would regulate the expression of at least 500 genes that participate in a variety of biological functions such as proliferation, apoptosis, cell differentiation, calcium homeostasis, and the immune response (Ascherio et al., 2010). Apart from the classical roles of vitamin D in calcium homeostasis, and bone health, it also has a regulatory function in immune system. Vitamin D deficiency is linked to many diseases like cancers, cardiovascular diseases, type 2 diabetes mellitus, infectious diseases, mental disorders, and autoimmune disorders such as type 1 diabetes mellitus, Crohn's disease, and MS. Since MS is an autoimmune disease, immune regulatory function of vitamin D plays a crucial role (Wacker et al., 2013; Hossein-nezhad and Holick, 2013). It has an essential part in lymphocyte activation and proliferation, T-helper cell differentiation, tissue-specific lymphocyte homing, the production of specific antibody isotypes, and regulation of the immune response

(Mora et al., 2008). Importantly, it promotes the differentiation of Th1 phenotype to Th2 phenotype (Bhalla et al., 1984; Boonstra et al., 2001; Mattner et al., 2000). An active vitamin D metabolite decreases the synthesis of inflammatory cytokines (IL-2, IL-17, and interferon-γ (IFNγ)) and decreases the cytotoxic activity and proliferation of CD4+ and CD8+ T cells. It prevents the B cell proliferation, plasma cell differentiation, and immunoglobulin production. In addition to these functions, active vitamin D metabolite diminishes the differentiation and maturation of dendritic cells which is important in the context of autoimmunity and self-tolerance. By preventing the maturation of Dendritic cells (DCs), tolerance is enhanced and autoimmune response against self-antigens is blocked as mature DCs induce antigen recognition of T cells and promote the immune response against antigens (Mora et al., 2008; Sintzel et al., 2018). As a result, vitamin D has a pivotal role in the cascade of events that would interpret with the mechanism of MS.

1.3 Cytochrome P450s

The cytochrome P450 (CYP) enzymes are membrane-bound superfamily of hemoproteins that have crucial parts in the detoxification of xenobiotics, cellular metabolism and homeostasis. Humans are subject to environmental chemicals, drugs, food additives, and pollutants on daily basis that could damage cellular metabolism with crucial harm on health. Defense system against these detrimental effects includes cytochrome P450 enzymes with pivotal roles (Manikandan and Nagini, 2018). Nomenclature of CYP term is attenuated as heme-containing proteins characterized by a maximum absorption wavelength of 450 nm in the reduced state in the presence of carbon monoxide (Omura et al., 1964). Cytochrome P450 enzymes are designated by 'CYP' letters followed with a numerical denotation for the superfamily (e.g., CYP24, and CYP27) and a capital letter (e.g., CYP24A, and CYP27B). Moreover, to specify the enzyme, another numerical is used (e.g., CYP24A1, and CYP27BA) (Guengerich et al., 2016; Nelson et al., 1996; Nelson et al., 2004). Therefore, CYP27B1 annotated as CYP family 27, subfamily of B and

enzyme 1 in the subfamily. In the family, amino acid sequences show 40% homology, however, within the subfamily this is raised to 55% (Coon et al., 1992). In humans, approximately 57 different genes and 58 pseudogenes are detected. Even though there are 18 different families and 44 subfamilies exist, only families of 1-3 are known to be hepatically active in metabolism of xenobiotics while the others participate in different endogenous functions. In fact, the whole family could be grouped into two major classes as some participating in detoxification of xenobiotics, and others involved in the biosynthesis of 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 (Porter et al., 1991).

Moreover, CYP enzymes are membrane-bound proteins and predominantly located in the endoplasmic reticulum and mitochondria which are mostly seen in eukaryotes. These enzymes are hemoproteins with 400-500 amino acid long sequences and single heme prosthetic group in the active site (Hill et al., 1970). In 1964, Omura and Sato concluded that the P450s are type-b hemoproteins which means they contain a protoporphyrin IX ring structure (Omura and Sato, 1984) (Figure 1.3).Iron is found in ferric form (Fe^{3+}) and it could exert two spin states as low spin (LS) in which five 3d electrons are maximally paired and high spin (HS) in which five 3d electrons are maximally unpaired. In substrate-free condition, LS state is stabilized by a water molecule. However, when a substrate binds to the enzyme, this iron-water complex is removed and then the iron Fe^{3+} changes the coordination structure from a six-fold to a five-fold coordination state so that Fe^{3+} could be released from the heme ring. Therefore, inhibitors are targeting Fe^{3+} and by binding to it, they are causing the removal of water molecule, and strengthening the LS state (Groves et al., 1998; Poulos et al., 1986).

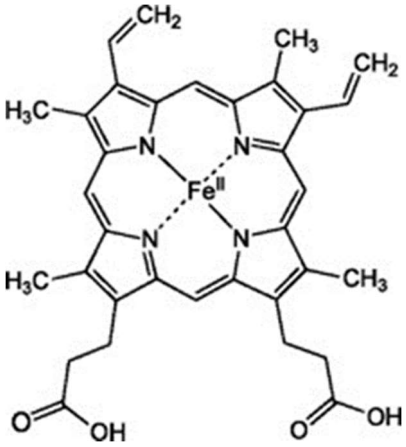

Figure 1.3 Type-b heme structure with protoporphyrin ring and an iron atom at the center (Omura and Sato, 1984).

CYPs are involved in a variety of oxidation and reduction reactions such as hydroxylation, heteroatom oxygenation, dealkylation, epoxidation, desaturation, and heme destruction (Figure 1.4). Classical catalytic cycle of CYPs start with binding of a substrate to the active site and then displacing the water molecule causes Fe^{3+} to change the spin movement, therefore, changing the LS state to the HS state. Then, electron transfer from NAPDH reduces the ferric CYP-substrate complex, so that Fe^{3+} reduced to Fe^{2+} by NADPH-P450 reductase enzyme. In the next step, O_2 binds to reduced enzyme-substrate complex and forms stable $Fe^{+3}O_2^-RH$ molecule which would be reduced to Fe^{3+}-O_2^{2-} by a second electron transfer from NADPH. After that, O-O bond is broken and H_2O is released in order to generate FeO^{3+} that would take H atom from the substrate and forms $FeOH^{3+}$-R. In the sixth step, OH group from $FeOH^{3+}$-R complex binds to the substrate and a hydroxylated product is generated. In the last step, product is released from the active site of the enzyme and the CYP becomes available for the other enzymatic reactions (Guengerich et al., 2008; Hrycay and Bandiera, 2015).

