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A Study on Vitamin D Metabolic Genes Single Nucleotide Polymorphisms and Their Linkage in Adult Asthmatic Emiratis

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A STUDY ON VITAMIN D METABOLIC GENES SINGLE NUCLEOTIDE POLYMORPHISMS AND THEIR LINKAGE IN ADULT ASTHMATIC EMIRATIS

Amneh Mohammad Ahmad Al Mousa

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in Molecular Biology and Biotechnology

Under the Supervision of Dr. Youssef Abouzaid

April 2019

Declaration of Original Work

I, Amneh Mohammad Ahmad Al Mousa, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*A Study on Vitamin D Metabolic Genes Single Nucleotide Polymorphisms and their Linkage in Adult Asthmatic Emiratis*." hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Yousef Abu Zaid, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Declaration of Original Work

I, Amneh Mohammad Ahmad Al Mousa, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*A Study on Vitamin D Metabolic Genes Single Nucleotide Polymorphisms and their Linkage in Adult Asthmatic Emiratis*." hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Yousef Abu Zaid, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Vitamin D normal levels are vital to many biological processes including its classical role in calcium and phosphate homeostasis, and regulation of immune and non-immune cells. Vitamin D mediates its actions via the vitamin D receptor (VDR) which is expressed in most tissues of the body including the airway smooth muscle. Asthma is a syndrome of chronic inflammatory airway disease. There is a genetic and immunological link between vitamin D and asthma. In this study, we aim to investigate whether asthma and its severity were associated with single nucleotide polymorphisms (SNPs) in vitamin D metabolic genes as well as vitamin D levels in asthmatic adult Emiratis. We conducted a case-control study that included 132 adult asthmatic patients and 164 non-asthmatic controls of both sexes. Four SNPs in *VDR* gene (rs731236, rs7975232, rs1544410, and rs2228570), one SNP in vitamin D 25-hydroxylase gene (*CYP2R1*; rs12794714) and two SNPs in vitamin D binding protein gene (*GC*; rs4588 and rs7041) were genotyped using TaqMan Real-Time PCR genotyping techniques, whereas vitamin D levels were measured using electrochemiluminescence binding assay.

Results revealed a statistically significant association between *VDR*; rs7975232 polymorphism and asthma severity in the homozygous recessive model (OR = 2.7, 95% CI = 1.12-6.56, P = 0.03) and in allele model (OR = 1.74, 95% CI = 1.04-2.9, P = 0.032). The ACG haplotype for *VDR*; rs731236, *VDR*; rs7975232, and *VDR*; rs1544410 was significantly linked with asthma severity (P = 0.045). There was no association between any of the SNPs and mild asthma. There was no statistically significant difference in vitamin D levels between asthmatic patients and controls, and 56.6% of asthmatic patients with normal vitamin D levels were taking vitamin D supplementation. In conclusion, *VDR*; rs7975232 is significantly associated with asthma severity, and the CC genotype increases the risk of severe asthma by 2.7-fold. Moreover, the ACG haplotype of three SNPs in the *VDR* gene showed a significant association with asthma severity. *VDR*; rs7975232 polymorphism may be considered as a biomarker for asthma severity and possibly play a role in the management of asthma in adult Emirati patients.

Keywords: Asthma, Vitamin D, Single nucleotide polymorphisms, Case-control study, United Arab Emirates, Haplotype.

Title and Abstract (in Arabic)

دراسة النيوكليوتيدات الأحادية المتعددة الأشكال في جينات فيتامين (د) الأيضية ومدى ارتباطها بالربو في الإماراتيين البالغين

الملخص

تعتبر المستويات العادية لفيتامين (د) حيوية للعديد من العمليات البيولوجية بما في ذلك دوره التقليدي في تنظيم توازن الكالسيوم والفوسفات، بالإضافة إلى تنظيم الخلايا المناعية وغير المناعية. ويقوم بعمله بواسطة مستقبلات فيتامين (د) (VRD) التي يتم التعبير عنها في معظم انسجه الجسم بما في ذلك العضلات الملساء في الجهاز التنفسي. الربو هو متلازمة امراض المسالك التنفسية التهابيه المزمنة. يوجد هنالك ارتباط من ناحية مناعية وجينية بين فيتامين (د) والربو. في هذه الدراسة نهدف إلى التحقق فيما إذا كانت النيوكليوتيدات الأحادية المتعددة الاشكال (SNPs) في الجينات الأيضية لفيتامين (د) مرتبطة بحدة الربو وكذلك مستويات فيتامين (د) في مرضي الربو البالغين الإماراتيين. أجرينا دراسة السيطرة على الحالة التي شملت 132 من المرضى البالغين المصابين بالربو و164 من غير المصابين (كعينة ضابطة) من كلا الجنسين. تم عمل التنميط الجيني لأربعه SNPs في الجينات *VDR* (rs731236، rs7975232، rs1544410 وrs2228570)، وSNP واحد في جين 25-هيدروكليز فيتامين (د) (*CYP2R1*; rs12794714) واثنين من الـ SNPs في جينات فيتامين (د) المرتبطة بالبروتين *DBP* (rs4588 وrs7041) -باستخدام تقنيات التنميط الجيني TapMan PCR، في حين تم قياس مستويات فيتامين (د) باستخدام electrochemiluminescence.

أظهرت النتائج وجود ارتباط إحصائي كبير بين *VDR* rs7975232 وشده الربو في النمط المتنحي المتماثل (p = 0.03، OR = 2.7, 95% CI = 1.12-6.56) وفي نموذج أليل (P = 0.032، OR = 1.74, 95% CI = 1.04-2.9) وقد ارتبط النمط الفرداني ACG ل *VDR* rs731236و *VDR* rs7975232و *VDR* rs1544410ارتباطا كبيرا بشده الربو(p = 0.045) . لم يكن هناك اي ارتباط بين أي SNPs والنوبات المعتدلة من الربو. لم يكن هناك فرق كبير إحصائيا في مستويات فيتامين (د) بين المرضي المصابين بالربو والأشخاص السليمين لأن 56.6% من المرضى المصابين بالربو وكان مستوى فيتامين(د) طبيعي-كانوا يتناولون مكملات فيتامين (د). نستنتج مما سبق أن *VDR*; rs7975232 يرتبط بشكل ملحوظ مع شده الربو ويزيد من خطر الربو الحاد 2.7 اضعاف مع وجود النمط الجيني.CC علاوة على ذلك، فان النمط ACG الفرداني لثلاثة SNPs في الجينات مستقبلات فيتامين (د) أظهرت ارتباطا كبيرا مع شده الربو. يمكن اعتبار *VDR* rs7975232 كمؤشر حيوي لشده الربو وقد يلعب دورا في السيطرة على الربو في المرضى الإماراتيين البالغين.

الكلمات المفتاحية: الربو، فيتامين (د)، دراسة رابطة-المرض، النيوكليوتيدات الأحادية المتعددة الاشكال، دراسة حالات-ضوابط، الأمارات العربية المتحدة، النمط الفرداني.

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Dedication

To my beloved husband and my children Ahmad and Malak who supported me and fill my life with happiness and joy

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List of Abbreviations

1α,25(OH)2D	1,25-dihydroxy vitamin D
25(OH)D	25-hydroxy vitamin D
7-DHC	7-dehydrocholesterol
AIF	Assay information file
CD40	Cluster of differentiation 40
CYP24A1	Cytochrome P450 family 24 subfamily A member 1
CYP27B1	Cytochrome P450 family 27 subfamily B member 1
CYP2R1	Cytochrome P450 Family 2 Subfamily R member 1
DBP	Vitamin D-binding protein
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
FAM	Fluorescein amidite
FEV1	Forced expiratory volume in the first second
GINA	Global Initiative for Asthma
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
IQR	Interquartile range
MAF	Minor allele frequency
MGB	Minor groove-binder
MHC-II	Major histocompatibility complex II
nmol/l	Nano-mole per liter

NTCs	No template controls
qPCR	Quantitative polymerase chain reaction
SD	Standard deviation
SKMC	Sheikh Khalifa Medical City
SNP	Single nucleotide polymorphism
TNFα	Tumor necrosis factor
UAE	United Arab Emirate
VDR	Vitamin D receptor
χ^2	Chi-square

Chapter 1: Introduction

1.1 Overview

Vitamin D is a fat-soluble steroid pro-hormone; it is made in the skin under the effect of ultraviolet light. It has several roles in many biological actions in our bodies, such as calcium and phosphate homeostasis, cell differentiation, proliferation, apoptosis, anti-inflammatory, and anti-microbial. Vitamin D makes the immune system smarter through its immunomodulatory effect on immune cells. Vitamin D actions are mediated via vitamin D receptor (VDR); a protein that expressed in most cell types including the airway smooth muscle (Hall & Agrawal, 2017). Vitamin D deficiency is one of the most prevalent health problems worldwide even in sunny countries (Wahl et al., 2012).

There is a group of genes that are involved in vitamin D metabolism. The most popular genes which have been studied in genetic polymorphism and their association with vitamin D levels are: vitamin D-binding protein gene (*DBP*; *GC*), vitamin D receptor (*VDR*), cytochrome P450 family 2 subfamily R member 1 (*CYP2R1*), cytochrome P450 family 24 subfamily A member 1 (*CYP24A1*), and 7-dehydrocholesterol reductase (Sadat-Ali, Al-Turki, Azam, & Al-Elq, 2016). In this study, four *VDR* gene polymorphisms (rs731236, rs7975232, rs1544410, and rs2228570), one vitamin D 25-hydroxylase gene polymorphism (*CYP2R1*; rs12794714) and two vitamin D binding protein gene polymorphisms (*GC*; rs4588 and rs7041) were genotyped.

Asthma is a complex inflammatory airway disorder and one of the most prevalent chronic respiratory diseases worldwide. It is characterized by elevated serum

immunoglobulin E (IgE) and airflow obstruction and bronchial hyper-responsiveness that leads to symptoms of wheezing, coughing, and dyspnea (Wenzel, 2006). It affects around 385 million people and cause the annual death of 0.4 million people worldwide (Soriano et al., 2017), and expected to affect 400 million people by 2025 (Ober & Yao, 2011). In general, the prevalence of allergic asthma is more than non-allergic asthma (Backman et al., 2017). In Arabic territories, about one –third of asthma patients in the Middle East and North Africa are still uncontrolled (Tarraf et al., 2018). Studying asthma and its related diseases began around 1900 (Cookson, 2004). In the following decades, more studies were done on asthma with more emphasis on genes related to asthma and its prevalence (Ober & Yao, 2011). A study in 1994 reported that the prevalence of asthma among children in the United Arab Emirates (UAE) was about 13.6 % (Bener, Abdulrazzaq, Debuse, & Al-Mutawwa, 1994). Another study in Al Ain reported a prevalence of asthma of 16% in adolescents and 12 % in adults (Alsowaidi, Abdulle, & Bernsen, 2010). On the other hand, 70% of the asthmatic patients in the UAE were under control (Hassan Mahboub, Santhakumar, Soriano, & Pawankar, 2010).

Many studies were reported worldwide on vitamin D levels and its metabolic genes and their associations with other diseases. In the UAE, there are studies about vitamin D and its related genes and its association with other conditions like diabetes (Al Safar et al., 2018) and obesity (Khan, Chehadeh, Abdulrahman, Osman, & Al Safar, 2018). To our knowledge, this study is the first investigating the association between asthma and single nucleotide polymorphisms (SNPs) in vitamin D metabolic genes as well as vitamin D levels in adult Emiratis. Genetic association studies play significant roles in understanding complex diseases like asthma and are considered as

the first step in studying the effect of SNPs on disease outcomes (Kumar & Ghosh, 2009).

We hypothesize that some SNPs in vitamin D metabolic genes are associated with asthma and may serve as biomarkers of the disease in asthmatic Emirati adults. Therefore, the primary objective of this study was to investigate if polymorphisms in vitamin D receptor (*VDR*), vitamin D binding protein *(DBP*) and vitamin D 25-hydroxylase (*CYP2R1*) genes are associated with asthma and its clinical severity. The secondary objectives was to study the linkage disequilibrium in the polymorphisms of vitamin D receptor and vitamin D binding protein genes in asthmatics and controls. Moreover, to investigate if the clinical asthma was associated with vitamin D levels in asthmatic Emirati adults.

1.2 Vitamin D

Vitamin D deficiency is one of the most prevalent health problems worldwide even in sunny countries (Wahl et al., 2012). A recent study in the United Arab Emirates (UAE) showed that vitamin D deficiency is more common in adult Emiratis compared with expatriates (Bani-Issa, Eldeirawi, Harfil, & Fakhry, 2017). The difference between the two populations was previously compared in Dubai (Yammine & Al Adham, 2016) and Al-Ain (Muhairi et al., 2013). On the other hand, another study in 2016 confirmed that vitamin D deficiency is common in all the multiethnic population of the UAE (Sridhar, Rao, Multani, & Jain, 2016). Vitamin D receptor was claimed to be a significant factor causing diseases related to vitamin D deficiency (Dastani, Li, & Richards, 2013).

There are two primary sources for vitamin D; Plants [Vitamin D2 (ergocalciferol)] and dermal [Vitamin D3 (cholecalciferol)] which is synthesized in

the skin from 7-dehydrocholesterol (7-DHC) under non-enzymatic process after exposure to ultraviolet B (UVB) (spectrum 280-320 nm) (Dastani et al., 2013). Vitamin D undergoes three significant hydroxylation steps, 25-hydroxylation, 1α-hydroxylation, and 24-hydroxylation. 25-hydroxylation occurs in the liver by 25-hydroxylase to produce 25-hydroxyvitamin D (25(OH)D), the most circulating form of vitamin D. 1α-hydroxylation occurs in kidney, under the influence of 1α-hydroxylase [known as cytochrome P450 family 27 subfamily B member 1 (CYP27B1)] to produce the active hormonal form 1,25-dihydroxy vitamin D (1,25(OH)2D or calcitriol). Finally, both 25(OH)D and 1,25(OH)2D are catabolized by 24-hydroxylase [known as cytochrome P450 family 24, subfamily A member 1 (*CYP24A1*)], which control vitamin D level and prevent the accumulation of vitamin D toxic levels (Bikle, 2014).

The most popular vitamin D related genes studied for genetic polymorphism and its association with vitamin D levels are presented in (Figure 1)*, including vitamin D-binding protein gene (*GC*), vitamin D receptor (*VDR*), *CYP2R1*, *CYP24A1*, and 7-dehydrocholesterol reductase. Vitamin D level is affected by skin pigmentation, age, gender, obesity, sun exposure and season, in addition to the genetic factors (Sadat-Ali et al., 2016).

* Figure 1 was adapted from Figure 1 in reference (Bossé et al., 2009) with few changes.

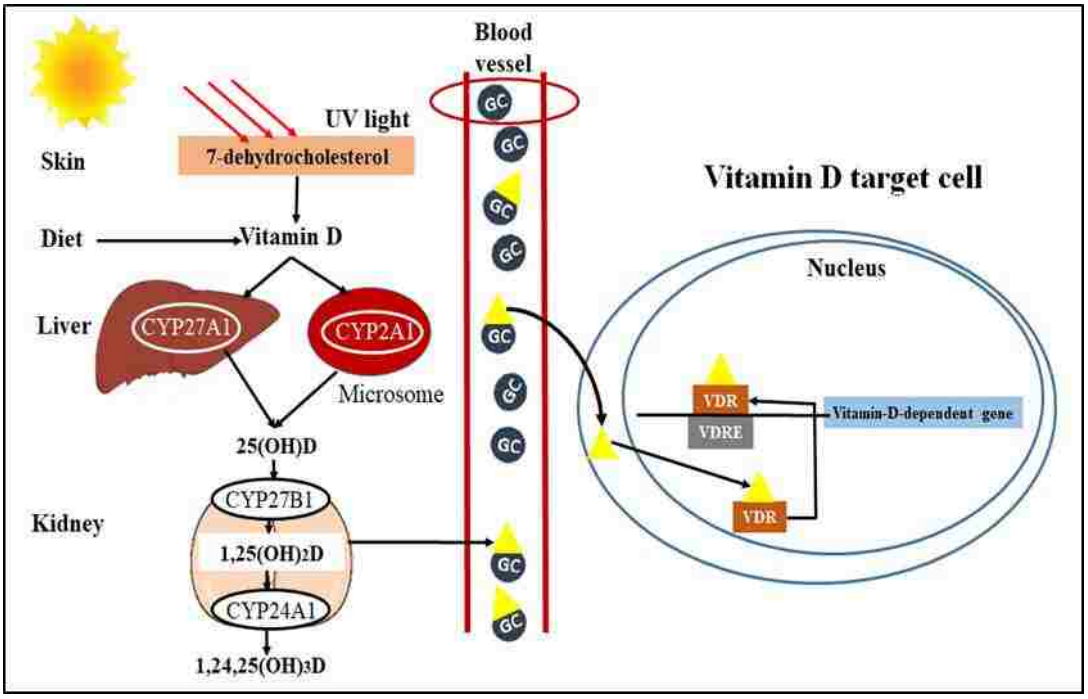

Figure 1: Genes involved in the vitamin D pathway

1.2.1 The Role of Vitamin D in Immunity

In addition to the classical role of vitamin D, it has an essential role as immuno-regulatory and anti-inflammatory, through its regulation on the growth and differentiation of innate and adaptive immune cells and non-immune cells (Figure 2) 1,25(OH)2D is produced in both immune and non-immune cells. Therefore, immune cells such as dendritic cells, macrophages, and T or B cells can hydroxylate 25(OH)D to 1,25(OH)2D. It means that these cells express CYP27B1. The hormonal actions of 1,25(OH)2D are mediated by VDR (Adorini & Penna, 2008; Mora, Iwata, & Von Andrian, 2008).