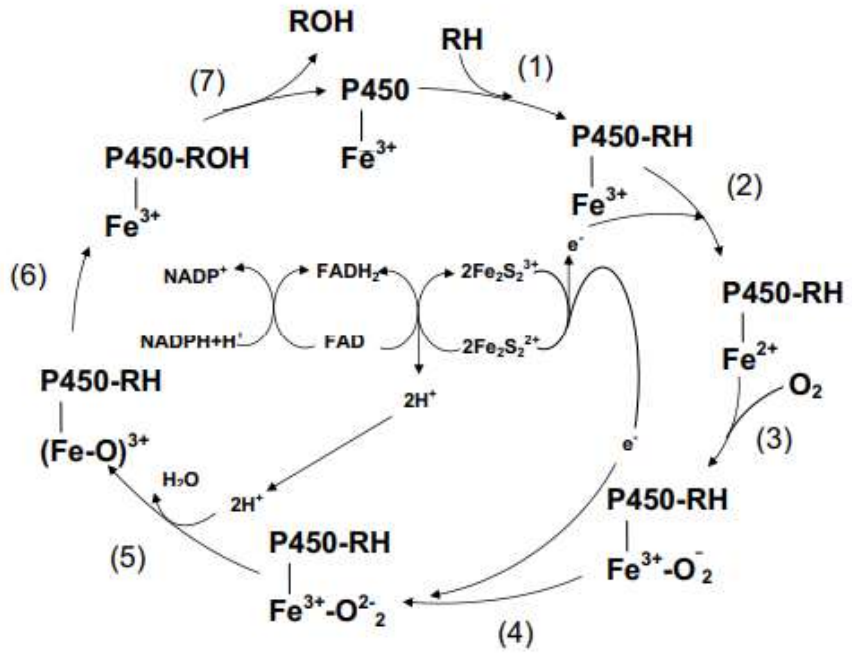

Figure 1.4 Cytochrome P450 enzymes' catalytic cycle (Manikandan and Nagini, 2018).

1.3.1 Vitamin D Metabolizing Cytochrome P450s

There are two major CYP isozymes that participate in the metabolism of vitamin D3 in the brain named as CYP27B1 (1α-hydroxylase) and CYP24A1 (24-hydroxylase).

1.3.1.1 CYP27B1

CYP27B1 belongs to mitochondrial (Type-1) cytochrome P450 class and mainly located in the proximal renal tubule of kidney, but, it is also found in different parts of human body including skin, brain, colon, prostate and breast. It carries out hydroxylation reactions by receiving electrons from NADPH via adrenodoxin reductase and adrenodoxin (Tang et al., 2010). CYP27B1 protein has a molecular mass of 56.5 kDa with an amino acid length of 508 (Fu et al., 1997; Yamamoto et al., 2004). Its 3D model of the structure shown in Figure 1.5 is proposed by

Yamamoto and colleagues. It is composed of 17 helices and 6 β-strands where F and G loops makes the entry site for the substrate. As a common feature to all cytochrome P450 enzymes, iron atom is present and is located in between the L and the I helices (Yamamoto et al., 2004).

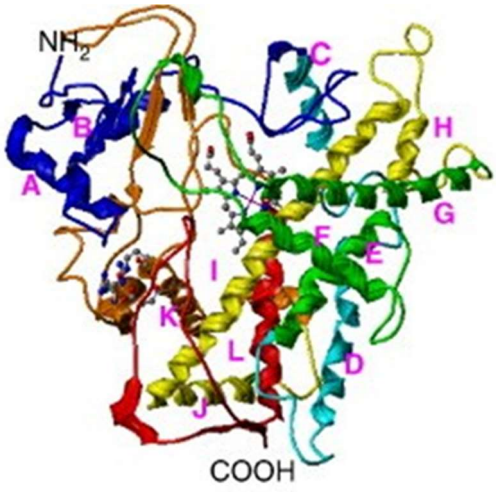

Figure 1.5 3D model of CYP27B1 that consist of ribbon loop structure and a heme group illustrated by ball and stick (Yamamoto et al., 2004).

In addition, CYP27B1 (25-Hydroxyvitamin D 1-hydroxylase) hydroxylates 25-hydroxyvitamin D3 (25(OH)D_3) in the 1-position producing 1,25 dihydroxyvitamin D_3 (Figure 1.6) (Sadie-Van Gijsen, 2019).

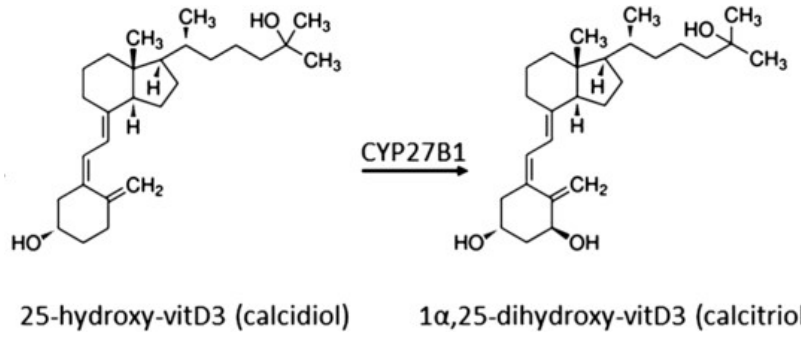

Figure 1.6 1α-Hydroxylation catalyzed by CYP27B1 (25-Hydroxyvitamin D 1-hydroxylase) that convert calcidiol into biologically active vitamin D form (calcitriol) (Sadie-Van Gijsen, 2019).

CYP27B1, apart from other functions in the body, it regulates the amount of biologically active form of vitamin D in the body. Therefore, dysregulation of this enzyme could bring out several diseases based on $1,25(OH)_2D_3$ level. For example, hypercalcemia and granulomatous disease are caused by excessive amount of circulating $1,25(OH)_2D_3$ (Chanakul et al., 2013). Therefore, CYP27B1 is also under the control of different factors. At transcriptional level, there are several transcription factors that control the transcription level of CYP27B1. One of them is reported as MAPK signaling via MEK/ERK1/2 and the other one cyclic AMP (cAMP) signaling pathway which controls the activation of MAPKs and also CYP27B1 gene expression by three cAMP response-element (CRE) sites in the promoter of the CYP27B1 (Brenza et al., 1998; Kong et al., 1999). Epidermal growth factor (EGF) and parathyroid hormone (PTH) are also participate in the pathway to control expression of CYP27B1 (T. C. Chen, 2015). In one study, it is found that expression of CYP27B1 mRNA was higher in active lesions of MS which concludes higher amount of biologically active $1,25(OH)_2D$ in order to exert anti-inflammatory effects of vitamin D (Smolders et al., 2013). Apart from the transcriptional level, CYP27B1 products and substrates also regulate its activity. Therefore, $1,25(OH)_2D_3$ has the ability to inhibit the enzymatic activity of CYP27B1 (Bikle, 2014).

1.3.1.2 CYP24A1

CP24A1 is a mitochondrial cytochrome P450 monooxygenase that catalyzes hydroxylation reactions at both carbons C-24 and C-23 of the side chain of hormonally active vitamin D metabolite ($1,25(OH)_2D_3$) to $1,24,25(OH)_3D_3$ mainly in kidney (Figure 1.7) (Jones et al., 2012).

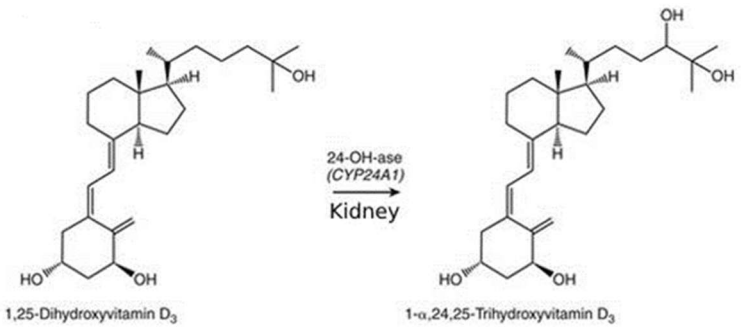

Figure 1.7 CYP24A1 catalyzes the hydroxylation of 1,25(OH)2D3 (Chemspider., 2017).

It has 58.9 kDa molecular mass with an amino acid length of 514. Its structure (Figure 1.8) consists of 12 α-helices (A-L) and four β-sheets (β1-β4), and substrate-binding site constructed by 2 β-sheets (β1 and β4) and 5 α-helices (E, F, G, I, and K). It has a heme at the center (Figure 1.8) (Annalora et al., 2010).

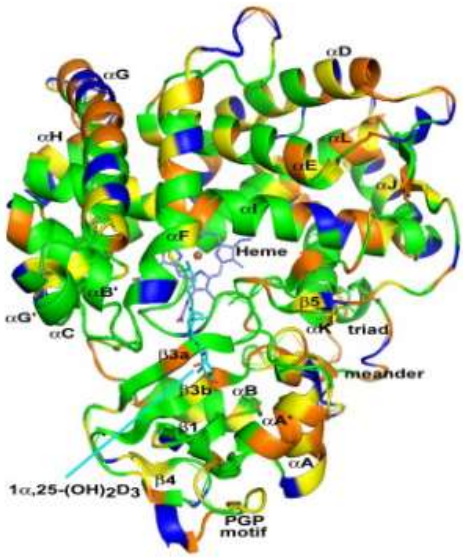

Figure 1.8 3D model of the structure of rat CYP24A1 (Jones et al., 2012).

In 1997, Akeno et al. concluded that CYP24A1 is expressed in the vitamin D target tissues mainly in kidneys, intestine, with smaller amounts present in skin, thymus, bone, lung, testis, spleen, pancreas, heart, and brain (Akeno et al., 1997). Moreover, it is under highly strict regulation like CYP27B1. CYP24A1 gene has two vitamin

D responsive elements (VDREs) in the promoter region. Active vitamin D metabolite when available makes a complex with VDR, then this complex can bind to RXR to recruit other activators to bind VDRE so that the promoter activity and gene expression of CYP24A1 is regulated at the transcriptional level (Meyer et al., 2007). CYP24A1 level is also regulated by $1,25(OH)_2D_3$. Its level could be upregulated by $1,25(OH)_2D_3$ in order to inactivate and eliminate the $1,25(OH)_2D_3$. The decrease in the amount of active vitamin D metabolite in turn reduces the CYP24A1 amount to basal level. Moreover, PTH and FGF-23 also regulates CYP24A1 (Petkovich and Jones, 2011). In fact, it is suggested that PTH inhibits renal CYP24A1 expression, on the other hand, induces renal CYP27B1 expression (Carpenter, 2017; Melo et al., 2020). It has been suggested that there are agents that can control CYP24A1 expression level. In one study, it is shown that AhR-ligand benzopyrene has the ability to induce vitamin D_3 synthesis by suppressing CYP24A1 (Matsunawa et al., 2009).

Similar to CYP27B1, dysregulation of CYP24A1 can induce several diseases. For example, increased expression and activity of CYP24A1 is linked to X-linked hypophosphatemia, chronic kidney disease, and type 1 diabetes. However, the major cause of these diseases is considered as being the decreased level of active vitamin D metabolite which is caused by increased level of CYP24A1 (Helvig et al., 2010; Hough et al., 1983; Tenenhouse et al., 1988). In one study, it is shown that tumor-derived endothelial cells represent reduced CYP24A1 gene expression. In addition to that study, several studies have shown that in various cancer types, tumor cells show elevated basal CYP24A1 expression. Those tumor cells, therefore, do not respond to vitamin D supplementation. Also, in lung cancer, it is presented that there is tumor-specific CYP24A1 gene up-regulation (Friedrich et al., 2003). Therefore, there are several strategies to prevent rapid degradation of $1,25(OH)_2D_3$ in these diseases which are the use of selective CYP24A1 inhibitors and vitamin D analogs such as VID-400, SDZ 89-443, CTA091, and CTA018 (Sakaki et al., 2014).

1.4 Aim of the Study

Multiple sclerosis is a complicated, repetitive, and often progressive chronic autoimmune, inflammatory neurological disease of the central nervous system (CNS). MS incidence increases with increasing latitude which negatively correlates with duration and intensity of sunlight and vitamin D concentrations. Even though there are many studies support MS-Vitamin D hypothesis, these studies weak to support the hypothesis as biochemical and molecular point of view is nearly absent. For now, literature is restricted to cell culture and mRNA expression studies which are insufficient to investigate regulation mechanism of vitamin D metabolizing enzymes as modifications at transcriptional and translational level could not be studied. Moreover, *in vitro* studies to understand the vitamin D metabolism is not a preferable way as the main sites of vitamin D metabolism are liver, and kidney. Although, there are studies approaching liver and kidney, brain is neglected even though all immune attacks and immune regulation occur in there. Moreover, there is no study examining the effects of vitamin D on the clinical course of MS and main metabolizing CYP450 enzymes at the protein level in the brain by using the Experimental Autoimmune Encephalomyelitis (EAE) mouse model.

In this study, effect of vitamin D on two major vitamin D metabolizing enzymes (CYP27B1 and CYP24A1) at the protein level with relation to the course of MS by using biochemical approach in the brain tissue of EAE mouse model is investigated. To the best of our knowledge, this is the first study investigating the effects of vitamin D on MS at the brain level. Consequently, we believe, this study might reveal a new point of view on immune regulatory function of vitamin D in the context of vitamin D metabolizing enzymes in the brain. In addition, this research may provide new findings that could explain MS progression and give rise to new approaches for the treatment of the disease

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 KitTM MOG35-55/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 chloride (NaCl; 1.06400), and sodium hydroxide (NaOH; 06462) were the products of E. Merck, Darmstadt, Germany.

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

Acetone (67-64-1) was obtained from DOP Organik Kimya, Ankara, Turkey.

Isopropanol (As040-L50) was the product of Atabay, Istanbul, Turkey.

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 CYP27B1 (sc-67260) and CYP24A1 (sc-66851) antibodies were purchased from Santa Cruz (Santa Cruz, CA).

Thermo Scientific™ PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa, was purchased from Thermo Fisher Scientific, Waltham, Massachusetts, USA.

2.2 Animal Studies

This thesis work is a continuation of the study of Dr. Emre Evin's Ph.D. thesis, therefore, procedures used present similarities (Evin E., 2021). All procedures of animal studies of this thesis work were approved by Bilkent University Animal Experimentation Ethics Committee. EAE model is constructed by using 10-12 weeks old female C57BL/6 mice weighing 20-25 g. Production and housing is carried out at the Animal Experimental Unit in Bilkent University. All 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 – MS Model)	12
Experimental	Group 4 (EAE – MS Model + Vitamin D)	12

Vitamin D deficient diet is used to fed all mice. Vitamin D supplementation to Group 2 and Group 4 was given via 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., 2002, it was reported that daily food and water intake of C57BL/6 mice were approximately 4 g and 6 mL, respectively. 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 was supplemented Group 4 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

According to the manufacturer's recommendations, C57BL/6J mice (10–12 weeks old) were inoculated with the Hooke Labs' Kit (Hooke Labs Inc., #EK-2110). Each mouse received two subcutaneous injections totaling 200 µL of the MOG_{35-55}/CFA emulsion (myelin oligodendrocyte glycoprotein/complete Freund's adjuvant) (lower and upper back of mice). Then, after 2 hours and 24 hours following the immunization, each mouse received an intraperitoneal injection of pertussis toxin (80 ng in 100 mL PBS/animal). The mice in Groups 1 and 2 received the identical care as those in the vaccinated groups, with the exception that PBS was used in place

of the MOG_{35-55} peptide (PBS). Mice were tracked individually using ear tags, and they were watched every day for 30 days following vaccination.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 brain and other 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