In innate immune systems, 1,25(OH)2D inhibits the differentiation and maturation of dendritic cells. In addition to decreasing the capacity of dendritic cells

as an immune-stimulatory factor by downregulating the expression of CD40, CD80, and CD86 as well as major histocompatibility complex-II (MHC-II) molecules. It also decreases the production of IL-12 and enhances IL-10 production, resulting in reducing T- cell activation as well as T helper-1 development (Adorini et al., 2004; Barragan, Good, & Kolls, 2015). A novel finding that vitamin D has an anti-microbial effect against inhaled pathogens, by increasing the expression of cathelicidin in the bronchial epithelial cells (Iqbal & Freishtat, 2011; Yim, Dhawan, Ragunath, Christakos, & Diamond, 2007). Also, it was found that the 1.25(OH)2D, as well as cathelicidin, are increased in the keratinocytes as a response to injury (Schauber et al., 2007). The above gives more evidence that vitamin D has a vital role in the innate immune system.

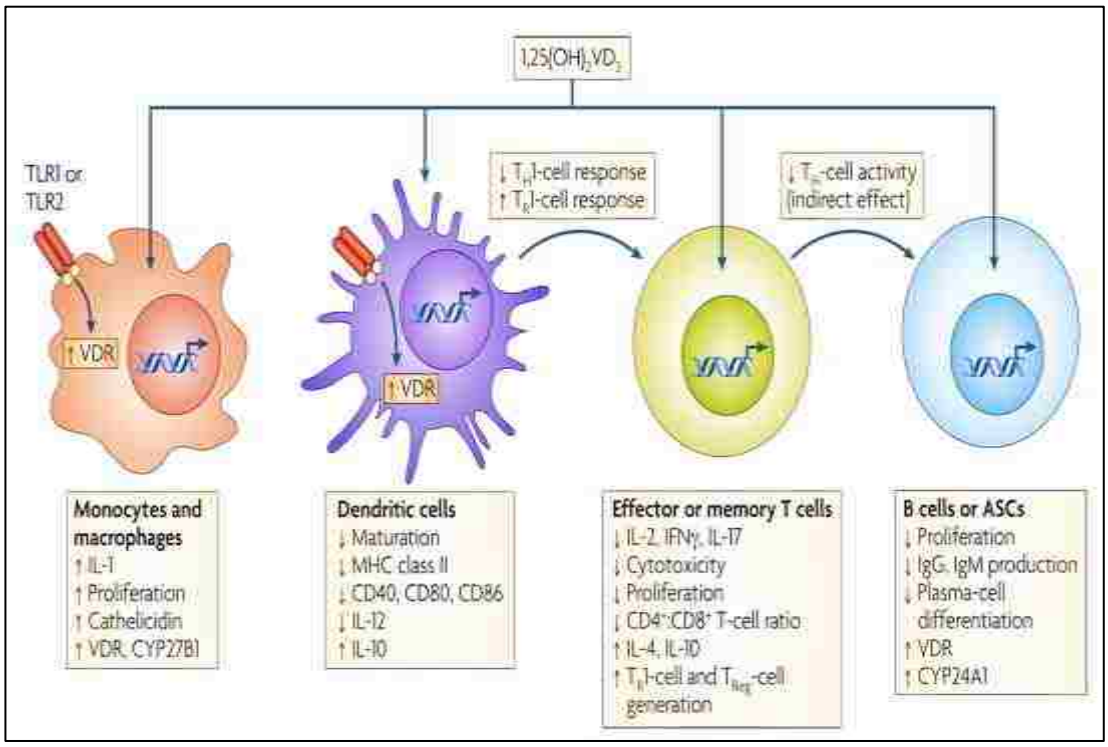

Figure 2: Mechanisms of vitamin D immunomodulation (Mora et al. 2008)

In adaptive immune system, vitamin D decreases plasma cell differentiation, IgG secretion and B cells proliferation, as well as its regulatory effect on CD4+ T cells differentiation by inhibiting the cell differentiation of both T helper-1 and T helper-17 and stimulate T helper-2 and T regulatory cell differentiation (Kongsbak et al., 2014). As the reflection of the anti-inflammatory effect of vitamin D, its deficiency shows association with an autoimmune and hypersensitivity diseases like type 1 diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus and asthma (Sun, 2010; Van Belle, Gysemans, & Mathieu, 2011). A study that examined the anti-inflammatory effects of 1,25(OH)2D3 in human airway smooth muscle (ASM) cell culture that treated with 1,25(OH)2D3, found that vitamin D modulates the expression of chemokines in ASM. However, the role of vitamin D as immune-regulatory in asthma remains unclear (Banerjee et al., 2008).

1.2.2 Single Nucleotide Polymorphisms (SNPs)

SNP is defined as the substitution of a single nucleotide in genome sequence with a frequency of greater than 1% at least in one population, SNP is considered as the most common genomic variation (Castle, 2011; Chanock, 2001; Wright, 2005). The human genome contains approximately 35,000 genes, and any two human genomes differ by about 0.1% (Chanock, 2001). There are 6 000 000 000 nucleotides and 3–10 million SNP variants in the human genome; it means that one nucleotide substitution every 1250 nucleotide base pairs (Wright, 2005).

There are two types of SNPs in coding regions; synonymous or silent SNPs that does not change the amino acid sequence and non-synonymous SNPs that cause change in amino acid substitution and can change the function of a protein, non-synonymous SNPs less common than synonymous SNPs in the coding region (Bali &

Bebok, 2015; Chanock, 2001; Wright, 2005). Synonymous mutations may occur in coding (exonic) and non-coding (intronic) regions (Bali & Bebok, 2015).

A study found that low SNPs rate occur in the sequences with high genomic sequence conservation like exon splice sites, polyadenylation sites and start codon, SNPs are more common in intron than exon regions (Castle, 2011) while the occurrences of synonymous codons differ within genes of the same genome and between species (Bali & Bebok, 2015).

Although nonsynonymous mutation within exons may have an impact on developing many diseases, a synonymous mutation also influence many diseases, mainly complex diseases like diabetes and cancer, because it is found that it might affect protein expression, conformation and function and mRNA splicing and stability (Bali & Bebok, 2015; Sauna & Kimchi-Sarfaty, 2011; Shabalina, Spiridonov, & Kashina, 2013). A study by Chen and coworkers found that non-synonymous and synonymous SNPs have similar effect size for disease association (Chen, Davydov, Sirota, & Butte, 2010).

1.2.3 Vitamin D Receptor

Vitamin D receptor (*VDR*) was discovered in 1969, cloned, and sequenced in 1987. It is expressed in virtually all cells of the body (skeletal and non-skeletal) (Rosen et al., 2012). *VDR* is a large pleiotropic gene. It regulates the expression of more than 900 genes (Kongsbak, Levring, Geisler, & Von Essen, 2013). Most tissues in our body have *VDR* even the airway tissue (Bozzetto, Carraro, Giordano, Boner, & Baraldi, 2012).

Vitamin D receptor (VDR) is a nuclear regulatory protein, which is activated by its ligand 1,25(OH)2D to form a transcription initiation complex. This interaction causes the binding of this complex with another protein called retinoid X receptors (RXR) to create another compound. The new complex binds to specific enhancer elements on the DNA, known as vitamin D response elements (VDRE). Co-activators such as histone acetyltransferase bind with VDR which cause the formation of the first transcription complex with ribonucleic acid polymerase II to activate gene expression, also vitamin D-RXR complex may recruit co-repressors that prevent the gene expression (Kato, 2000).

VDR gene locates on chromosome 12q13.11 with nine exons and eight introns and encodes 427 amino acids. There are more than 100 Single Nucleotide Polymorphisms (SNPs) in the *VDR* gene (Wjst, 2005). The most studied single nucleotide polymorphisms (SNPs) in Caucasians are those located at 3´ end of the *VDR* gene in exon 9 (*TaqI*-rs731236 T/C) and intron 8 (*ApaI*-rs7975232 G/T, *BsmI*-rs1544410 A/G), and at 5´ end in exon 2 (*FokI*-rs2228570 C/T). They are named according to the restriction enzyme used in SNP detection as shown in (Figure 3).

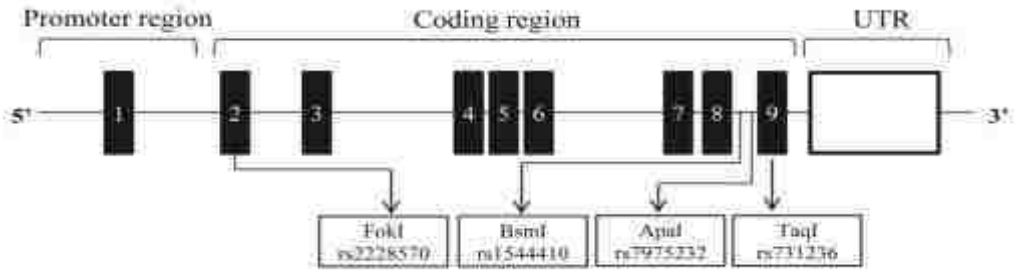

Figure 3: *VDR* polymorphisms location on chromosome 12 (Triantos et al., 2018) The black boxes show the exons of the *VDR* gene. Arrows designate the locations of the tested polymorphisms.

The *VDR*; rs731236 polymorphism causes synonymous codon that produces more stable mRNA but not changing the amino acid sequence in VDR protein. *VDR*; rs2228570 polymorphism cause missense in start codon that produces more active smaller size protein with 424 amino acids (Tizaoui et al., 2014). The produced protein known as M4 were methionine at the fourth position. Whereas, the wild-type protein called M1 where methionine at first position. *VDR*; rs2228570 polymorphism is considered as functional and an independent polymorphism with no linkage disequilibrium (LD) with other *VDR* polymorphisms (Uitterlinden, Fang, van Meurs, Pols, & van Leeuwen, 2004). On the other hand, *VDR*; rs731236, *VDR*; rs7975232, and *VDR*; rs1544410 polymorphisms are nonfunctional polymorphism with LD, but this LD is weak in some ethnic groups. Despite that these three polymorphisms cause variation in a single (A) length in the ninth exon, none of them affect the VDR protein structure (Uitterlinden et al., 2002; Whitfield et al., 2001).

1.2.4 Vitamin D Binding Protein

Vitamin D binding protein *(*DBP*)* is a hepatic alpha globulin. It was named as GC- globulin (Group-specific component of serum) that carries 85-90% of both 25-hydroxyvitamin D (25(OH)D and 1,25-dihydroxy vitamin D3 (1,25(OH)2D3) in blood circulation while the remaining vitamin D is bound to albumin. There are more than 120 genetic polymorphic sites on *DBP* gene which affect the binding affinity between vitamin D and DBP (Bhan, 2014). DBP plays a role in 25(OH)D delivery to target tissues and protects vitamin D from degradation (Bhan, 2014). DBP circulate in high concentration compared to vitamin D with 300-600 µg/ml. DBP concentration does not vary with seasonal variation, and it has short plasma half-life (2.5 days) compared

to vitamin D, which has 12 days half-life (Speeckaert, Huang, Delanghe, & Taes, 2006).

In humans, *GC* gene is located on the long arm of chromosome 4 (4q11-13). It consists of 474 amino acid with three domains. Due to the combination of the two non-synonymous SNPs (*GC*; rs7041 and *GC* rs4588) in the *DBP* gene, three main phenotypic alleles were identified *GC*1F, *GC*IS, and *GC*2 as shown in (Table 1). These phenotypic alleles distinguished by their amino acid at position 432 and 436 in strong linkage disequilibrium. The combination of the three phenotypic alleles results in six diplotypes (1F-1F, 1F-1S, IF-2, 1S-1S, 1S-2, and 2-2). These *DBP*-SNPs are associated with serum vitamin D concentration (Gozdzik et al., 2011; Lafi, Irshaid, El-Khateeb, Ajlouni, & Hyassat, 2015). *GC* has 13 exons and 12 introns (*GC*; rs4588 on exon 11, *GC*; rs7041on exon 4) (Speeckaert et al., 2006).

Table 1: Summary of *DBP* phenotypic alleles

DBP type	**Nucleotide replacement**	**a.a. position 432**	**a.a. position 436**	**a.a. transversion**	**SNP**	**Homozygous Haplotype**
*GC*1F (Wild type)	No	Aspartic acid GAT	Threonine ACG	No	NO	TT: CC
*GC*1S	T→G	Glutamic acid GAG	Threonine	a.a. 432	rs7041	GG: CC
*GC*2	C→A	Aspartic acid	Lysine AAG	a.a. 436	rs4588	TT: AA

DBP= Vitamin D binding protein, a.a= Amino acid, SNP= Single nucleotide polymorphism

1.2.5 Vitamin D Hydroxylase

24-hydroxylase enzyme (*CYP24A1*) is a mitochondrial enzyme that catalyzes both 25(OH)D3 and 1,25(OH)2D3 into 24-hydroxylated products to keep vitamin D

homeostasis. It is expressed in almost all cells that have *VDR* (Jones, Prosser, & Kaufmann, 2012). Cytochrome P450, family 2, subfamily R, polypeptide 1 (CYP2R1) and Cytochrome P450, family 27, subfamily A, polypeptide 1(CYP27A1) are 25-hydroxylase enzymes that are responsible for vitamin D hydroxylation to form 25-hydroxy vitamin D. CYP27A1 is a mitochondrial enzyme that expressed in all tissues and it hydroxylates only vitamin D3. While CYP2R1 is an endoplasmic reticulum enzyme expressed mostly in the liver and catalyze 25-hydroxylation of both vitamin D2 and D3. A study confirmed that CYP2R1 is the major enzyme for 25-hydroxylation of vitamin D because when they knocked out both *CYP2R1*and *CYP27A1* in mice, the concentration of 25-hydroxyvitamin D remained similar to knockout *CYP2R1* only (Zhu, Ochalek, Kaufmann, Jones, & DeLuca, 2013).

CYP2R1 gene is located on chromosome 11p15.2 with five exons and four introns. There are many SNPs on this gene. In this study, the focus is on one polymorphism (*CYP2R1*; rs12794714 T/C) located on exon 1. This polymorphism causes a synonymous codon (serine→serine) (Ramos-Lopez, Brück, Jansen, Herwig, & Badenhoop, 2007). The CYP2R1 protein contains 509 amino acid and the polymorphism *CYP2R1*; rs12794714 occurs in amino acid number 59 (Thacher & Levine, 2017).

1.3 Asthma

Asthma is a syndrome of chronic inflammatory complex disease in the airways of the lung. It is characterized by elevated serum immunoglobulin E (IgE) and airflow obstruction and bronchial hyper-responsiveness that leads to symptoms of wheezing, coughing, and dyspnea (Wenzel, 2006). The etiology of asthma is multifactorial; however, it can be generally attributed to genetic and environmental factors. Asthma

is a complex disease, and no clear etiological agent can be found in many patients (Borish & Culp, 2008).

There are different criteria to classify asthma phenotype. Generally, allergic and non-allergic asthma are the most common studied phenotype. Asthma phenotypes can also be classified according to the age, clinically, response to the treatment, inflammatory response and causal triggers (Wenzel, 2006).

Allergic (atopic) asthma phenotype is the most common phenotype in childhood and adolescence, which is characterized with less severity, more genetic origin; more associated with seasonal variation, increase in mast cell count in the airway and more total IgE concentration compared with non-allergic asthma. Allergic asthma is more common in male patients, and non-allergic asthma is more common in female patients (Schatz & Rosenwasser, 2014). Non-allergic asthma phenotype is the most common phenotype among adult. It is a non-immune phenotype and without any atopic history (Kumar & Ghosh, 2009).

Early-onset asthma phenotype that started in ages less than 12 years that identified with more atopic and allergic conditions with mostly mild severity (Wenzel, 2012). Late-onset asthma phenotype that began in adulthood, which identified with more heterogeneity, more severity and more probability in loss lung function compared with childhood-onset asthma. The mechanisms of adult-onset asthma phenotype are not well known. Late-onset asthma is non-atopic, non-allergic and mostly associated with specific triggers such as respiratory tract infections, obesity, aspirin treatment, and smoking and more associated with eosinophilic inflammation (Amelink et al., 2013). Amelink and coworkers classified adult-onset asthma into two groups according to eosinophil concentration in sputum; the first group is

characterized by increased sputum eosinophil, whereas the second group, with low sputum eosinophil. Well-controlled asthma with mild-to-moderate severity was clustered in one group (Amelink et al., 2013).

Clinical phenotypes define asthma according to the level of severity into four categories: mild-intermittent, mild-persistent, moderate-persistent, and severe-persistent, based on clinical symptoms, treatment response and lung function forced expiratory volume (FEV) (Dalen et al., 2008).

1.3.1 Immunopathology of Asthma

The immunopathology of asthma involves many cells with different functions such as dendritic cells, macrophages, lymphocytes, and mast cells. The dendritic cell (DC) in the asthmatic airway is mature, while healthy airway has immature DC which cannot activate the T helper lymphocytes (Borish & Culp, 2008).

In an asthmatic case, the allergens are engulfed by the DC, which presents allergens to T cell. T cell differentiates to T helper-2, which is stimulated to secret a panel of cytokines, such as IL -13, IL-4 and IL-5. The first two cytokines encourage B cells to produce IgE. IgE binds to its high-affinity receptor on the surface of mast cells, and then allergens bind to IgE. The binding of allergens was resulting in secretion of mediators of acute and chronic inflammation from mast cell. IgE-mediated responses, and also activate eosinophils, neutrophils, T and B lymphocytes as illustrated in (Figure 4), (Cookson, 2004).

When repeated allergen exposure occurs, T helper-2-like cells and their cytokines, particularly interleukins IL-4, IL-5, IL-9, and IL-13, cause chronic airway inflammation (Cookson, 2004). The migration of lung mast cells into the airway and

derive mast cell progenitors from bone marrow cause significantly increase the mast cell number within airway in allergic asthma that increases the inflammation (Borish & Culp, 2008).

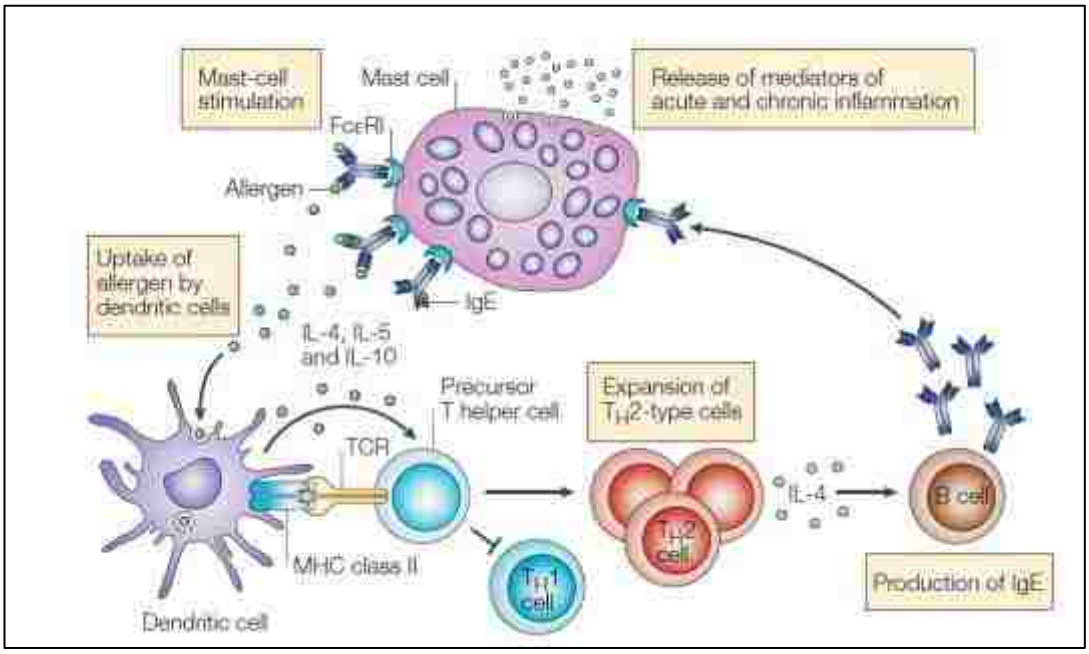

Figure 4: Mechanism of allergic asthma (Cookson, 2004)

In non-allergic asthma which is known as intrinsic asthma is associated with cigarette smoking, prior noninfectious rhinitis, as well as genetic factors, the mechanism of non-allergic asthma is not well known as allergic asthma (Borish & Culp, 2008). The T cells differentiation toward T helper-1(Th-1) or T helper-2 (Th-2) will determine asthma pathogenicity to develop as allergic asthma or non-allergic asthma (Kumar & Ghosh, 2009) and as illustrated in (Figure 5).

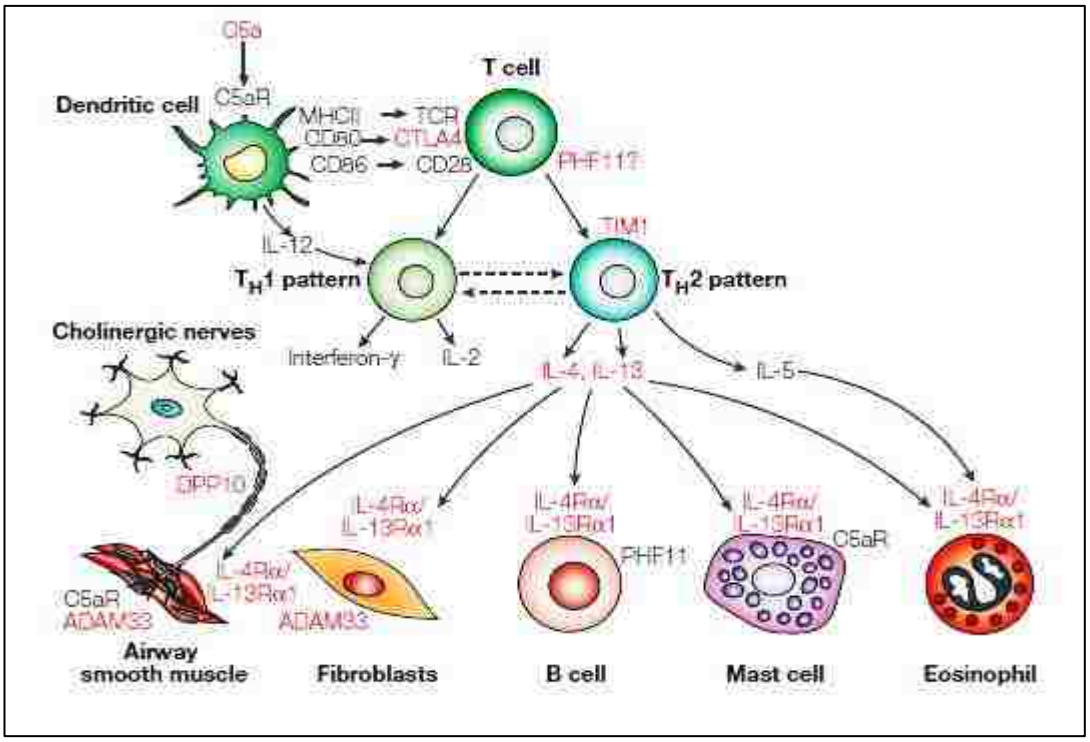

Figure 5: T cell differentiation towards Th-1 or Th-2 determines asthma pathogenicity (Wills-Karp & Ewart, 2004)

Not all patients with allergy to any trigger develop asthma, and not all asthmatic patients have allergens to trigger an asthmatic episode, so no clear answer, what causes asthma, it is a combination between genetics and environment despite that most of the asthmatics are allergic in origin (Cho et al., 2016).

1.3.2 Multigenic of Asthma

Because of the multi-genic nature of asthma, it is challenging to identify the specific genes of asthma. There are more than 20 chromosomal regions that have associations with asthma (Wills-Karp & Ewart, 2004), and more than 100 genes have relation with asthma (March, Sleiman, & Hakonarson, 2013). It is found that some of the asthma susceptibility genes are regulated by Vitamin D, mainly the genes located within the major histocompatibility complex region (MHC-II). For example, Vitamin

D down-regulates the expression of MHC-II antigens, which play a crucial role in presenting antigens to T cells in case of allergic asthma. Moreover, Vitamin D suppresses the expression of disintegrin and metalloprotein-33 (ADAM-33) protein, which is one of the novel asthmatic related genes that express in airway smooth muscle (Lương & Hoàng Nguyễn, 2012).

1.4 Association of Vitamin D Related Genes Polymorphisms with Asthma

Several studies link between vitamin D metabolic genes polymorphisms and asthma susceptibility, depending on the fact that asthma is an inflammatory disease, whereas vitamin D is considered as an anti-inflammatory prohormone. Vitamin D has a vital role as immuno-regulatory and anti-inflammatory, through its regulation for the growth and differentiation of innate and adaptive immune cells and non-immune cells (Mora et al., 2008).

Finding that vitamin D deficiency increases airway remodeling, IgE and eosinophilia and reduce the T regulatory cells in blood, as well as increase the expression of pro-inflammatory cytokines like IL-17 and decrease the expression of anti-inflammatory cytokines like IL-10 (Pfeffer et al., 2014). IL-10 has a role in steroid sensitivity in asthma, in which the steroid resistance patients have low IL-10 level, and the immunosuppressive effect of glucocorticoid will be more effective in the presence of 1,25(OH)2D3 and IL-10 (Mann, Chambers, Pfeffer, & Hawrylowicz, 2014; Poon, Mahboub, & Hamid, 2013).

Also that Vitamin D reduces the airway smooth muscle (ASM) mass (Pfeffer et al., 2014; Poon et al., 2013) and inhibits the differentiation and maturation of dendritic cells which is essential for initiating adaptive immune responses and promote activation of Th-2 responses (Beghé, Fabbri, Contoli, & Papi, 2017). Vitamin D also

regulates the transcription of genes that are involved in airway remodeling in ASM cells like ADAM33 (Foley et al., 2007). The various roles of vitamin D in asthma are shown in Figure 6 (Mann et al. 2014).

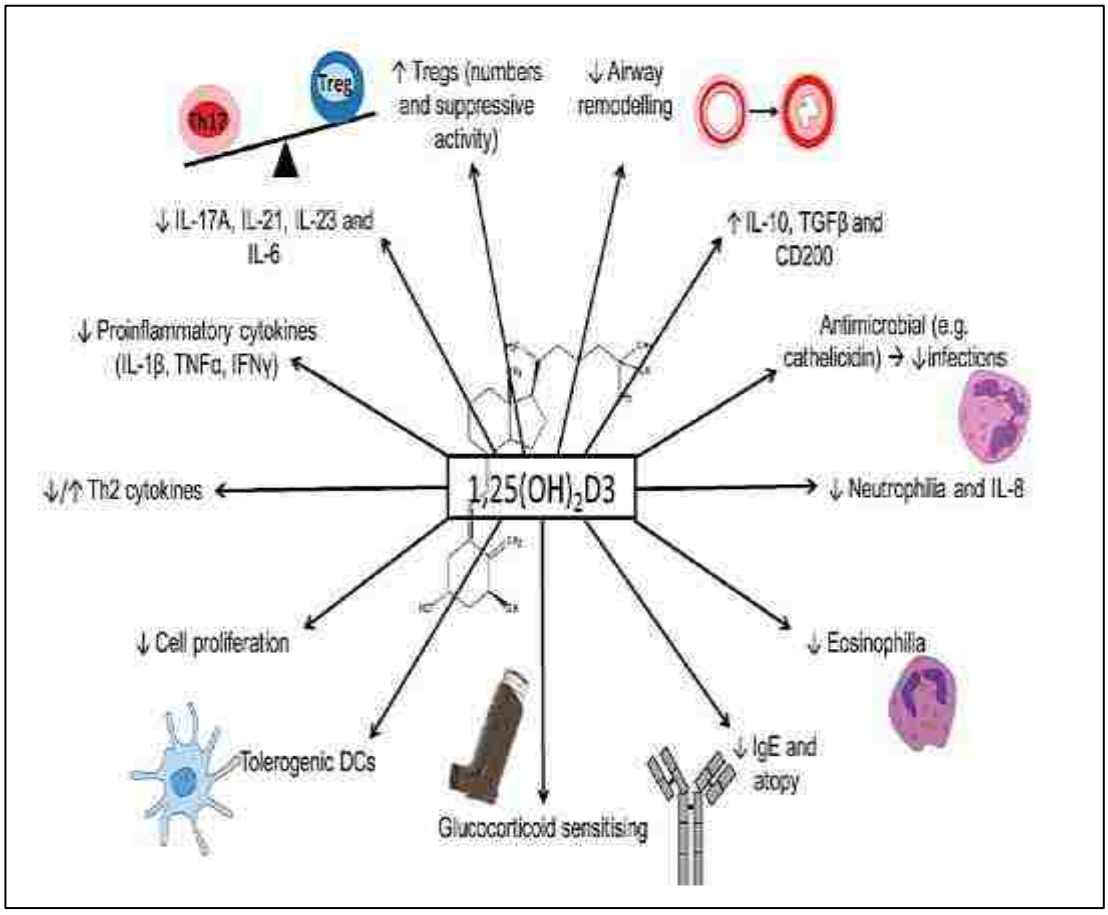

Figure 6: The roles of vitamin D in asthma (Mann et al. 2014)

In asthma, there is an activation for Th-2 cells in the airway wall, which produces IL-5, IL-4, IL-13, and tumor necrosis factor (TNF). IL-9 and IL-13 affect bronchial smooth cells, by increasing bronchial hyper-reactivity. Vitamin D downregulates inflammatory responses by reducing IL-4 secretion, suppresses Th-2 mediated allergic airway and inhibits IL-9 production (Van Belle et al., 2011). It was

reported that vitamin D increases the expression of VDR and CYP2A1 and inhibits chemokines expression in airway smooth muscle treated with TNFα (Banerjee et al., 2008).

Late-onset asthma differs from early-onset asthma; the first one is more heterogeneous (Amelink et al., 2013). A study verified significant associations between serum vitamin D status and IgE level and steroid requirement in the pediatric-only but not in adults (Goleva, Searing, Jackson, Richers, & Leung, 2012). An *in vitro* study reported that 1,25(OH)2D level is associated with improving steroid responsiveness (Searing et al., 2010). A study found a significantly lower serum 25(OH)D3 level in severe treatment-resistant asthmatic children compared to the moderate asthmatic and non-asthmatic children (Gupta et al., 2011). Persistent asthma is associated with vitamin D deficiency in early childhood (Hollams et al., 2017). Moreover, a study on adults reported no association between serum 25(OH)D concentrations and asthma phenotype (Jolliffe et al., 2018) while a study found that most severe and uncontrolled adult asthma were vitamin D insufficient compared with patients with intermittent, mild or moderate and controlled asthma (Korn, Hübner, Jung, Blettner, & Buhl, 2013).

In an epidemiological study assessment of asthma control in adult asthma population in the Middle East and North Africa found that asthma control in the Middle East and North Africa is unsatisfactory emphasizing the need to improve treatment and the follow up for the asthmatic patients (Tarraf et al., 2018). There are contradictions in the results of previous studies on the association between asthma and vitamin D metabolic genes in both children and adults because of the difference in study design, methodologies, ethnicity and the heterogeneity in adults.

Finding in meta-analysis about the association of vitamin D metabolic genes and asthmatic childhood suggested that childhood asthma was significantly associated with *VDR* gene rs7975232 polymorphism in Asians and marginally associated with *VDR*; rs1544410. Moreover, pediatric asthma might be correlated with *VDR*; rs2228570 polymorphism in the Caucasian population. However, no significant association between childhood asthma and *VDR*; rs731236 polymorphism (Zhao et al., 2017).