Heart blood samples were taken at the start of the mice's sacrifice process by PBS perfusion. The blood samples of the mice were centrifuged at 2500 rpm for 5 minutes at 4 ºC to obtain serum. According to the manufacturer's instructions, the ELISA kit (ab213966, Abcam) was used to test the levels of serum $25(OH)D_3$. Both the standards and the samples underwent duplicate analyses. Each well received 90 µL of dissociation buffer, to put it briefly. Following that, 10 µL of the standards (0.5,

4.8, 24.4, 71.6, 279, and 1010 ng/mL) and samples were pipetted into the proper wells, and 10 µL of sample diluent solution was added to the wells for maximum binding and non-specific binding (NSB). 50 mL of the 1X conjugate and 50 mL of the conjugate diluent were pipetted into each well after 5 minutes of incubation at room temperature on a plate shaker. The plate was sealed and incubated at room temperature for an hour on a plate shaker. The plate was then washed three times with wash buffer before the wash buffer was aspirated. Each well was then filled with 200 µL of pNpp substrate solution, and the sealed plate was incubated at room temperature for 30 minutes on a plate shaker. 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

Following the perfusion of mice with PBS solution, the brain was removed. It was homogenized with a pestle in a ceramic mortar that was filled with liquid nitrogen to create homogeneity using the cryogenic grinding technique. The T-PER™ tissue protein extraction reagent was mixed with the 1% Halt™ protease inhibitor cocktail before use. The homogenate was then weighed at 50 mg, and 500 µL of the T-PER™ reagent were then added in a microcentrifuge tube. After that, a 5-minute centrifugation at 10,000 x g was performed on the homogenate-reagent mixture. The

whole protein-containing supernatant was collected after centrifugation and kept at -80 ºC until needed.

2.5 Determination of Protein Concentration

By using the BCA (Bicinchoninic Acid) assay, the protein concentrations of the total protein extract produced from the mouse brain samples were determined. The basic idea behind this procedure is that the protein reduces Cu^{+2} to Cu^{+} under alkaline conditions, which reacts with BCA and results in a color shift (Smith et al., 1985). The intensity of the color change, which is inversely related to protein concentration, can be detected by a spectrophotometer at a wavelength of 562 nm.

The samples' protein concentrations were determined using the PierceTM BCA protein assay kit in accordance with the instructions provided by the manufacturer. With a range of varied values (25, 125, 250, 500, 750, 1000, 1500, 2000 g/mL), bovine serum albumin was utilized as the standard. Every measurement, including the ones on the standards, samples, and blanks, was carried out twice. The 96 well plate's wells were filled with 25 µL of standards and samples. The working reagent (reagent A:B, 50:1) was then added to each well and stirred for 30 seconds on a plate shaker. The plate was sealed and heated to 37 ºC for 30 minutes. Using a microplate spectrophotometer (MultiskanTM GO, Thermo Fisher Scientific, Waltham, Massachusetts, USA), absorbance was measured after incubation at 562 nm. By deducting the OD_{562} of the blank from the OD_{562} of the standards and samples, one may determine the net OD562 of the standards and samples. The concentration of samples was calculated by plotting Net OD_{562} versus concentration of bovine serum albumin standards.

2.6 Determination of Protein Expressions by Western Blotting Technique

Effects of vitamin D and EAE immunization on protein expression of CYP27B1 and CYP24A1 in the brain of the mice were analyzed by the Western blot method

(Towbin et al., 1979). GAPDH was used as a loading control. Prior to western blotting, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). For the SDS-PAGE method, system of 4% stacking gel and 10% separating gel in a discontinuous buffer system were used and gels are prepared freshly. Components of separating and stacking gel solutions were given in Table 2.3.

Table 2.3 Components of separating and stacking gel solutions.

Components	Separating Gel Solution	Stacking Gel Solution
Monomer Concentration	10%	4%
Gel Solution	5 mL	650 µL
dH_2O	6,02 mL	3,05 mL
Separating Buffer	3,75 mL	-
Stacking Buffer	-	1,25 mL
10% SDS	150 µL	50 µL
10% APS	75 µL	25 µL
TEMED	15 µL	5 µL
Total Volume	15 mL	5 mL

The gel sandwich unit was prepared tightly so that there would be no space in between glass plates. Then, the solution was prepared according to the Table 2.3. From the 10% separating gel solution, 4250 µL was poured into the glass plates on the casting stand. Immediately, 1000 µL butanol was poured top of the separating gel solution in order to block the interaction with oxygen, so that polymerization process is accelerated with a flat gel surface. After polymerization of separating gel,

butanol was poured out and then 1500 µL of 4% stacking gel solution was dispense onto the separating gel, then 15 well comp was inserted right away. After the polymerization, the comb was taken out and 1X Electrophoresis Running Buffer (ERB) was added to the system. Wells were cleaned out with a syringe in order to prevent any air bubbles and gel particles if left which would prevent smooth running of samples. Vertical gel electrophoresis was performed using Mini-PROTEAN tetra cell mini trans blot module (Bio-Rad, Richmond, CA).

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 4X in the sample dilution buffer, which contains the following ingredients: pH 6.8, 0.25 M Tris-HCl, 8% SDS, 40% glycerol, 20% -mercaptoethanol, and 0.1% bromophenol blue. The samples were then incubated for three minutes on a heat block at 100 ºC. 20 µg of the samples were loaded into wells, while 3 µL of protein ladder was loaded as the 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 prewetted by submerging it in 100% methanol for 20 seconds after being cut to the same size as the gel. 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.

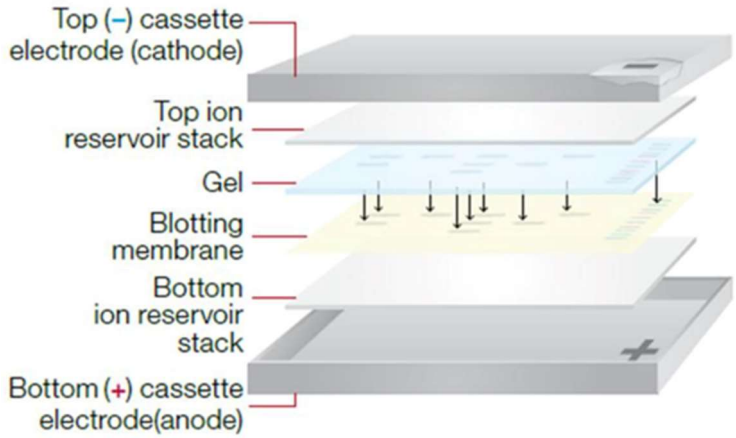

Figure 2.1 Preparation of western blot sandwich (Bio-Rad, 2021).

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 long. 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 long. 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 Lab visualization software developed by NIH.

Table 2.4 Primary and secondary antibody dilutions.

Protein	*1st Antibody*	*2nd Antibody*
GAPDH	1/5000	1/2000
CYP27B1	1/1000	1/2000
CYP24A1	1/500	1/2000

2.7 Statistical Analysis

The statistical software tool GraphPad Prism version 9 for Windows was used to conduct the statistical analysis. For multiple comparisons, one-way ANOVA (Tukey's test) was employed. Two groups were compared using the student's t-test. The mice's recovery was examined using the chi-square test. The level of significance was set at p0.05, and all data were reported as means with their Standard Deviations (mean SD).

CHAPTER 3

RESULTS

3.1 Animal Studies

This study is a continuation of work of doctorate thesis of Dr. Emre Evin, therefore, parts of results (section 3.1) includes information from the previous study (Evin E., 2021).

3.1.1 Examination and Clinical Scoring

Among 4 groups presented in "Methods" Table 2.1, just Gr 3 and Gr 4 were inoculated with MOG_{35-55} peptide. After the induction of the EAE mouse model, clinical scores were collected daily taken the immunization as a first day which was previously presented in "Methods" Table 2.2. Clinical scores were taken into the consideration in order to figure out if EAE model was successful. According to the results, EAE model construction was accomplished for all mice belonging to Gr 3 and Gr 4 (Evin E., 2021).

Moreover, Figure 3.1 shows the effect of vitamin D supplementation on EAE mouse MS model. There was no considerable change between Gr 3 and Gr 4 ($p= 0.8397$).

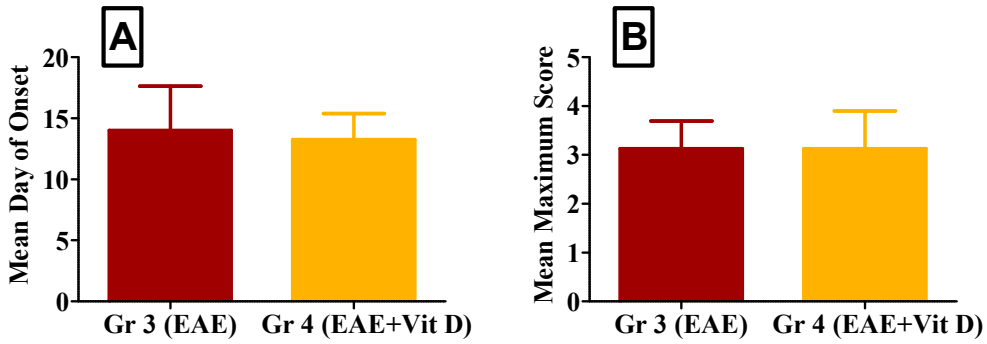

Figure 3.1 Comparison of mean day of onset (A) and mean maximum score (B) of Gr 3 (EAE-MS Model) and Gr 4 (EAE-MS Model + Vitamin D) (Evin E., 2021).

3.1.2 Serum Vitamin D Quantification

According to the manufacturer's instructions, the ELISA kit (ab213966, Abcam) was used to test the levels of serum 25(OH)D_3. Both the standards and the samples underwent duplicate analyses. By applying curve fitting with four parameters and standards of 0.5, 4.8, 24.4, 71.6, 279, and 1010 ng/mL vitamin D, the standard curve was created. Interpolating the standard curve yielded the mice's serum 25(OH)D_3 concentration (R2 = 0.9973). According to Figure 3.2, supplementation of vitamin D had a major effect as seen with Gr 2 and Gr 4 ($p<0.0001$).

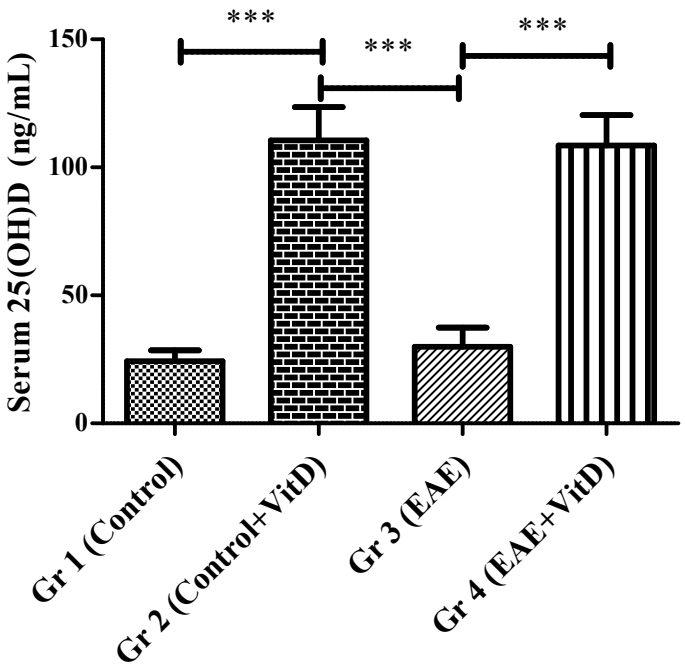

Figure 3.2 Blood serum 25(OH) vitamin D quantification of Gr 1 (6 mice), Gr 2 (6 mice), Gr 3 (12 mice), and Gr 4 (12 mice). Whole measurements were displayed as duplicates. ***Significantly different ($p \leq 0.001$) (Evin E., 2021).

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

Each mouse's clinical scores were monitored for 30 days beginning on the day of the EAE immunization. To ascertain the therapeutic impact of vitamin D supplementation, two groups (Gr 3 (EAE) and Gr 4 (EAE + Vitamin D)) were contrasted. Moreover, the outcomes were examined using the chi-square test. In Gr 3 (EAE), only two out of twelve mice were recovered, whereas in Gr 4 (EAE + vitamin D), eight out of twelve mice were recovered, and this difference was statistically significant ($p=0.013<0.05$) (Evin E., 2021).

3.2 Protein Concentration of Brain

In accordance with the instructions provided by the manufacturer, a Pierce™ BCA protein assay kit was used to measure the samples' protein concentrations. Different concentrations of bovine serum albumin (25, 125, 250, 500, 750, 1000, 1500, and 2000 g/mL) were employed as the standard. Every measurement, including the ones on the standards, samples, and blanks, was carried out twice. Figure 3.3 shows the total protein concentration in brain tissue homogenate.

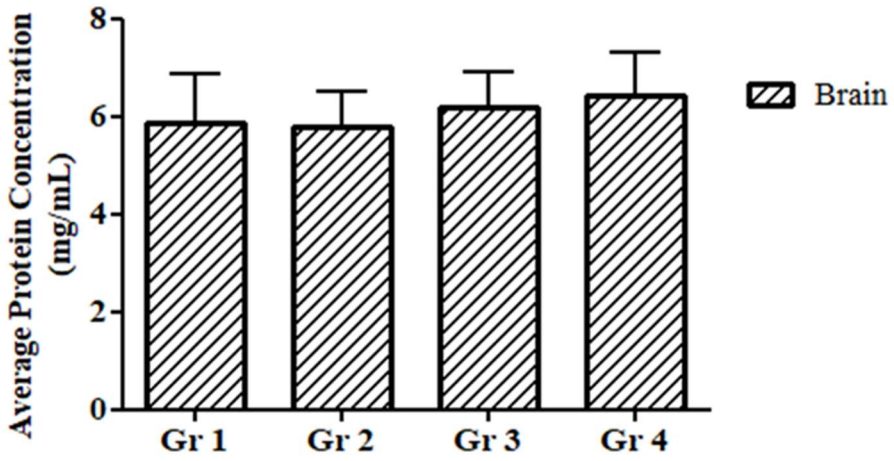

Figure 3.3 Protein concentrations of mouse brain homogenate.