A study on Egyptian asthmatic children found that genotype CC of *VDR*; rs2228570 polymorphism is associated with increased susceptibility for asthma development (Ismail, Elnady, & Fouda, 2013). Also, a case-control study in asthmatic children found a correlation between vitamin D sufficiency and *VDR*; rs2228570 polymorphism in the genotype CC with more requirement for treatment to control asthma (Einisman et al., 2015). In contracts a case-control study on Serbian adult patients that found that the *VDR*; rs2228570 polymorphism (CC) associated with protection from asthma development while the other polymorphisms in the *VDR* gene (rs731236, rs7975232, and rs1544410) showed no significant difference between asthmatics and controls (Despotovic, Jevtovic Stoimenov, Stankovic, Basic, & Pavlovic, 2017; Maalmi et al., 2013). Also, Tizaoui and colleagues supported the finding that *VDR*; rs2228570 polymorphism (CC) is associated with protection from asthma development; but in female patients and asthmatics with low Vitamin D levels (Tizaoui et al., 2014).

A case-control study on Tunisian children found that *VRD*; rs1544410, *VDR*; rs731236, and *VDR*; rs2228570 polymorphisms were highly associated with asthma and no association between *VDR*-SNPs with Vitamin D concentration (Maalmi et al.,

2013). In Chinese adults, no genetic association between gene polymorphisms *VDR*; rs2228570 and *CYP2R1*; rs1279714 with asthma risk was found, whereas, *GC*1/2 and *GC*2/2 were significantly associated with asthma risk (Li, Jiang, Willis-Owen, Zhang, & Gao, 2011). In Tunisian adults, *CYP2R1*; rs1279714 (AA) polymorphism protects from asthma, and *GC*1/2 and *GC*2/2 polymorphisms did not show significant association with asthma (Lahmar et al., 2018). A meta-analysis study found that *VDR*; rs7975232, *VDR*; rs2228570, and *VDR*; rs731236 SNPs are associated with increased asthma susceptibility (Han et al., 2016).

Chapter 2: Methods

2.1 Participants

All participants were aged 18 years and above and included both males and females. 132 Emirati patients with asthma were recruited from the respiratory clinic in Al Ain Hospital from March 2016 to April 2017. One hundred sixty-four controls with no asthma or allergy and no family history of asthma were enrolled in the study from Al Ain Hospital outpatient clinic in the same period.

2.1.1 Exclusion Criteria

Patients with other lung diseases including patients with obstructive lung diseases, cystic fibrosis and pneumonia were excluded in this study. Moreover, expatriates, Emiratis aged below 18 years and patients with incomplete questionnaires or missing information were also not included (one patient and ten controls were excluded).

2.2 Study Design

The severity of asthma was categorized based on clinical symptoms and lung function into four groups according to the Global Initiative for Asthma (GINA) as shown in (Table 3), mild intermittent, mild-persistent, moderate-persistent and severe-persistent (Koshak, 2007).

The study is a case-control investigating the association between vitamin D metabolic genes polymorphisms and its levels with asthma severity in adult Emiratis. A trained nurse filled a detailed structured interview based on the responses of the participant. The information includes age, gender and previous hospitalization with asthma, family history of asthma, In addition to the results of the lung function test

(FEV) for asthmatic patients. The data were entered into an excel database. Only patients and controls that provide written consent to participate in the study were enrolled.

Table 2: GINA classification of asthma severity

Group	Symptoms/Day	Symptoms/Night	FEV1
Mild Intermittent	< 1 time a week Asymptomatic and normal PEF between attacks	</= 2 times a week	>/= 80%
Mild Persistent	>1 time a week but < 1 time a day Attacks may affect the activity	> 2 times a month	>/= 80%
Moderate Persistent	Daily Attacks affect activity	>1 time a week	60%-80%
Severe Persistent	Continuous Limited physical activity	Frequent	</=60%

FEV1, Forced expiratory volume in the first second*

* Forced expiratory volume in the first second: It is a pulmonary function test to measure the volume of air in one second that can be forced out after taking a deep breath that measure by a peak flow meter (Seema & Damayanthi, 2016).

2.3 Samples

Whole blood was obtained in EDTA-containing tubes, and genomic DNA was extracted from whole blood (lymphocyte buffy coat) using a manual column-based method (Qiagen) then stored at −20°C until analyzed. The rest of the blood was centrifuged at 3000 round per minute for 10 minutes, and plasma was used to measure total vitamin D.

2.4 DNA Extraction

Genomic DNA extraction was carried out using QIAamp genomic DNA kits from QIAGEN (QIAamp genomic DNA kits). DNA extraction passes through many steps; starting with DNA extraction from buffy coat using silica matrices based on a rapid lyse-wash-elute principle. Finally, quantity and purity assessment performed for isolated DNA samples by spectrophotometer (Nanodrop ND1000, Wilmington, Delaware, USA). Ideally, the purified DNA sample should have an A260/A280 ratio of (1.7-1.9) to indicate the absence of protein contamination.

2.5 Genotyping

2.5.1 Reagents

TaqMan Genotyping requires the following components for PCR (TaqMan SNP Genotyping Assays, Catalog Number 4351379, 4351384, 4351376, 4351382, 4351374, 4351380, 4362691, 4331349, 4332077, 4332072, 4332075, 4332073, and 4332076, Publication Number MAN0009593. 2014).

1. 1–20 ng purified genomic DNA per well (final concentration of ≥ 0.2 ng/μL).

2. 2X TaqMan Genotyping GTX-press Master Mix.

3. SNP 20X was working stock.

4. DNase-free, sterile water for sample dilution.

5. AE buffer for SNP dilution.

Master GTX-press Mix contains a buffer, dNTPs, ROX passive reference dye (an internal reference to normalize the reporter-dye signal during data analysis) and DNA polymerase.

2.5.2 Principle of TaqMan SNP Genotyping Assay

An allelic discrimination assay using the 5′ Nuclease with two primers and two fluorogenic probes for detecting known mutants. Whereas, each probe consists of an oligonucleotide with a 5′ reporter dye and a 3′ non-fluorescent quencher dye minor groove-binder (MGB).

TaqMan probes; for one allele have VIC dye, whereas, the TaqMan probes for the other allele have FAM dyes. This combination of both probes allows genotyping of the SNP in the template. Homozygotes genotype contain only one type of allele, while heterozygote contains both alleles.

The MGB molecule binds to DNA helix minor groove improves hybridization-based assay by stabilizing the MGB probe-template complex. This increase the stability of the binding of probes as short as 13 bases and increase the melting temperature.

Genotyping was performed according to the protocol (www.applieds Stem.com) using Applied Biosystems TaqMan 5’ nuclease qPCR assays and QuantStudio3 from Thermo Fisher as shown in (Figure 7).

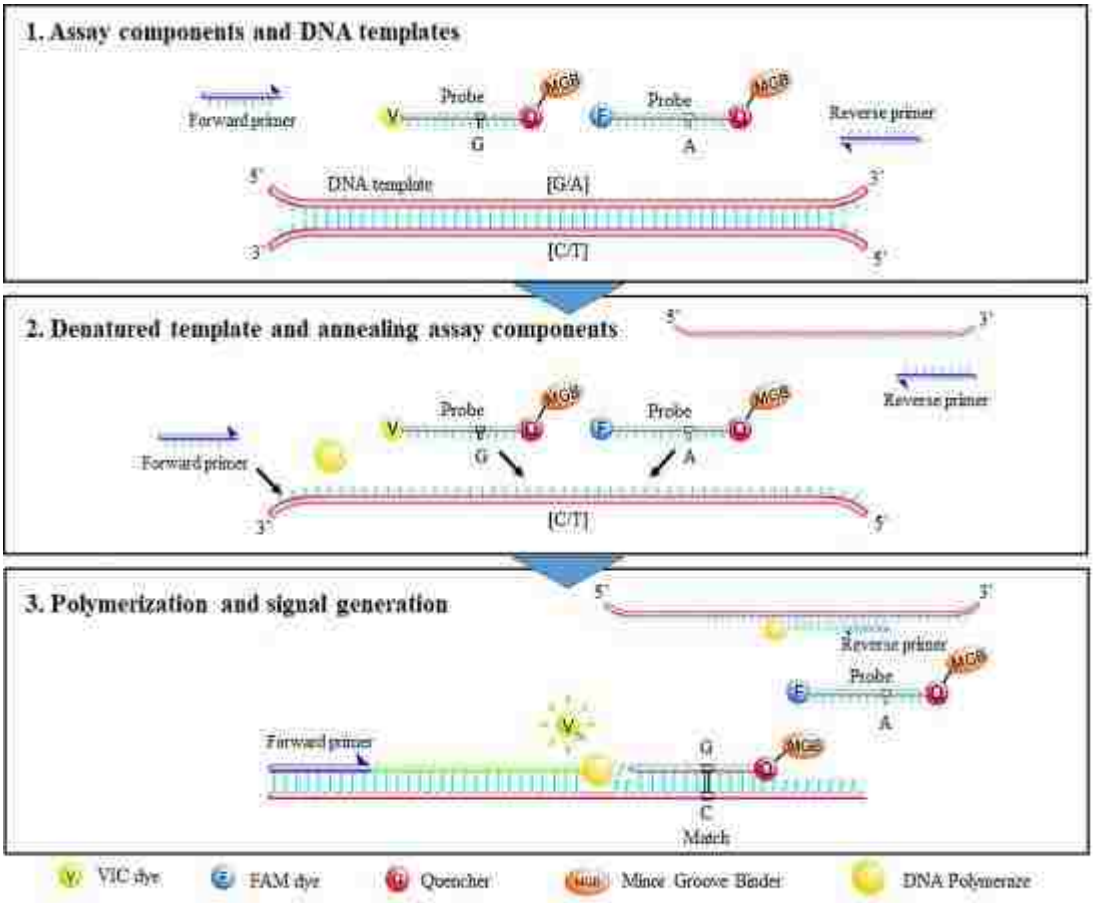

Figure 7: Allelic discrimination assay using the 5′ nuclease

SNP assays were ordered from Applied Biosystems, six pre-designed and one customized. The sequence for the customized assay *VDR*; rs1544410 was retrieved from NCBI database.

Genotyping for known SNPs namely, *VDR*; rs731236, *VDR*; rs7975232, *VDR*; rs2228570, *GC*; rs7041, *GC*; rs4588 and *CYP2R1*; rs12794714 was done using predesigned TaqMan probes (Life Technologies) as shown in (Table 3) and for one customized SNP *VDR*; rs1544410 using primers and reported sequences as listed in (Table 4). SNPs Assay summary information file (AIF) from Applied Biosystems for the studied SNPs is shown in (Table 5).

Table 3: Probe sequence of six SNPs used in this study

SNP	Context Sequence [VIC/FAM]
VDR; rs731236	GCGGTCCTGGATGGCCTC**[A/G]**ATCAGCGCGGCGTCCTGCACCCCAG
VDR; rs7975232	AGGAGCTCTCAGCTGGGC**[A/C]**CCTCACTGCTCAATCCCACCACCCC
VDR; rs2228570	CTGGCCGCCATTGCCTCC**[A/G]**TCCCTGTAAGAACAGCAAGCAGGCC
DBP; rs7041	CAGTTCCGTGGGTGTGGC**[A/C]**TCAGGCAATTTTGCTTTTAGTCGCT
DBP; rs4588	ACCAGCTTTGCCAGTTCC**[G/T]**TGGGTGTGGCATCAGGCAATTTTGC
CYP2R1; rs12794714	GTAGACATGGGGAAGCTC**[A/G]**GATGAGGCTGCCAGGGAATAGATG

Bold and underline are the bases at the SNP

Table 4: Custom TaqMan SNP VDR; rs1544410 sequence

Forward Primer Seq.	AGTGTGCAGGCGATTCGTA
Reverse Primer Seq.	GCAAGAGCAGAGCCTGAGTAT
Reporter VIC Sequence	CCTGC**A**CATTCCCA
Reporter FAM Sequence	CTGC**G**CATTCCCA

Bold and underline are the bases at the SNP

Table 5: Single nucleotide polymorphisms assay summary

Assay ID	Catalog Number	SNP	SNP Name	a.a. Change	SNP Position	Gene Name	SNP Type
C___2404008_10	4351379	rs731236	*Taq1*	ATC to ATT Iso. to Iso.	Exon 9	*VDR*	Synonymous
C__28977635_10	4351379	rs7975232	*Apa1*	C to A	Intron 8	*VDR*	Synonymous
C__12060045_20	4351379	rs2228570	*Fok1*	ATG to ACG Met to Thr	Exon 2	*VDR*	Non-synonymous-missense
C___3133594_30	4351379	rs7041	*GC1*	GAT to GAG Asp. to Glu	Exon 4	*DBP*	Non-synonymous-missense
C___8278879_10	4351379	rs4588	*GC2*	ACG to AAG Thr. to Lys	Exon 11	*DBP*	Non-synonymous-missense
C___1131665_10	4351379	rs12794714	*CYP2R1*	CGA to CGG Ser to Ser.	Exon 1	*CPY2R 1*	Synonymous
Custom	4331349	rs1544410	*Bsm1*	G to A	Intron 8	*VDR*	Synonymous

Iso= Isoleucine, SNP= Single nucleotide polymorphism, a.a= Amino acid, Met= Methionine, Thr= Threonine, Asp= Aspartic acid, Ser= Serine, *VDR*= Vitamin D receptor, *DBP*= Vitamin D binding protein, *CYP2R1*= Cytochrome P450 Family 2 Subfamily R member 1

The Real-Time PCR consists of five steps, starts with pre-read stage 60°C for 30 seconds, then DNA polymerase activation at 95°C for 20 seconds, followed by 40 cycles at 95°C for 3 seconds and 40 cycles at 60°C for 30 seconds, finally post-read stage 60°C for 30 seconds. Fluorescence detection takes place at a temperature of 60°C. All assays were performed in 10-µl reactions using TaqMan GTXpress master mix on 96-well plates by using applied bio-system QuatStudio-3 fast real-Time PCR System with universal cycling condition for all assays.

Control samples representing all possible genotypes and the negative control was included in each reaction with one PCR amplification step.

40X SNP genotyping assays were stored at –15 to –25°C in the dark with less than ten freeze-thaw cycles. SNP genotyping assays were diluted to a 20X working stock with AE buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0) from QIAamp, then aliquoted for routine use to minimize freeze-thaw cycles and the reagents exposure to light. Before use, TaqMan genotyping master mix carefully mixed by swirling the bottle and re-suspended SNP genotyping assay by vortexing, then centrifuged the tube briefly before use. The recommended final reaction volume per well is ten µl for a 96-well.

After pipetting the reaction mix (genotyping assay and master mix) into each well of the reaction plate pipette, 4.5 µl from the DNA sample was then added as listed in (Table 6). The plate was covered with MicroAmp optical adhesive film, then centrifuged briefly to spin down the contents and eliminate any air bubbles from the solutions and then the PCR was carried out.

Table 6: Components of the reaction mix

Component	96 well plate (10 µl reaction)
2X TaqMan Master Mix	Five µl
20X Assay Working Stock (SNP)	0.5 µl
Sample (1–20 ng) ≥ 0.2 ng/µl)	4.5 µl
Final Volume	Ten µl

To ensure optimal analysis and troubleshooting of the TaqMan SNP genotyping assays, in each reaction plate, we include DNA samples with known genotype (as a positive control) at the polymorphism of interest and two no template controls (NTCs), repeated genotyping for random selected SNPs for each genotype on about 19.26% of the samples to ensure genotype calling. We accepted only the genotype with calling > 95%.

2.6 Total Vitamin D

2.6.1 Principle

Plasma is used to measure 25-hydroxy vitamin D using electrochemiluminescence binding assay by Cobas 602 from Roche Germany, a fully automated equipment in Sheikh Khalifa Medical City (SKMC) laboratory.

2.6.2 Reagents

All reagents that used for measuring total 25-hydroxyvitamin D were barcoded ready to use and stable at 2-8°C. The reagent contains rackpack and the pretreatment reagents that are labeled as vitamin D and cannot be separated.

2.6.3 Calibration

This method is standardized according to the National Institute of Standards and Technology Standard. The results are determined depending on the standard curve generated by two-point calibration, with calibrator lot number 243104.

2.6.4 Quality Control

Preci-control varia kits were used, and the quality control (QC) interval attached with each kit in nmol/l are presented in (Table 7). Based on the methodology reference range that is used in SKMC patients, the Vitamin D results for the patients were categorized into sufficient, insufficient and deficient, > 75 nmol/l, 50-75 nmol/l and < 50 nmol/l, respectively.

Table 7: Quality control of preci-control varia kit of vitamin D assay

Control	Lot Number	Mean	2 SD	Result	QC interval
Preci-control level 1	276842	44.1	9.8	40.4	34.3-53.9
Preci-control level 2	276843	98.3	19.66	92.0	78.64-117.96

SD = Standard deviation, QC = Quality control

2.7 Statistical Analysis

Seven SNPs (*GC*; rs4588, *GC*; rs7041, *VDR*; rs731236, *VDR*; rs7975232, *VDR*; rs1544410 and *VDR*; rs2228570, and *CYP2R1*; rs12794714) were studied for allelic, genotypic and haplotype association with asthma among asthmatic and control participants.