3.3 Impact of EAE Immunization and Vitamin D Supplementation on Protein Expressions

By using specific antibodies and Western blotting technique, the effects of EAE vaccination and vitamin D supplementation on the expression of the vitamin D metabolizing CYPs (CYP27B1 and CYP24A1) in brain of female C57BL/6 mice were identified. The protein loading control was GAPDH. For the immunochemical detection of the GAPDH protein, primary rabbit monoclonal anti-GAPDH (1/10000

dilution) and monoclonal anti-rabbit alkaline phosphatase conjugated secondary (1/2000 dilution) antibodies were used. Image Lab program used an arbitrary measurement called relative peak area (RPA) to measure the intensity of each band. For Gr 1 (the control), this RPA was roughly adjusted to 1.00, and the protein expression of the other groups was estimated relative to Gr 1. One-way ANOVA was used to compare the protein expression of each group numerous times. The criterion of significance was set at $p<0.05$, and the quantifications were expressed as the mean SD of the relative protein expression from three independent experiments.

3.3.1 Impact of EAE Immunization and Vitamin D Supplementation on CYP27B1 Protein Expression in Brain

Western blotting was used to evaluate the expression of the brain CYP27B1 protein, which has a size of 56.5 kDa. Each well was filled with 20 μg of protein. For the immunochemical detection of the CYP27B1 protein, primary rabbit polyclonal anti-CYP27B1 antibodies (1/1000 dilution) and secondary anti-rabbit antibodies (1/2000 dilution) coupled to alkaline phosphatase (AP) were utilized (Figure 3.4/A). With the aid of the visualization program Image Lab, band intensities were calculated. The relative brain CYP27B1 protein expressions are shown in Figure 3.4/B. There was no significant difference in brain CYP27B1 protein expressions among each group.

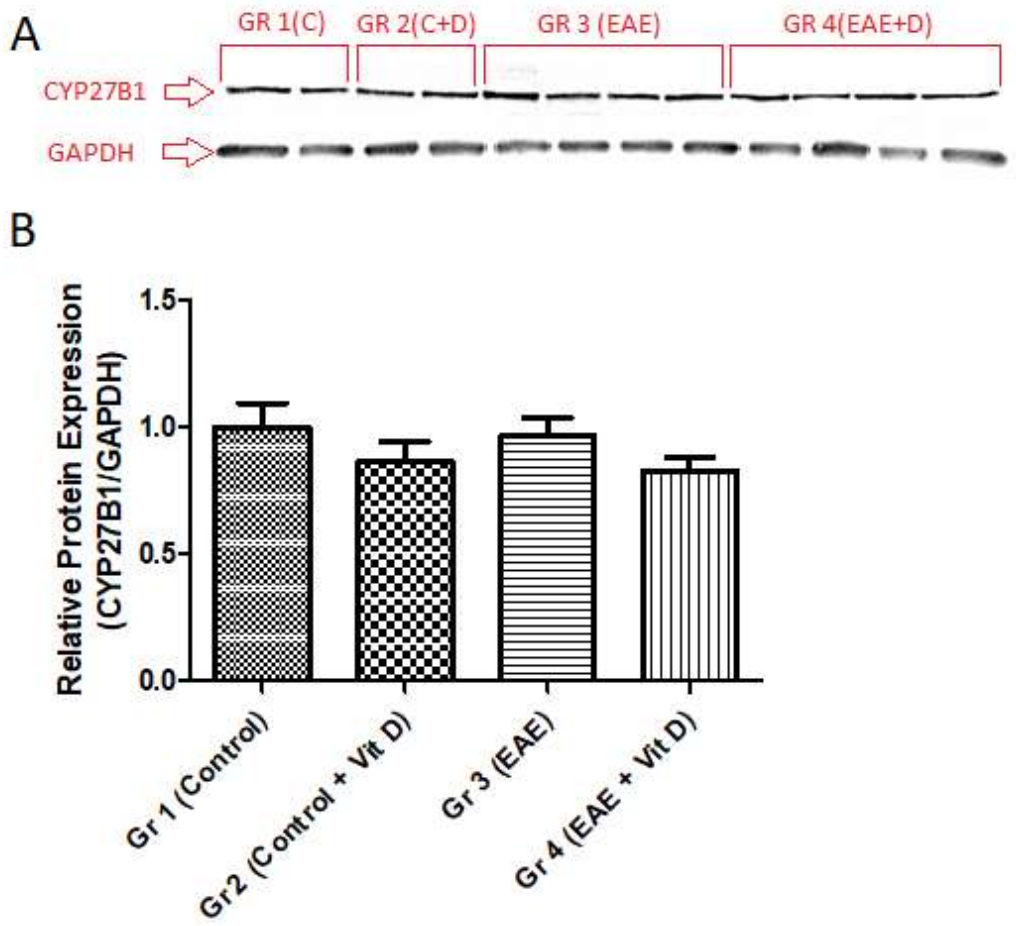

Figure 3.4 CYP27B1 protein expression levels of mouse brain in relation to EAE immunization and vitamin D supplementation. A) Exemplary immunoblot image of brain CYP27B1 in Gr 1 (Control), Gr 2 (Control + Vitamin D), Gr 3 (EAE), and Gr 4 (EAE + Vitamin D). B) Brain CYP27B1 protein expression comparison in the four groups. Experiments were repeated at least 3 times.

3.3.2 Impact of EAE Immunization and Vitamin D Supplementation on CYP24A1 Protein Expression in Brain

The brain expression level of the CYP24A1 (58.9 kDa) protein was assessed using the Western blotting method. Each well was filled with 20 μg of protein. For the immunochemical detection of the CYP24A1 protein, primary rabbit polyclonal anti-CYP24A1 antibodies (1/500 dilution) and secondary anti-rabbit antibodies (1/2000 dilution) coupled to alkaline phosphatase (AP) were utilized (Figure 3.5/A). With the aid of the visualization program Image Lab, band intensities were calculated. The relative protein expressions are depicted in Figure 3.5/B. The results were not statistically significant, despite the fact that vitamin D supplementation (Gr 2 and Gr 4) decreased brain CYP24A1 protein expression in comparison to the control groups (Gr 1 and Gr 3).