The chi-square (χ^2) test using SPSS version 25 software was used to compare between asthmatics and controls.

SNPs (*GC*; rs4588, *GC*; rs7041, *VDR*; rs731236, *VDR*; rs7975232, *VDR*; rs1544410, and *VDR*; rs2228570) were evaluated with Hardy-Weinberg Equilibrium (HWE) by comparing the genotype frequency within the asthmatic and control groups using chi-square through the web-based software Haploview package version 4.2. HWE was tested in control and asthmatic cases separately.

Hardy-Weinberg Equilibrium (HWE) is based on the frequencies of allele and genotype are the same between generations if we assumed that no mutation, no migration, random mating, and no selection. Deviations from HWE means that there are genotyping or population structure problems (heterogeneous population). Deviations from HWE in case samples may indicate loci association with disease. Therefore, testing for departure from HWE mainly used only for control samples but the cases can be tested (Anderson et al., 2010; Lewis, 2002; Wigginton, Cutler, & Abecasis, 2005). HWE test is considered as one of the data quality control tools in case-control association study (Anderson et al., 2010; Clarke et al., 2011).

Descriptive statistic of SPSS software (version 25) was used to explore the levels of vitamin D in the three major groups (mild intermittent/persistent asthmatics, moderate/severe asthmatics and controls) and within the genotype in each group. Shapiro-Wilk test was used to study the distribution of vitamin D between the three groups and within genotype in each group separately. Vitamin D difference among the three groups described as mean, standard deviation and interquartile range. Based on Shapiro-Wilk test results for data normality distribution, we found that data was not normally distributed. Accordingly, we used Kruskal Wallis to test the difference in vitamin D mean between and within the group.

Odds ratios (OR) and 95% confidence interval (CI) were calculated by web-based software SNPstats (https://www.snpstats.net/start.htm) to study the genotypic association of each SNP with asthma severity in four genetic models, codominant, dominant, recessive and additive (Lewis, 2002).

* Codominant: Each genotype has an independent effect.
* Dominant: The risk of the disease increase even if one allele is polymorphic.
* Recessive: The risk of the disease increase only if both alleles are polymorphic.
* Log-additive: The disease severity increase with the number of polymorphic (the risk of the disease increase with homozygous polymorphic)

Allele or genotype odds ratio: Measure the strength of association between the allele and the genotype with the disease.

Haploview package version 4.2 web-based software was used to; analyze minor allele frequency (MAF) for each allele. Analyze linkage disequilibrium (LD) and study the haplotype distribution and allelic and haplotype association for each SNP with asthma (Barrett, Fry, Maller, & Daly, 2004). Haplotype: A group of alleles that are inherited together on the same chromosome (Clarke et al., 2011).

The solid spin of LD was used for haplotype block determination with an exclusion for any marker with MAF < 0.05 and the extended spin if D` > 0.8.

Linkage disequilibrium (LD): A non-random association between genetic loci at same chromosome which is measured as r^2 or D' multiplied by 100, r^2 or D' = 0 means no LD and Strong evidence of recombination. r^2 or D' = 1 Strong evidence of LD with no evidence of recombination between markers (Clarke et al., 2011; Song et al., 2016).

D` is more useful to study the historical recombination while r^2 for association mapping (Mueller, 2004).

Genomic position for each SNPs determined according to Single Nucleotide Polymorphism Database (dbSNP) hosted by the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/projects/SNP/) (Table 8).

For all tests, a p-value < 0.05 was considered significant, p-value (0.05-0.08) was considered marginally significant and p-value > 0.08 was considered non-significant.

Table 8: Genomic positions of SNPs

SNP	Genomic position
DBP; rs4588	71752606
DBP; rs7041	71752617
VDR; rs731236	47844974
VDR; rs7975232	47845054
VDR; rs1544410	47846052
VDR; rs2228570	47879112

Chapter 3: Results

3.1 Participants

The total number of subjects in this study were 296 (132 asthmatics and 164 non –asthmatic (controls) aged 18 years and above with an average age of 47.58±17.27 and 38.41±13.34 for asthmatic patients and controls respectively (Table 9). There was a difference in age and gender distribution between asthmatic patients and controls. Therefore, age and gender were considered as covariates in statistical analyses, and the adjusted p-values were used.

The 132 asthmatic participants were categorized according to asthma severity into four groups (mild intermittent, mild-persistent, moderate-persistent and severe-persistent). As the number of severe cases was not enough for statistical analysis, the four groups were merged into two groups; mild intermittent and mild-persistent were combined as a (mild group, 79 patients), while moderate-persistent and severe-persistent were merged as a (moderate/severe group, 53 patients).

Table 9: Age and gender in asthmatics and controls and the distribution of asthmatics according to asthma severity

Variables		Asthmatics n (%)	Controls n (%)	p-value
Age		47.58 (17.27)	38.41 (13.34)	0.000
Gender	Females	100 (75.8)	158 (96.3)	0.000
	Males	32 (24.2)	6 (3.7)	
Asthma severity	Mild intermittent	40 (30.3)		
	Mild persistent	39 (29.5)		
	Moderate persistent	48 (36.4)		
	Severe persistent	5 (3.8)		
Total		132	164	

3.2 Genotypes

As a quality control, the results of duplicate genotyping was the same whenever repeated and the call rate for all genotyping was > 95%, which support the accuracy and the precision of genotyping assay. The graph for genotype cluster plots for each SNP represented the data of two probes across all subjects in the run together. For examples, the intensity of probe C is plotted against the intensity of probe A in allelic discrimination plot. Each genotype is represented by a different color (Figure 8).

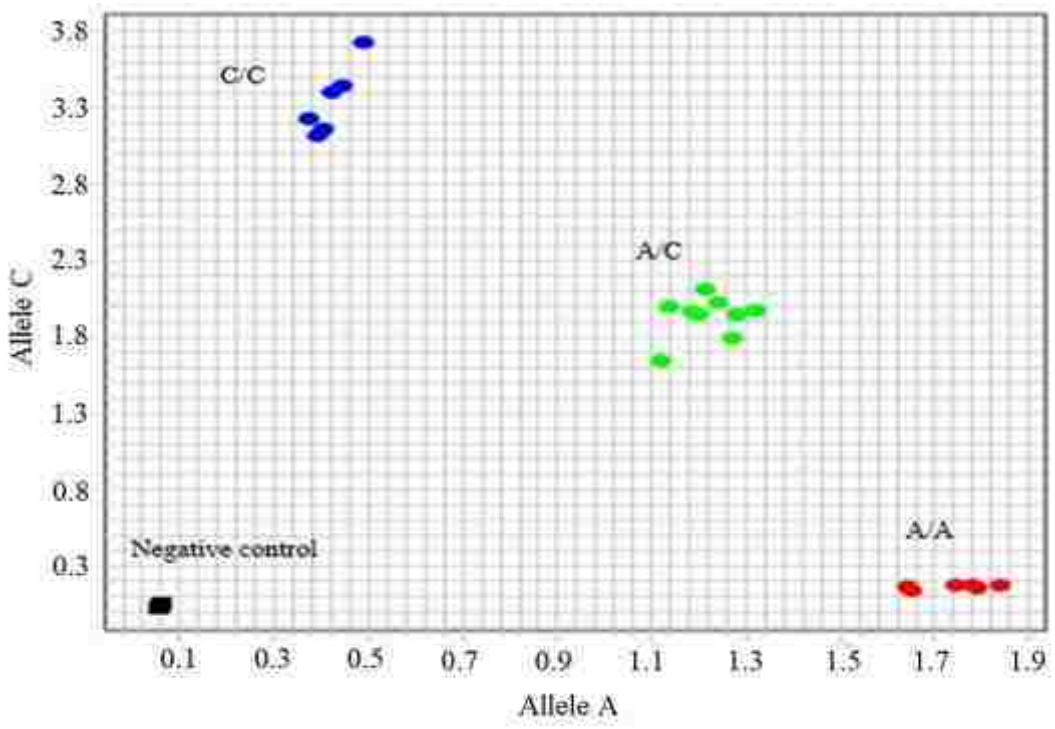

Figure 8: Allelic discrimination plot for SNPs assay

3.2.1 Genotypes of Asthmatics and Controls

The genotyping distributions of seven SNPs in *DBP*, *VDR*, and *CYP2R1* genes in all asthmatic cases and controls are presented (Tables 10-13). Generally, no statistically significant difference was found between asthmatic patients and controls.

Table 10: Genotypes distribution of DBP; rs4588 and DBP; rs7041 in asthmatics and controls

Model	Genotype/ allele	Asthmatics n (%)	Controls n (%)	OR (95% CI)*	p-value*
DBP; rs4588					
Codominant	G/G	92 (69.7)	114 (69.9)	1.00	
	G/T	35 (26.5)	45 (27.6)	1.08 (0.6-1.93)	0.96
	T/T	5 (3.8)	4 (2.5)	0.89 (0.19-4.21)	
Dominant	G/G	92 (69.7)	114 (69.9)	1.00	0.85
	G/T-T/T	40 (30.3)	49 (30.1)	1.06 (0.6-1.86)	
Recessive	G/G-G/T	127 (96.2)	159 (97.5)	1.00	0.86
	T/T	5 (3.8)	4 (2.5)	0.87 (0.19-4.08)	
Over-dominant	G/G-T/T	97 (73.5)	118 (72.4)	1.00	0.80
	G/T	35 (26.5)	45 (27.6)	1.08 (0.6-1.93)	
Log-additive	-----	--	--	1.03 (0.63-1.68)	0.91
Allele	G	219 (83.0)	273 (84)	--	--
	T	45 (17.0)	53 (16)	--	--
DBP; rs7041					
Codominant	C/C	45 (34.1)	66 (40.5)	1.00	
	A/C	61 (46.2)	71 (43.6)	0.82 (0.46-1.45)	0.49
	A/A	26 (19.7)	26 (15.9)	0.64 (0.30-1.35)	
Dominant	C/C	45 (34.1)	66 (40.5)	1.00	0.33
	A/C-A/A	87 (65.9)	97 (59.5)	0.76 (0.45-1.31)	
Recessive	C/C-A/C	106 (80.3)	137 (84)	1.00	0.33
	A/A	26 (19.7)	26 (15.9)	0.71 (0.36-1.40)	
Over-dominant	C/C-A/A	71 (53.8)	92 (56.4)	1.00	0.83
	A/C	61 (46.2)	71 (43.6)	0.95 (0.56-1.59)	
Log-additive	-----	--	--	0.8 (0.56-1.15)	0.23
Allele	C	151 (57)	203 (62)	--	--
	A	113 (43)	123 (38)	--	--

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

Table 11: Genotypes distribution of rs731236 and VDR; rs7975232 in asthmatics and controls

Model	Genotype/ allele	Asthmatics n (%)	Controls n (%)	OR (95% CI)*	p-value*
VDR; rs731236					
Codominant	A/A	53 (40.1)	61 (37.2)	1.00	
	A/G	62 (47.0)	77 (47.0)	1.13 (0.65-1.98)	0.66
	G/G	17 (12.9)	26 (15.8)	1.44 (0.66-3.14)	
Dominant	A/A	53 (40.1)	61 (37.2)	1.00	0.50
	A/G-G/G	79 (59.9)	103 (62.8)	1.2 (0.71-2.03)	
Recessive	A/A-A/G	115 (87.1)	138 (84.2)	1.00	0.42
	G/G	17 (12.9)	26 (15.8)	1.34 (0.61-1.71)	
Over-dominant	A/A-G/G	70 (53.0)	87 (53.0)	1.00	0.93
	A/G	62 (47.0)	77 (47.0)	1.02 (0.61-1.71)	
Log-additive	-----	--	--	1.18 (0.82-1.71)	0.37
Allele	A	168 (64.0)	199 (61.0)	--	--
	G	96 (36.0)	129 (39.0)	--	--
VDR; rs7975232					
Codominant	A/A	37 (28.2)	62 (37.8)	1.00	
	A/C	70 (53.4)	78 (47.6)	0.76 (0.43-1.35)	0.27
	C/C	24 (18.3)	24 (14.7)	0.53 (0.25-1.15)	
Dominant	A/A	37 (28.2)	62 (37.8)	1.00	0.19
	A/C-C/C	94 (71.8)	102 (62.2)	0.69 (0.4-1.2)	
Recessive	A/A-A/C	107 (81.7)	140 (85.4)	1.00	0.19
	C/C	24 (18.3)	24 (14.7)	0.63 (0.31-1.25)	
Over-dominant	A/A-C/C	61 (46.6)	86 (52.4)	1.00	0.78
	A/C	70 (53.4)	78 (47.6)	0.93 (0.56-1.55)	
Log-additive	-----	--	--	0.73 (0.50-1.07)	0.11
Allele	A	144 (55.0)	202 (62.0)	--	--
	C	118 (45.0)	126 (38.0)	--	--

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

Table 12: Genotypes distribution of VDR; rs2228570 and VDR; rs1544410 in asthmatics and controls

Model	Genotype /allele	Asthmatics n (%)	Controls n (%)	OR (95% CI)*	p-value*
VDR; rs2228570					
Codominant	G/G	82 (62.1)	96 (58.5)	1.00	
	A/G	46 (34.9)	59 (36.0)	1.07 (0.62-1.85)	0.96
	A/A	4 (3.0)	9 (5.5)	0.98 (0.27-3.58)	
Dominant	G/G	82 (62.1)	96 (58.5)	1.00	0.82
	A/G-A/A	50 (37.9)	68 (41.5)	1.06 (0.63-1.80)	
Recessive	G/G-A/G	128 (97.0)	155 (94.5)	1.00	0.95
	A/A	4 (3.0)	9 (5.5)	0.96 (0.27-3.44)	
Over-dominant	G/G-A/A	86 (65.2)	105 (64.0)	1.00	0.79
	A/G	46 (34.9)	59 (36.0)	1.08 (0.63-1.84)	
Log-additive	-----	--	--	1.04 (0.66-1.63)	0.86
Allele	G	210 (80.0)	251 (77.0)	--	--
	A	54 (20.0)	77 (23.0)	--	--
VDR; rs1544410					
Codominant	G/G	50 (37.9)	54 (32.9)	1.00	
	A/G	64 (48.5)	83 (50.6)	1.16 (0.66-2.05)	0.72
	A/A	18(13.6%)	27(16.5%)	1.37 (0.63-2.98)	
Dominant	G/G	50 (37.9)	54 (32.9)	1.00	0.49
	A/G-A/A	82 (62.1)	110 (67.1)	1.21 (0.71-2.07)	
Recessive	G/G-A/G	114 (86.4)	137 (83.5)	1.00	0.53
	A/A	18 (13.6)	27 (16.5)	1.25 (0.62-2.54)	
Over-dominant	G/G-A/A	68 (51.5)	81 (49.4)	1.00	0.84
	A/G	64 (48.5)	83 (50.6)	1.05 (0.63-1.76)	
Log-additive	-----	--	--	1.17 (0.80-1.70)	0.41
Allele	G	164 (62.0)	191(58.0)	--	--
	A	100 (38.0)	137 (42.0)	--	--

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

Table 13: Genotypes distribution of CYP2R1; rs12794714 in asthmatics and controls

Model	Genotype/ allele	Asthmatics n (%)	Controls n (%)	OR (95% CI)*	p-value*
CYP2R1; rs12794714					
	G/G	48 (36.4)	64 (39)	1.00	
Codominant	A/G	56 (42.4)	70 (42.7)	1.08 (0.61-1.92)	0.92
	A/A	28 (21.2)	30 (18.3)	0.94 (0.46-1.93)	
	G/G	48 (36.4)	64 (39.0)	1.00	
Dominant	A/G-A/A	84 (63.6)	100 (61.0)	1.03 (0.61-1.76)	0.90
	G/G-A/G	104 (78.8)	134 (81.7)	1.00	
Recessive	A/A	28 (21.2)	30 (18.3)	0.9 (0.47-1.73)	0.76
	G/G-A/A	76 (57.6)	94 (57.3)	1.00	
Over-dominant	A/G	56 (42.4)	70 (42.7)	1.1 (0.66-1.85)	0.71
Log-additive	-----	--	--	0.99 (0.69-1.40)	0.93
	G	152 (58.0)	198 (60.0)	--	--
Allele	A	112 (42.0)	130 (40.0)	--	--

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

3.2.2 Genotypes of Mild Intermittent/Persistent Asthmatics and Controls

The genotyping distribution of seven polymorphisms in vitamin D binding protein, vitamin D receptors, and cytochrome P450 family 2 subfamily R member 1 genes between mild asthmatics and controls is presented in (Tables 14-17). No statistically significant differences were found between mild asthmatics and controls.

Table 14: Genotypes distribution of DBP; rs4588 and DBP; rs7041in mild intermittent/persistent asthmatics and controls

Model	Genotype/ Allele	(MILD) n (%)	Controls n (%)	OR (95% CI)*	p-value*
DBP; rs4588					
Codominant	G/G	58 (73.4)	114 (69.9)	1.00	
	G/T	17 (21.5)	45 (27.6)	0.65 (0.32-1.33)	0.39
	T/T	4 (5.1)	4 (2.5)	1.57 (0.31-7.84)	
Dominant	G/G	58 (73.4)	114 (69.9)	1.00	0.35
	G/T-T/T	21 (26.6)	49 (30.1)	0.73 (0.37-1.43)	
Recessive	G/G-G/T	75 (94.9)	159 (97.5)	1.00	0.49
	T/T	4 (5.1)	4 (2.5)	1.75 (0.36-8.63)	
Over-dominant	G/G-T/T	62 (78.5)	118 (72.4)	1.00	0.21
	G/T	17 (21.5)	45 (27.6)	0.64 (0.31-1.30)	
Log-additive	-----	--	--	0.85 (0.48-1.51)	0.58
Allele	G	133 (84.0)	273 (84.0)	--	--
	T	25 (16.0)	53 (16.0)	--	--
DBP; rs7041					
Codominant	C/C	30 (38.0)	66 (40.5)	1.00	
	A/C	35 (44.3)	71 (43.6)	1.05 (0.55-2.03)	0.78
	A/A	14 (17.7)	26 (15.9)	1.35 (0.57-3.21)	
Dominant	C/C	30 (38.0)	66 (40.5)	1.00	0.70
	A/C-A/A	49 (62.0)	97 (59.5)	1.13 (0.61-2.08)	
Recessive	C/C-A/C	65 (82.3)	137 (84.0)	1.00	0.50
	A/A	14 (17.7)	26 (15.9)	1.32 (0.60-2.89)	
Over-dominant	C/C-A/A	44 (55.7)	92 (56.4)	1.00	0.90
	A/C	35 (44.3)	71 (43.6)	0.96 (0.53-1.75)	
Log-additive	-----	--	--	1.14 (0.75-1.74)	0.53
Allele	C	95 (60.0)	203 (62.0)	--	--
	A	63 (40.0)	123 (38.0)	--	--

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

Table 15: Genotypes distribution of VDR; rs731236 and VDR; rs7975232 in mild intermittent/persistent asthmatics and controls

Model	Genotype/ Allele	(MILD) n (%)	Controls n (%)	OR (95% CI)*	p-value*
VDR; rs731236					
Codominant	A/A	27 (34.2)	61 (37.2)	1.00	
	A/G	41 (51.9)	77 (47.0)	1.19 (0.62-2.29)	0.83
	G/G	11 (13.9)	26 (15.8)	0.96 (0.39-2.37)	
Dominant	A/A	27 (34.2)	61 (37.2)	1.00	0.70
	A/G-G/G	52 (86.1)	103 (62.8)	1.13 (0.61-2.1)	
Recessive	A/A-A/G	68 (86.1)	138 (84.2)	1.00	0.74
	G/G	11 (13.9)	26 (15.8)	0.87 (0.38-1.97)	
Over-dominant	A/A-G/G	38 (48.1)	87 (53.0)	1.00	0.54
	A/G	41 (51.9)	77 (47.0)	1.2 (0.66-2.18)	
Log-additive	-----	--	--	1.02 (0.66-1.56)	0.93
Allele	A	95 (60.0)	199 (61.0)	--	--
	G	63 (40.0)	129 (39.0)	--	--
VDR; rs7975232					
Codominant	A/A	24 (30.8)	62 (37.8)	1.00	
	A/C	43 (55.1)	78 (47.6)	1.35 (0.70-2.61)	0.66
	C/C	11 (14.1)	24 (14.7)	1.31 (0.51-3.38)	
Dominant	A/A	24 (30.8)	62 (37.8)	1.00	0.36
	A/C-C/C	54 (69.2)	102 (62.2)	1.34 (0.71-2.52)	
Recessive	A/A-A/C	67 (85.9)	140 (85.4)	1.00	0.83
	C/C	11 (14.1)	24 (14.7)	1.1 (0.47-2.59)	
Over-dominant	A/A-C/C	35 (44.9)	86 (52.4)	1.00	0.47
	A/C	43 (55.1)	78 (47.6)	1.25 (0.69-2.27)	
Log-additive	-----	--	--	1.19 (0.76-1.85)	0.45
Allele	A	91 (58.0)	202 (62.0)	--	--
	C	65 (42.0)	126 (38.0)	--	--

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

Table 16: Genotypes distribution of VDR; rs2228570 and VDR; rs1544410 in mild intermittent/persistent asthmatics and controls

Model	Genotype/ Allele	(MILD) n (%)	Controls n (%)	OR (95% CI)*	p-value*
VDR; rs2228570					
Codominant	G/G	49 (62)	96 (58.5)	1.00	0.96
	A/G	28 (35.4)	59 (36)	0.92 (0.49-1.73)	
	A/A	2 (2.5)	9 (5.5)	0.89 (0.17-4.56)	
Dominant	G/G	49 (62.0)	96 (58.5)	1.00	0.79
	A/G-A/A	30 (38.0)	68 (41.5)	0.92 (0.50-1.69)	
Recessive	G/G-A/G	77 (97.5)	155 (94.55)	1.00	0.92
	A/A	2 (2.5)	9 (5.5)	0.92 (0.18-4.62)	
Over-dominant	G/G-A/A	51 (64.6)	105 (64.0)	1.00	0.81
	A/G	28 (35.4)	59 (36.0)	0.93 (0.50-1.73)	
Log-additive	-----	--	--	0.93 (0.55-1.58)	0.79
Allele	G	126 (80.0)	251 (77.0)	--	--
	A	32 (20.0)	77 (23.0)	--	--
VDR; rs1544410					
Codominant	G/G	26 (32.9)	54 (32.95)	1.00	0.97
	A/G	41 (51.9)	83 (50.6)	1.09 (0.56-2.12)	
	A/A	12 (15.2)	27 (16.5)	1.02 (0.42-2.48)	
Dominant	G/G	26 (32.9)	54 (32.9)	1.00	0.84
	A/G-A/A	53 (67.1)	110 (67.1)	1.07 (0.57-2.02)	
Recessive	G/G-A/G	67 (84.8)	137 (83.5)	1.00	0.94
	A/A	12 (15.2)	27 (16.5)	0.97 (0.44-2.14)	
Over-dominant	G/G-A/A	38 (48.1)	81 (49.4)	1.00	0.80
	A/G	41 (51.9)	83 (50.6)	1.08 (0.6-1.96)	
Log-additive	-----	--	--	1.02 (0.66-1.57)	0.92
Allele	G	93 (59.0)	191(58.0)	--	--
	A	65 (41.0)	137 (42.0)	--	--

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

Table 17: Genotypes distribution of CYP2R1; rs12794714 mild intermittent/persistent asthmatics and controls

Model	Genotype/ Allele	(MILD) n (%)	Controls n (%)	OR (95% CI)*	p-value*
CYP2R1; rs12794714					
Codominant	G/G	31 (39.2)	64 (39.0)	1.00	
	A/G	33 (41.8)	70 (42.7)	0.83 (043-1.61)	0.86
	A/A	15 (19.0)	30 (18.3)	0.93 (0.40-2.14)	
Dominant	G/G	31 (39.2)	64 (39.0)	1.00	0.63
	A/G-A/A	48 (60.8)	100 (61.0)	0.86 (0.47-1.58)	
Recessive	G/G-A/G	64 (81.0)	134 (81.7)	1.00	0.95
	A/A	15 (19.0)	30 (18.3)	1.02 (0.48-2.20)	
Over-dominant	G/G-A/A	46 (58.2)	94 (57.3)	1.00	0.6
	A/G	33 (41.8)	70 (42.7)	0.85 (0.46-1.56)	
Log-additive	-----	--	--	0.94 (0.62-1.42)	0.77
Allele	G	95 (60.0)	198 (60.0)	--	--
	A	63 (40.0)	130 (40.0)	--	--

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

3.2.3 Genotypes of Moderate/Severe Asthmatics and Controls

The genotype distributions of seven polymorphisms in vitamin D binding protein, vitamin D receptor, and vitamin D 25-hydroxylase are studied for moderate/severe asthmatics and controls. The only SNP that presented a statistically significant association with asthma severity was *VDR*; rs7975232, while *VDR*; rs731236 and *VDR*; rs1544410 polymorphisms showed a marginal association with asthma severity.

For *VDR*; rs7975232, the additive model revealed that the disease severity increases 1.74 times in the presence of C allele (p-value = 0.032). In codominant

model, the genotype C/C (OR = 3.2, 95% CI = 1.16-8.78, p-value = 0.05) increases the risk of asthma by 3.2 times. In the recessive model, the homozygous genotype C/C (OR = 2.7, 95% CI = 1.12-6.56, p = 0.03) increase the risk of asthma 2.7 times. The homozygous genotype CC was over-represented in severe asthmatics (24.5%) compared to controls (14.7 %). The allele C frequency for severe asthmatics and controls was 50% and 38% respectively (Table 19).

For *VDR*; rs731236, in a dominant model, the genotype A/G and G/G (OR = 0.52, 95% CI 0.26-1.07, P = 0.074) adjusted by age and sex showed marginal risk reduction with severe asthma. A trend was observed in the log-additive model that allele G reduce the risk of severe asthma (P = 0.075, Table 19). For SNP *VDR*; rs1544410 dominant model, the genotype A/G and A/A (OR = 0.52, 95% CI = 0.25-1.07, P = 0.075) showed marginal significant association with a lower risk of asthma severity. A trend was observed in the log-additive model the allele A reduce the risk of severe asthma (p-value = 0.063, Table 20).

For the other SNPs (*GC*; rs4588, *GC*; rs7041, *VDR*; rs2228570, and rs12774714) no statistically significant association was observed between these polymorphisms and risk of asthma severity (Tables 18, 20 and 21).

Table 18: Genotypes distribution of DBP; rs4588 and DBP; rs7041in moderate/severe asthmatics and controls

Model	Genotype/ allele	Moderate/ Severe asthmatics n (%)	Controls n (%)	OR (95% CI)*	p-value*
DBP; rs4588					
Codominant	G/G	34 (64.2)	114 (69.9)	1.00	0.33
	G/T	18 (34.0)	45 (27.6)	1.55 (0.73-3.33)	
	T/T	1 (1.9)	4 (2.5)	0.33 (0.02-4.81)	
Dominant	G/G	34 (64.2)	114 (69.9)	1.00	0.36
	G/T-T/T	19 (35.9)	49 (30.1)	1.42 (0.67-2.99)	
Recessive	G/G-G/T	52 (98.1)	159 (97.5)	1.00	0.33
	T/T	1 (1.9)	4 (2.5)	0.28 (.02-4.04)	
Over-dominant	G/G-T/T	35 (66.0)	118 (72.4)	1.00	0.22
	G/T	18 (34.0)	45 (27.6)	1.61 (0.76-3.43)	
Log-additive	-----	--	--	1.21 (0.62-2.36)	0.59
Allele	G	86 (81.0)	273 (84.0)	--	--
	T	20 (19.0)	53 (16.0)	--	--
DBP; rs7041					
Codominant	C/C	15 (28.3)	66 (40.5)	1.00	0.32
	A/C	26 (49.1)	71 (43.6)	1.65 (0.74-3.69)	
	A/A	12 (22.6)	26 (15.9)	2.02 (0.72-5.64)	
Dominant	C/C	15 (28.3)	66 (40.5)	1.00	0.14
	A/C-A/A	38 (71.7)	97 (59.5)	1.74 (0.82-3.73)	
Recessive	C/C-A/C	41 (77.4)	137 (84)	1.00	0.37
	A/A	12 (22.6)	26 (15.9)	1.51 (0.61-3.73)	
Over-dominant	C/C-A/A	27 (50.9)	92 (56.4)	1.00	0.47
	A/C	26 (49.1)	71 (43.6)	1.3 (0.64-2.63)	
Log-additive	-----	--	--	1.45 (0.88-2.38)	0.14
Allele	C	56 (53.0)	203 (62.0)	--	--
	A	50 (47.0)	123 (38.0)	--	--

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

Table 19: Genotypes distribution of VDR; rs731236 and VDR; rs7975232 in moderate/severe asthmatics and controls

Model	Genotype/ Allele	Moderate/ Severe Asthmatics n (%)	Controls n (%)	OR (95% CI)*	p-value*
VDR; rs731236					
Codominant	A/A	26 (49.1)	61 (37.2)	1.00	
	A/G	21 (40)	77 (47.0)	0.56 (0.26-1.19)	0.18
	G/G	6 (11.3)	26 (15.8)	0.44 (0.15-1.31)	
Dominant	A/A	26 (49.1)	61 (37.2)	1.00	0.074
	A/G-G/G	27 (50.9)	103 (62.8)	0.52 (0.26-1.07)	
Recessive	A/A-A/G	47 (88.7)	138 (84.2)	1.00	0.30
	G/G	6 (11.3)	26 (15.8)	0.59 (0.21-1.66)	
Over-dominant	A/A-G/G	32 (60.4)	87 (53.0)	1.00	0.31
	A/G	21 (40.0)	77 (47.0)	0.69 (0.34-1.41)	
Log-additive	-----	--	--	0.63 (0.38-1.06)	0.075
Allele	A	73 (69.0)	199 (61.0)	--	--
	G	33 (31.0)	129 (39.0)	--	--
VDR; rs7975232					
Codominant	A/A	13 (24.5)	62 (37.8)	1.00	
	A/C	27 (50.9)	78 (47.6)	1.34 (0.59-3.04)	
	C/C	13 (24.5)	24 (14.7)	3.2 (1.16-8.78)	0.055
Dominant	A/A	13 (24.5)	62 (37.8)	1.00	0.16
	A/C-C/C	40 (75.5)	102 (62.2)	1.71 (0.80-3.68)	
Recessive	A/A-A/C	40 (75.5)	140 (85.4)	1.00	0.03
	C/C	13 (24.5)	24 (14.7)	2.7 (1.12-6.56)	
Over-dominant	A/A-C/C	26 (49.1)	86 (52.4)	1.00	0.73
	A/C	27 (50.9)	78 (47.6)	0.89 (0.44-1.79)	
Log-additive	-----	--	--	1.74 (1.04-2.90)	0.032
Allele	A	53 (50.0)	202 (62.0)	--	--
	C	53 (50.0)	126 (38.0)	-- --	

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

Table 20: Genotypes distribution of VDR; rs2228570 and VDR; rs1544410 in moderate/severe asthmatics and controls

Model	Genotype/ Allele	Moderate/ Severe Asthmatics n (%)	Controls n (%)	OR (95% CI)*	p-value*
VDR; rs2228570					
Codominant	G/G	33 (62.3)	96 (58.5)	1.00	
	A/G	18 (34.0)	59 (36.0)	0.95 (0.45-2.00)	0.97
	A/A	2 (3.8)	9 (5.5)	1.17 (0.21-6.53)	
Dominant	G/G	33 (62.3)	96 (58.5)	1.00	0.93
	A/G-A/A	20 (37.7)	68 (41.5)	0.97 (0.47-1.99)	
Recessive	G/G-A/G	51 (96.2)	155 (94.5)	1.00	0.84
	A/A	2 (3.8)	9 (5.5)	1.19 (0.22-6.53)	
Over-dominant	G/G-A/A	35 (66.0)	105 (64.0)	1.00	0.87
	A/G	18 (34.0)	59 (36.0)	0.94 (0.45-1.97)	
Log-additive	-----	--	--	1.00 (054-1.84)	1.0
Allele	G	84 (79.0)	251 (77.0)	--	--
	A	22 (21.0)	77 (23.0)	--	--
VDR; rs1544410					
Codominant	G/G	24 (45.3)	54 (32.9)	1.00	
	A/G	23 (43.4)	83 (50.6)	0.56 (0.26-1.21)	0.17
	A/A	6 (11.3)	27 (16.5)	0.4 (0.13-1.22)	
Dominant	G/G	24 (45.3)	54 (32.9)	1.00	0.075
	A/G-A/A	29 (54.7)	110 (67.1)	0.52 (0.25-1.07)	
Recessive	G/G-A/G	47 (88.7)	137 (83.5)	1.00	0.24
	A/A	6 (11.3)	27 (16.5)	0.55 (0.20-1.55)	
Over-dominant	G/G-A/A	30 (56.6)	81 (49.4)	1.00	0.39
	A/G	23 (43.4)	83 (50.6)	0.73 (0.36-1.48)	
Log-additive	-----	--	--	0.61 (0.36-1.04)	0.063
Allele	G	71 (67.0)	191 (58.0)	--	--
	A	35 (33.0)	137 (42.0)	--	--

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

Table 21: Genotypes distribution of CYP2R1; rs12794714 moderate/severe asthmatics and controls

Model	Genotype/ allele	Controls n (%)	Moderate/ Severe Asthmatics n (%)	OR (95% CI)*	p-value*
CYP2R1; rs12794714					
	G/G	17 (32.1)	64 (39.0)	1.00	
Codominant	A/G	23 (43.4)	70 (42.7)	1.13 (0.51-2.54)	0.63
	A/A	13 (24.5)	30 (18.3)	1.59 (0.61-4.15)	
Dominant	G/G	17 (32.1)	64 (39.0)	1.00	0.54
	A/G-A/A	36 (67.9)	100 (61.0)	1.26 (0.60-2.67)	
Recessive	G/G-A/G	40 (75.5)	134 (81.7)	1.00	0.36
	A/A	13 (24.5)	30 (18.3)	1.48 (0.64-3.43)	
Over-dominant	G/G-A/A	30 (56.6)	94 (57.3)	1.00	0.88
	A/G	23 (43.4)	70 (42.7)	0.95 (0.47-1.91)	
Log-additive	-----	--	--	1.25 (0.77-2.02)	0.36
Allele	G	57 (54.0)	198 (60.0)	--	--
	A	49 (46.0)	130 (40.0)	--	--

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

3.2.4 Genotypes of Mild Intermittent/Persistent and Moderate/Severe Asthmatics

No significant statistical difference in genotyping between the mild intermittent/persistent group and moderate/severe asthmatic group for the seven SNPs in vitamin D binding protein, vitamin D receptor, and vitamin D 25-hydroxylase (Tables 22-25).