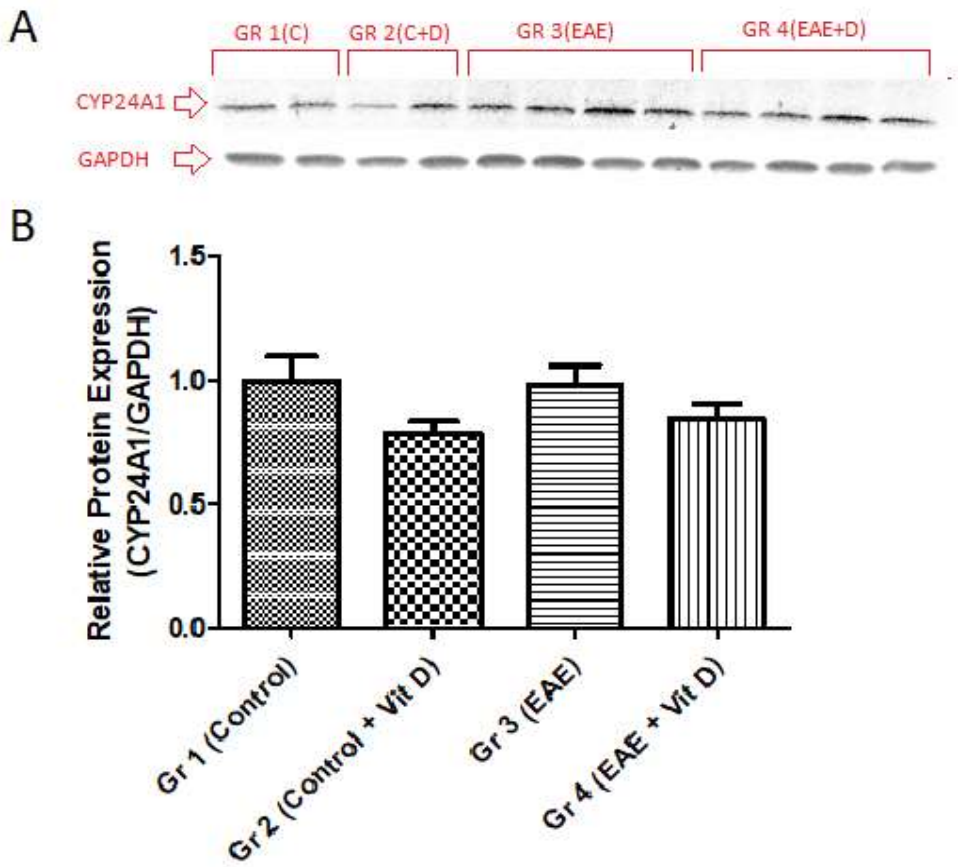

Figure 3.5 CYP24A1 protein expression levels of mouse brain in relation to EAE immunization and vitamin D supplementation. A) Exemplary immunoblot image of brain CYP24A1 in Gr 1 (Control), Gr 2 (Control + Vitamin D), Gr 3 (EAE), and Gr 4 (EAE + Vitamin D). B) Brain CYP24A1 protein expression comparison in the four groups. Experiments were repeated at least 3 times.

CHAPTER 4

DISCUSSION

Multiple Sclerosis (MS) is an inflammatory demyelinating autoimmune disease of the central nervous system that is complex, repetitious, and typically progressive (Noseworthy et al., 2000). Although the etiopathogenesis of MS is not fully understood, environmental factors promote genetically and immunologically based inflammation and demyelination in the myelin sheath (Ghasemi et al., 2017). The prevalence of MS disease was also shown to rise with distance from the equator, leading to the idea that MS is negatively linked with the length and intensity of sunshine exposure as well as vitamin D concentrations (Compston et al., 2008; Goldberg, 1974). The MS-Vitamin D theory has drawn more attention in the years since the discovery of immunomodulatory properties of Vitamin D. However, these research loose a biochemical and molecular viewpoint to fully explore this association, despite the fact that many studies demonstrate a strong inverse relation between MS and vitamin D. The literature also focuses only on mRNA and cell culture investigations, which are inadequate to comprehend the regulation machinery of enzymes that participate in vitamin D system because various alterations take place at the post-transcriptional and translational stages. Additionally, considering the brain as a primary site for vitamin D to exert its immunomodulatory effect on immune cells, an *in vitro* research of vitamin D metabolism is not a useful way to comprehend the connection between multiple sclerosis and vitamin D metabolizing enzymes. Moreover, no research has been done utilizing the EAE mice model to examine how vitamin D affects the clinical course of MS, and the effect of CYP450 enzymes of brain that metabolize vitamin D at the protein level. Therefore, by examining MS, vitamin D, and vitamin D metabolizing enzymes in the mouse EAE model using molecular methods, this study may fill a gap in the literature by examining the link between MS and vitamin D. Therefore, it is certain that two major

enzymes (CYP27B1 and CYP24A1) in the machinery of vitamin D should be examined in the brain to see the correlation with MS.

The immunomodulatory properties of vitamin D are widely established. Vitamin D in high levels decreases the percentage of immune cells that produce the inflammatory cytokine (IL-17), including CD4+ T cells (Pierrot-Deseilligny et al., 2017). Additionally, vitamin D administration promotes the production of the anti-inflammatory cytokine IL-10 while decreasing the release of pro-inflammatory cytokines from Th1 and Th17 cells, such as IL-12 and IL-23 (Bhargava et al., 2017; Häusler et al., 2019). Vitamin D supplementation has immunomodulatory effects in addition to causing oligodendrocyte lineage differentiation, which leads to an increase in remyelination in rats (Gomez-Pinedo et al., 2020). Moreover, the objective of this study was to explore the potential impact of vitamin D metabolizing enzymes on the development of MS.

As a secosteroid, vitamin D is known for its traditional effects on calcium and phosphorus balance and bone health (Omdahl et al., 2002; Zalewski et al., 2016). Despite the fact that humans can get some vitamin D through their diet, most of it is created in their skin by ultraviolet B radiation from the sun. Cholecalciferol, also known as vitamin D_3, and ergocalciferol, often known as vitamin D_2, are the two main forms of vitamin D that can be obtained from both plant and animal sources. Vitamin D is carried to the brain via the vitamin D binding protein (DBP) in the blood, whether it is produced in the skin or consumed through diet (Bouillon et al., 2020). In the brain, cytochrome P450 enzymes first hydroxylate vitamin D at its 25th carbon to create 25-hydroxyvitamin D ($25(OH)D_3$) (Sakaki et al., 2005). In addition, 25-hydroxyvitamin D ($25(OH)D_3$) undergoes 1α-hydroxylation by CYP27B1 enzyme to form biologically active 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Moreover, this metabolite could be inactivated and eliminated via hydroxylation at 24th carbon to make 1,24,25-trihydroxyvitamin D_3 by CYP24A1 enzyme.

In this study, protein expression levels of CYP27B1 and CYP24A1 enzymes were analyzed by Western blotting analysis. In the brain, $25(OH)D_3$ is converted into

$1,25(OH)_2D_3$ (calcitriol), a physiologically active form of vitamin D, by the action of cytochrome P450 (CYP27B1) enzyme (Holick et al., 1987). In order to determine if CYP27B1 plays a part in the development of MS, the regulation of CYP27B1 protein expression by vitamin D supplementation and EAE vaccination was examined. This was done since CYP27B1 plays a vital role in vitamin D activation. However, the levels of protein expression in any group did not significantly alter. Therefore, neither vitamin D supplementation nor EAE induction did not alter the CYP27B1 protein expression levels in the brain. In contrast to this outcome, higher levels of CYP27B1 protein expression should have boosted $1,25(OH)_2D_3$ synthesis, which might have resulted in a negative feedback system whereby calcitriol would has suppressed CYP27B1 expression (Brenza et al., 2000). However, it cannot be claimed that the groups receiving vitamin D supplements had high amounts of active vitamin D at the end of the trial because to the role of CYP24A1 in the breakdown and inactivation of $1,25(OH)_2D_3$ molecules. Additionally, the suppressive effects of the product can be offset by the upregulating effects of high substrate concentration. Since the EAE vaccination did not significantly alter the protein expression of CYP27B1 in the brain, it may be assumed that CYP27B1 may not be a key player in the explanation of the association between MS and vitamin D, in addition to the effects of vitamin D supplementation. However, it was noted in the literature that a decrease in CYP27B1 expression in tolerogenic dendritic cells (DC2) is linked to an increased risk of MS (Shahijanian et al., 2014). Morever, there are numerous studies in the literature that link the CYP27B1 enzyme to various disorders, in addition to MS. For instance, a research by Broyna et al. (2013) found that CYP27B1 expression declines as melanoma progresses. Another study found that schizophrenia patients had higher levels of CYP27B1 expression, which was thought to be a defense mechanism against the disease's potentially detrimental side effects (Manjili et al., 2018).