Table 22: Genotypes distribution of DBP; rs4588 and DBP; rs7041 in mild intermittent/persistent and moderate/severe asthmatics

Model	Genotype/ allele	(MILD) n (%)	Moderate/ Severe Asthmatics n (%)	OR (95% CI)*	p-value*
DBP; rs4588					
Codominant	G/G	58 (73.4)	34 (64.2)	1.00	
	G/T	17 (21.5)	18 (34.0)	1.86 (0.84-4.12)	0.21
	T/T	4 (5.1)	1 (1.95)	1.00	
Dominant	G/G	58 (73.4)	34 (64.2)	1.6 (0.75-3.43)	0.23
	G/T-T/T	21 (26.6)	19 (35.9)	1.00	
Recessive	G/G-G/T	75 (94.9)	52 (98.1)	0.39 (0.04-3.59)	0.37
	T/T	4 (5.1)	1 (1.9)	1.00	
Over-dominant	G/G-T/T	62 (78.5)	35 (66.0)	1.92 (0.87-4.25)	0.10
	G/T	17 (21.5)	18 (34.0)	1.00	
Log-additive	-----	--	--	1.26 (0.67-2.39)	0.48
Allele	G	133 (84.0)	86 (81.0)	--	--
	T	25 (16.0)	20 (19.0)	--	--
DBP; rs7041					
Codominant	C/C	30 (38.0)	15 (28.3)	1.00	
	A/C	35 (44.3)	26 (49.15)	1.36 (0.60-3.09)	0.59
	A/A	14 (17.7)	12 (22.6)	1.64 (06-4.46)	
Dominant	C/C	30 (38.0)	15 (28.3)	1.00	0.34
	A/C-A/A	49 (62.0)	38 (71.7)	1.44 (0.67-3.11)	
Recessive	C/C-A/C	65 (82.3)	41 (77.4)	1.00	0.48
	A/A	14 (17.7)	12 (22.6)	1.37 (0.57-3.27)	
Over-dominant	C/C-A/A	44 (55.7)	27 (50.9)	1.00	0.75
	A/C	35 (44.3)	26 (49.1)	1.12 (0.55-2.29)	
Log-additive	-----	--	--	1.29 (0.79-2.11)	0.31
Allele	C	95 (60.0)	56 (53.0)	--	--
	A	63 (40.0)	50 (47.0)	--	--

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

Table 23: Genotypes distribution of VDR; rs731236 and VDR; rs7975232 in mild intermittent/persistent and moderate/severe asthmatics

Model	**Genotype/ allele**	**(MILD) n (%)**	**Moderate/ Severe Asthmatics n (%)**	**OR (95% CI)***	**p-value***
VDR; rs731236					
Codominant	A/A	27 (34.2)	26 (49.1)	1.00	
	A/G	41 (51.9)	21 (39.6)	0.55 (0.26-1.19)	0.29
	G/G	11 (13.9)	6 (11.3)	0.6 (0.19-1.89)	
Dominant	A/A	27 (34.2)	26 (49.1)	1.00	0.12
	A/G-G/G	52 (86.1)	27 (50.9)	0.56 (0.27-1.16)	
Recessive	A/A-A/G	68 (86.1)	47 (88.7)	1.00	0.72
	G/G	11 (13.9)	6 (11.3)	0.82 (0.28-2.4)	
Over-dominant	A/A-G/G	38 (48.1)	32 (60.4)	1.00	0.19
	A/G	41 (51.9)	21 (39.6)	0.62 (0.31-1.27)	
Log-additive	-----	--	--	0.7 (0.41-1.2)	0.19
Allele	A	95 (60.0)	73 (69.0)	--	--
	G	63 (40.0)	33 (31.0)	--	--
VDR; rs7975232					
Codominant	A/A	24 (30.8)	13 (24.5)	1.00	
	A/C	43 (55.1)	27 (50.95)	1.09 (0.47-2.54)	0.38
	C/C	11 (14.1)	13 (24.5)	2.00 (0.69-5.79)	
Dominant	A/A	24 (30.8)	13 (24.5)	1.00	0.54
	A/C-C/C	54 (69.2)	40 (75.5)	1.28 (0.58-2.86)	
Recessive	A/A-A/C	67 (85.9)	40 (75.5)	1.00	0.17
	C/C	11 (14.1)	13 (24.5)	1.89 (076-4.69)	
Over-dominant	A/A-C/C	35 (44.9)	26 (49.1)	1.00	0.59
	A/C	43 (55.1)	27 (50.9)	0.82 (04-1.68)	
Log-additive	-----	--	--	1.38 (0.81-2.35)	0.23
Allele	A	91 (58.0)	53 (50.0)	--	--
	C	65 (42.0)	53 (50.0)	--	--

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

Table 24: Genotypes distribution of VDR; rs2228570 and VDR; rs1544410 in mild intermittent/persistent and moderate/severe asthmatics

Model	Genotype/ Allele	(MILD) n (%)	Moderate/ Severe Asthmatics n (%)	OR (95% CI)*	p-value*
VDR; rs2228570					
Codominant	G/G	49 (62.0)	33 (62.3)	1.00	
	A/G	28 (35.4)	18 (34.0)	0.94 (0.45-1.98)	0.86
	A/A	2 (2.5)	2 (3.8)	1.71 (0.22-13.26)	
Dominant	G/G	49 (62.0)	33 (62.3)	1.00	0.97
	A/G-A/A	30 (38.0)	20 (37.7)	0.99 (0.48-2.03)	
Recessive	G/G-A/G	77 (97.5)	51 (96.2)	1.00	0.59
	A/A	2 (2.5%)	2 (3.8)	1.74 (0.23-13.32)	
Over-dominant	G/G-A/A	51 (64.6)	35 (66.0)	1.00	0.82
	A/G	28 (35.4)	18 (34.0)	0.92 (0.44-1.92)	
Log-additive	-----	--	--	1.04 (0.55-1.98)	0.89
Allele	G	126 (80.0)	84 (79.0)		
	A	32 (20.0)	22 (21.0)		
VDR; rs1544410					
Codominant	G/G	26 (32.9)	24 (45.3)	1.00	
	A/G	41 (51.9)	23 (43.4)	0.66 (0.30-1.42)	0.49
	A/A	12 (15.2)	6 (11.3)	0.6 (0.19-1.88)	
Dominant	G/G	26 (32.9)	24 (45.3)	1.00	0.24
	A/G-A/A	53 (67.1)	29 (54.7)	0.64 (0.31-1.34)	
Recessive	G/G-A/G	67 (84.8)	47 (88.7)	1.00	0.61
	A/A	12 (15.2)	6 (11.3)	0.76 (0.26-2.19)	
Over-dominant	G/G-A/A	38 (48.1)	30 (56.6)	1.00	0.43
	A/G	41 (51.9)	23 (43.4)	0.75 (0.37-1.53)	
Log-additive	-----	--	--	0.74 (0.43-1.26)	0.27
Allele	G	93 (59.0)	71 (67.0)		
	A	65 (41.0)	35 (33.0)		

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

Table 25: Genotypes distribution of CYP2R1; rs12794714 in mild intermittent/persistent and moderate/severe asthmatics

Model	Genotype/ allele	(MILD) n (%)	Moderate/ Severe Asthmatics n (%)	OR (95% CI)*	p-value*
CYP2R1; rs12794714					
	G/G	31 (39.2)	17 (32.1)	1.00	
Codominant	A/G	33 (41.8)	23 (43.4)	1.23 (0.55-2.74)	0.74
	A/A	15 (19.0)	13 (24.5)	1.45 (0.55-3.8)	
Dominant	G/G	31 (39.2)	17 (32.1)	1.00	0.49
	A/G-A/A	48 (60.8)	36 (67.9)	1.3 (0.62-2.72)	
Recessive	G/G-A/G	64 (81.0)	40 (75.5)	1.00	0.56
	A/A	15 (19.0)	13 (24.5)	1.29 (0.55-3.04)	
Over-dominant	G/G-A/A	46 (58.2)	30 (56.6)	1.00	0.85
	A/G	33 (41.8)	23 (43.4)	1.07 (0.53-2.17)	
Log-additive	-----	--	--	1.21 (0.75-1.94)	0.44
Allele	G	95 (60.0)	57 (54.0)	--	--
	A	63 (40.0)	49 (46.0)	--	--

* = Adjusted for age and gender, OR = odds ratio, CI = confidence interval

3.3 Linkage Disequilibrium and Haplotypes

3.3.1 Linkage Disequilibrium and Haplotypes of Asthmatics and Controls

Linkage disequilibrium (LD) analysis was used to identify the combined function of genes. The association between *VDR* SNPs were assessed by measuring the LD between polymorphisms using r^2 and D' (0-1). The location of each tested SNPs on the chromosome was indicated on the top of the diamond (Figures 9, 10 and 11). The number in each diamond indicates the LD (D') divided on 100 (Diamond without value means D' is equal 1). The legs of each triangle show the LD for two markers. The haplotype in each block was listed with their frequency below each diamond block (Haploview package version 4.2). Diamond colors indication in figures for D' values: white 0-0.2, pink 0.2-0.5, light red 0.5-0.8 and dark red 0.8-1.0. Linkage disequilibrium (LD) was calculated for two SNPs (rs4588 and rs7041) in the *DBP*

gene. The calculated LD for all cases (asthmatics and controls), asthmatic patients and controls D'=1, while r^2 values were 0.29, 0.27 and 0.31, respectively (Figure 10).

LD was calculated for four SNPs (rs731236, rs7975232, rs1544410, and rs2228570) in the *VDR* gene. The disequilibrium coefficient r^2 between *VDR*; rs731236 and *VDR*; rs1544410 was higher in asthmatic cases compared with controls. The disequilibrium coefficient D'(r^2) for all cases (asthmatics and controls), asthmatic patients, and controls, as well as severe, were 0.89(0.74), 0.91(0.78), 0.88(0.71) and 0.95(0.83), respectively (Figure 9 and 11). The presence of rs2228570 outside the *VDR* block means that it was not in LD with any of the other three SNPs.

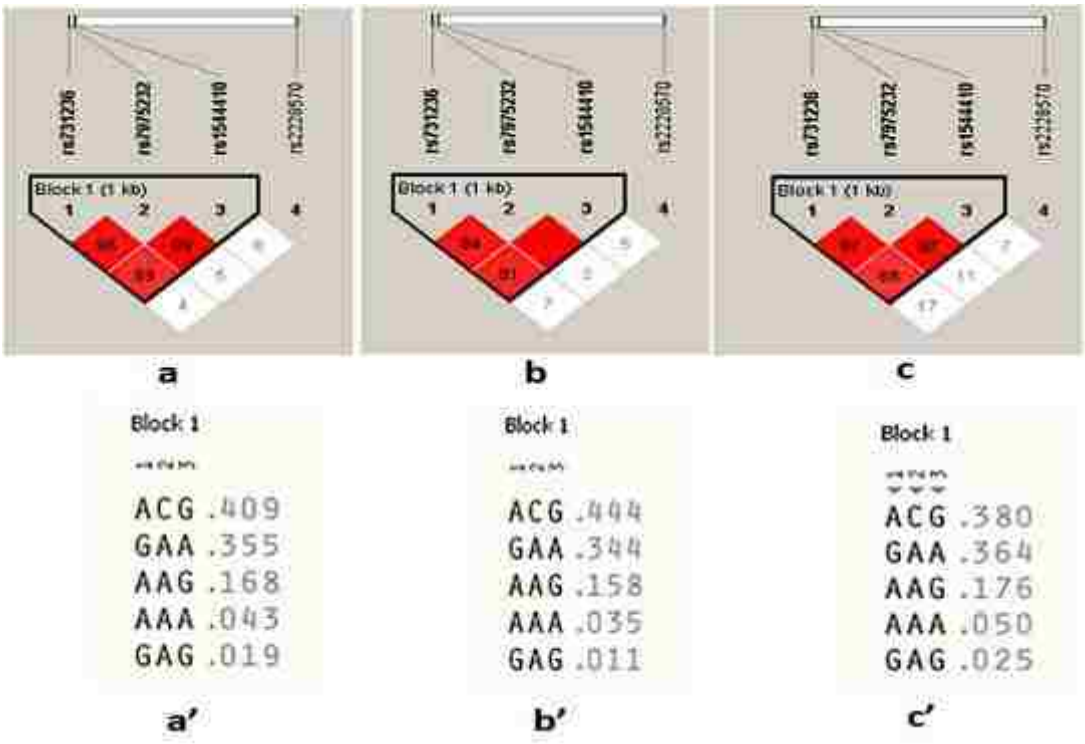

Figure 9: Linkage disequilibrium and haplotype block structure in the VDR gene

D' multiplied by 100 for all controls and asthmatic cases (a) haplotype frequency in all controls and asthmatic cases (a`), asthma cases (b) haplotype frequency in asthmatic cases (b`) controls (c) haplotype frequency in Controls (c`).

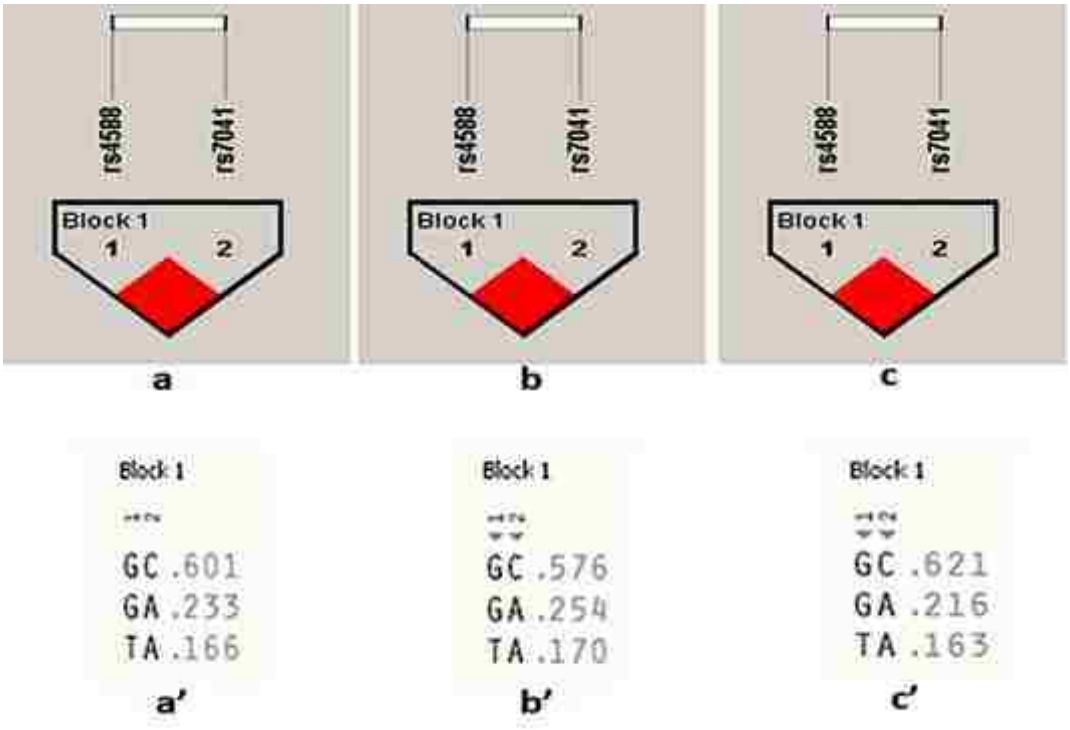

Figure 10: Linkage disequilibrium and haplotype block structure in the DBP gene D' multiplied by 100 for all controls and asthmatic cases (a) haplotype frequency (a`), asthma cases (b) haplotype frequency in asthmatic cases (b`) controls (c) haplotype frequency in Controls (c`).