The active vitamin D metabolite ($1,25(OH)_2D_3$) which was produced by the CYP27B1 enzyme is inhibited and removed by the CYP24A1 enzyme by hydroxylating to $1,24,25(OH)_3D_3$ (calcitroic acid). Hormones and other metabolites,

as well as the active vitamin D metabolite ($1,25(OH)_2D_3$), strongly control CYP24A1 expression and activity (Jones et al., 2012; Zierold et al., 1994). The metabolite $1,25(OH)_2D_3$ promotes the expression of the CYP24A1 gene by binding to the VDR, it enables the receptor-ligand complex to connect with the VDRE in the promoter region of the CYP24A1 gene and recruit transcription factors (Meyer et al., 2020). For instance a study shown that CYP24A1 mRNA expression was upregulated in immune cells by $1,25(OH)_2D_3$ (Smolders et al., 2013). Therefore, it was assumed, in light of this knowledge, that the vitamin D-treated groups (Gr 2 and Gr 4) would exhibit higher CYP24A1 protein expression. However, in our results, there was a decrease in CYP24A1 protein expression in vitamin D supplemented groups (Gr 2 and Gr 4), but, it was not significant compared to control groups (Gr 1 and Gr 3). Therefore, this means that there was a suppression of CYP24A1 enzyme with vitamin D supplementation even though it was not at a considerable level. It seems that low doses of vitamin D supplementation would not be effective to suppress CYP24A1 enzyme significantly. In addition, post-transcriptional and post-translational regulations might be under the influence of vitamin D, therefore, investing the changes on those pathways would be beneficial. The initial goal of this work was to identify a potential target in the route of vitamin D metabolism in order to enhance its therapeutic benefits. Therefore, this enzyme is a possible target for the treatment of MS due to the link between the expression of the CYP24A1 protein and the clinical score of EAE.

Numerous studies in the literature link elevated CYP24A1 expression and activity to a wide range of diseases, including type 1 diabetes, chronic kidney disease, and cancer types such as human breast cancer and colorectal cancer (Helvig et al., 2010; Hough et al., 1983; Tenenhouse et al., 1988; Christakos et al., 2016). These diseases could be brought on by the increased rate of $1,25(OH)_2D_3$ breakdown by CYP24A1, which results in lower active vitamin D concentrations. Additionally, tumor cells that do not respond to treatment with $1,25(OH)_2D_3$ exhibit increased CYP24A1 expression (M. G. Anderson et al., 2006; Friedrich et al., 2003; N. King et al., 2012; Townsend et al., 2005). Moreover, there are recent studies in the literature using

CYP24A1 inhibitors in order to diminish the breakdown of $1,25(OH)_2D_3$. Therefore, based on the outcome of this study, these agents could be used in MS treatments with vitamin D supplementation.

CONCLUSION

One of the most prevalent autoimmune diseases of the central nervous system is multiple sclerosis (MS), although the cause of the disease's etiopathogenesis is still unknown. The likelihood of MS rises with latitude, leading to a theory that MS is negatively associated with vitamin D levels and sunlight length and intensity. Researchers from all around the world are working more intensively to gain insight on the etiopathogenesis of MS and the role that vitamin D plays in the disease. These studies, however, do not take a comprehensive strategy that takes into account vitamin D metabolizing CYP enzymes, and vitamin D supplementation in the EAE model. In this investigation, we examined the protein expression levels of two major vitamin D-metabolizing CYP enzymes, CYP27B1 and CYP24A1 in the brain in connection to vitamin D supplementation, MS, and these enzymes. Determining the roles of these enzymes in the etiopathogenesis of MS may help us to identify a viable target for the treatment of the disease because active vitamin D production, degradation, and function depend on them. Based on our findings, vitamin D can greatly aid in the recovery of mice with MS disease, even while it cannot prevent disease from developing. Additionally, since there is an adverse relation with the expression of the CYP24A1 protein and serum vitamin D levels, it is possible to boost the effectiveness of vitamin D in MS by targeting the CYP24A1 enzyme. Therefore, a CYP24A1 inhibitor that keeps high vitamin D concentrations in circulation and prevents the breakdown of active vitamin D may be useful in the treatment of multiple sclerosis. In fact, particular inhibitors for the CYP24A1 enzyme is being actively researched. It is necessary to look at the regulatory mechanism that underlies this regulation and identify new targets within it. It can be suggested to increase the amount of vitamin D in the blood. However, the negative effects of sustained high levels of circulation vitamin D should be considered.

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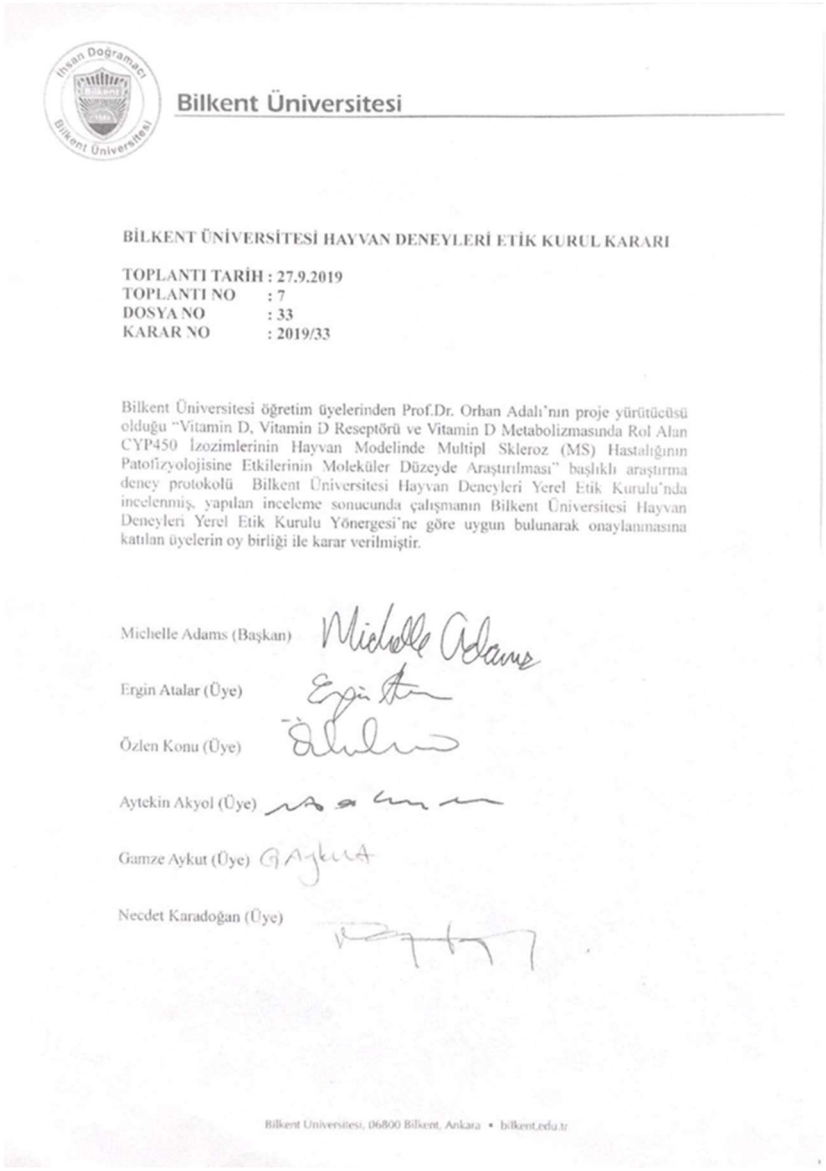
APPENDICES

A. Animal Experimentation Ethics Committee Approval Document



Bilkent Üniversitesi

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TOPLANTI TARİH : 27.9.2019
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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.

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