3.3.2 Linkage Disequilibrium and Haplotypes of Moderate/Severe Asthmatics and Controls

Strong LD was observed at 3` end of the *VDR* gene. In moderate/severe cases the LD between *VDR*; rs7975232 and the other two SNPs rs731236 and *VDR*; rs1544410) were D' (r^2) = 0.92(0.38) and D'(r^2) = 1(0.49) respectively. The SNP *VDR*; rs2228570 was not linked to the other three SNPs (Figure 11). The LD between *DBP*; rs4588 and *DBP*; rs7041 was D' (r^2) = 1(0.26).

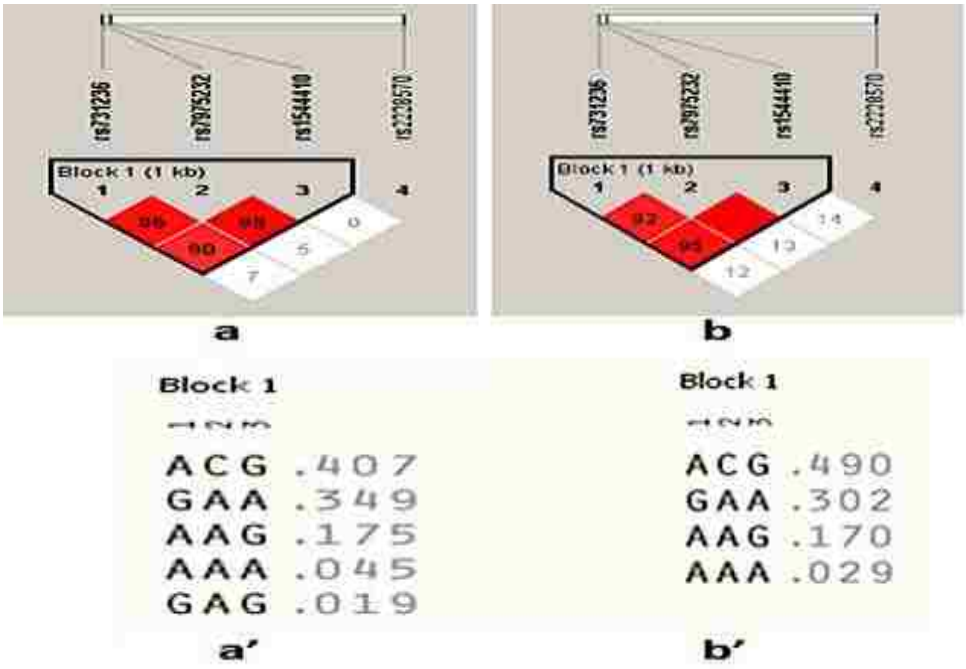

Figure 11: Linkage disequilibrium and haplotype block structure in moderate/severe asthmatics and controls.

D' multiplied with 100 for all controls and moderate/severe asthmatics (a) haplotype frequency (a`), moderate/severe asthmatics (b) haplotype frequency in moderate/severe asthmatics (b`).

3.4 Haplotypes

Haplotype analysis for *DBP* and *VDR* gene polymorphisms was carried out because of the observed LD between the SNPs in each gene. The association between haplotype and risk of asthma was listed in (Tables 26 and 27). The haplotype frequency between asthmatic cases and controls, no statistically significant difference between asthmatic cases and controls haplotypes (Table 26). In contrast, the haplotype frequency and association between severe cases and controls, ACG haplotype for polymorphisms rs731236, rs7975232 and rs1544410 in the *VDR* gene were more dominant in moderate/severe cases compared with controls (Table 27). The presence of ACG haplotype increased the risk of asthma severity ($p = 0.045$).

Table 26: Haplotype frequency in asthmatics and controls

SNP	Haplotype	Frequency	Frequency		χ^2	p-value
			Asthmatics	Controls		
DBP						
rs4588 rs7041	GC	0.601	0.576	0.621	1.254	0.2628
	GA	0.233	0.254	0.216	1.166	0.2801
	TA	0.166	0.170	0.163	0.061	0.8050
VDR						
rs731236 rs7975232 rs1544410	ACG	0.409	0.444	0.380	2.464	0.1165
	GAA	0.355	0.343	0.365	0.288	0.5917
	AAG	0.168	0.158	0.177	0.393	0.5307
	AAA	0.043	0.035	0.050	0.772	0.3797
	GAG	0.019	0.012	0.025	1.293	0.2555

SNP = single nucleotide polymorphism, χ^2 = Chi-square, *VDR* = Vitamin D receptors, *DBP* = Vitamin D binding protein.

Table 27: Haplotype frequency in moderate/severe asthmatics and controls

SNP	Haplotype	Frequency	Frequency		χ^2	p-value
			Moderate/ Severe asthmatics	Controls		
DBP						
rs4588 rs7041	GC	0.598	0.528	0.621	2.871	0.090
	GA	0.232	0.283	0.216	2.015	0.155
	TA	0.169	0.189	0.163	0.380	0.537
VDR						
rs731236 rs7975232 rs1544410	ACG	0.407	0.490	0.380	4.007	**0.045**
	GAA	0.349	0.301	0.364	1.436	0.230
	AAG	0.174	0.170	0.177	0.030	0.863
	AAA	0.043	0.029	0.050	0.797	0.372
	GAG	0.019	0.001	0.025	2.520	0.112

SNP = single nucleotide polymorphism, χ^2 = Chi-square, *VDR* = Vitamin D receptors, *DBP* = Vitamin D binding protein.

3.5 Minor Allele Frequencies

Based on the data obtained from the Haploview software, there was a highly significant difference between severe asthmatic cases and controls in the frequency of allele C in *VDR*; rs7975232 polymorphisms ($p = 0.035$, $\chi^2 = 4.437$, Table 28). MAF

was more than 16% for all alleles and SNPs conformed to HWE with p-value > 0.05. There was no statistically significant difference between all asthmatics and controls or between moderate/severe and mild intermittent/persistent asthmatics (Table 29 and 30)

Table 28: Alleles frequency in moderate/severe asthmatics and controls

SNP	Allele	Frequency		χ^2	p-value	Allele	HWE p-value	MAF
		Moderate/ Severe asthmatics	Controls					
DBP								
rs4588	T	0.189	0.163	0.388	0.533	G:T	0.797	0.169
rs7041	A	0.472	0.377	2.969	0.084	C:A	0.397	0.400
VDR								
rs731236	A	0.689	0.607	2.300	0.129	A:G	0.686	0.373
rs7975232	C	0.500	0.384	4.437	0.035	A:C	1.000	0.412
rs1544410	G	0.670	0.582	2.563	0.109	G:A	0.897	0.396
rs2228570	G	0.792	0.765	0.337	0.561	G:A	1.000	0.228

SNP = single nucleotide polymorphism, χ^2 = Chi-square, HWE = Hardy-Weinberg Equilibrium, MAF = minor allele frequency, *VDR* = Vitamin D receptors, *DBP* = Vitamin D binding protein.

Table 29: Allele frequency in asthmatics and controls

SNP	Allele	Frequency		χ^2	p-value	Allele	HWE p-value	MAF
		Asthmatics	Controls					
DBP								
rs4588	T	0.170	0.163	0.065	0.7982	G:T	0.8321	0.166
rs7041	A	0.424	0.377	1.341	0.2468	C:A	0.2414	0.398
VDR								
rs731236	A	0.636	0.607	0.546	0.4600	A:G	1.0000	0.380
rs7975232	C	0.450	0.384	2.635	0.1045	A:C	0.6604	0.414
rs1544410	G	0.621	0.582	0.922	0.3370	G:A	0.6602	0.400
rs2228570	G	0.795	0.765	0.775	0.3788	G:A	0.7716	0.221

SNP = single nucleotide polymorphism, χ^2 = Chi-square, HWE = Hardy-Weinberg Equilibrium, MAF = minor allele frequency, *VDR* = Vitamin D receptors, *DBP* = Vitamin D binding protein.

Table 30: Allele frequency in mild intermittent/persistent and moderate/severe asthmatics

SNP	Allele	Frequency		χ^2	p-value
		Moderate/ Severe Asthmatics	(Mild) Asthmatics		
DBP; rs4588	T	0.189	0.158	0.416	0.5189
DBP; rs7041	A	0.472	0.392	1.633	0.2013
VDR; rs731236	A	0.689	0.601	2.095	0.1478
VDR; rs7975232	C	0.500	0.417	1.771	0.1833
VDR; rs1544410	G	0.670	0.589	1.778	0.1824
VDR; rs2228570	G	0.792	0.797	0.010	0.9211

SNP = single nucleotide polymorphism, X^2 = Chi-square

Then the MAF was calculated for control and asthmatic cases separately, and all the tested SNPs conformed to HWE in both controls and asthmatic cases with value > 0.05 (Table 31).

Table 31: Minor allele frequency comparison between controls and asthmatics

SNP	Allele	Controls		Asthmatics	
		HWE p-value	MAF	HW p-value	MAF
DBP; rs4588	G:T	1.0000	0.163	0.6230	0.1700
DBP; rs7041	C:A	0.4195	0.377	0.5051	0.4240
VDR; rs731236	A:G	0.9321	0.393	1.0000	0.3640
VDR; rs7975232	A:C	1.0000	0.385	0.4912	0.4500
VDR; rs1544410	G:A	0.7515	0.418	0.9082	0.3790
VDR; rs2228570	G:A	1.0000	0.235	0.6311	0.2050

SNP = single nucleotide polymorphism, HWE = Hardy-Weinberg equilibrium, MAF = minor allele frequency.

3.6 Vitamin D Levels

Descriptive statistic was used to study vitamin D level in mild intermittent/persistent, moderate/severe and controls. Vitamin D level < 50 nmol/l was considered indicative of vitamin D insufficiency were presented as mean, standard deviation (SD) and interquartile range (IQR) for vitamin D (Table 32). The frequency of sufficient vitamin D levels was higher in moderate/severe asthmatics than controls, 50.9% versus 44.0% respectively. There was no statistically significant difference between mild intermittent/persistent asthmatics, moderate/severe asthmatics and controls p-value > 0.05. This difference in vitamin D levels distribution among the three groups is presented as a boxplot graph (Figure 12), that showed the outliers who had vitamin D levels > 100 nmol/l.

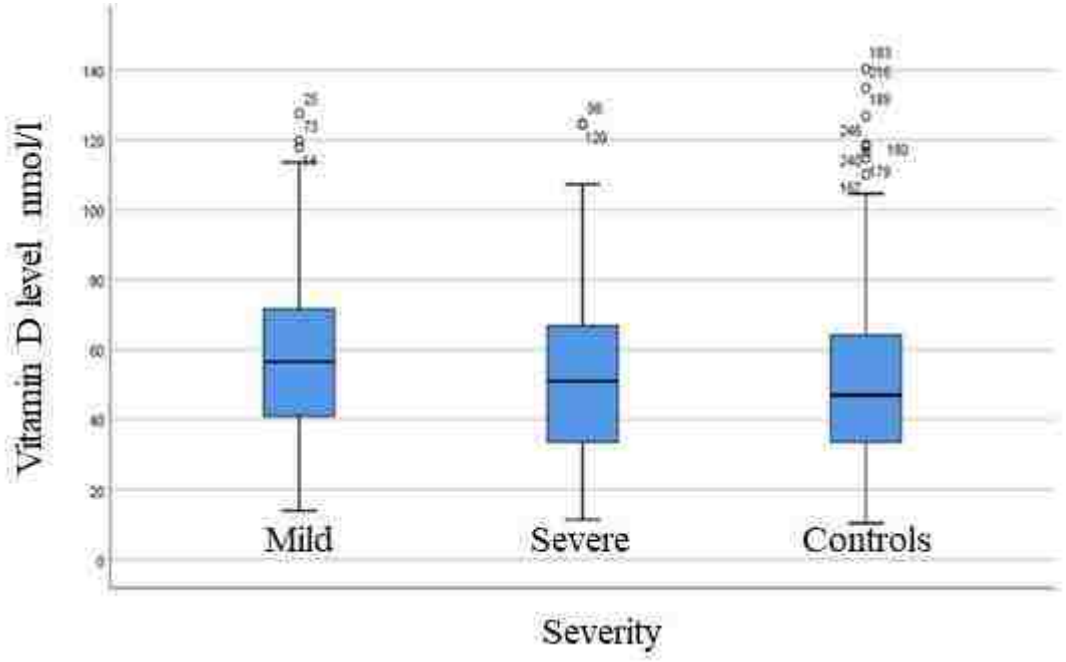

Figure 12: Vitamin D concentrations (nmol/l) in mild intermittent/persistent [Mild] asthmatics, moderate/severe asthmatics [Severe] and controls

Table 32: Vitamin D concentrations (nmol/l) in mild intermittent/persistent (MILD) asthmatics, moderate/severe asthmatics and controls

Group	**Males n (%)**	**Females n (%)**	**Vitamin D concentration mean (SD)**	**IQR**	**95% CI**	**50%**	**Vitamin D deficient* n (%)**	**Vitamin D non-deficient** n (%)**
(MILD)	20 (28)	59 (75)	58 (25)	32	52-63	56.60	30 (38.0)	49 (62.0)
Moderate/Severe	12 (23)	41 (77)	54 (26)	36	46-61	51.10	26 (49.1)	27 (50.9)
Controls	6 (4)	158 (96)	52 (26)	31	48-56	47.00	91 (56.0)	73 (44.0)

IQR = interquartile range, CI = confidence interval, * = < 50 nmol/l, ** = >50 nmol/l

To verify the association of vitamin D metabolic genes polymorphisms and vitamin D levels; the SNPs were studied per genotype and vitamin D levels in controls, mild intermittent/persistent (MILD) and moderate/severe asthmatics. There was no statistically significant difference in vitamin D levels between the three groups (Tables 33-35). Vitamin D supplementation was frequent among the asthmatics as 56.6% with sufficient vitamin D levels were under vitamin D supplementation, no information about Vitamin D supplementation for the controls. Therefore, because of Vitamin D supplementation, the results of vitamin D levels were of no value in testing for the association of Vitamin D levels with asthma.

Table 33: Vitamin D concentrations (nmol/l) according to SNPs genotypes in controls

SNP	Genotyping	n	Vitamin D mean (SD)	IQR	75%	50%
	GG	114	52.68 (26.85)	30	63.90	47.30
DBP; rs4588	GT	44	49.20 (22.97)	28	59.18	43.35
	TT	4	70.18 (40.83)	74	111.30	62.20
	AA	26	53.20 (25.30)	29	64.83	49.55
DBP; rs7041	AC	70	52.19 (25.50)	33	65.85	47.55
	CC	66	51.74 (27.70)	29	62.55	45.80
	AA	59	52.37 (27.00)	25	59.10	46.50
VDR; rs731236	AG	77	51.13 (27.30)	33	64.20	44.40
	GG	25	54.79 (21.50)	32	69.55	55.50
	AA	62	55.78 (27.40)	33	69.55	52.75
VDR; rs7975232	AC	76	51.06 (26.20)	30	63.25	46.55
	CC	24	46.37 (22.65)	20	52.57	43.35
	AA	29	53.79 (28.33)	34	67.40	44.00
CYP2R1; rs12794714	AG	70	50.44 (24.34)	36	67.20	48.80
	GG	63	53.35 (27.58)	27	65.60	46.40
	AA	27	55.60 (27.06)	36	70.00	54.00
VDR; rs1544410	AG	83	50.16 (25.15)	30	61.70	44.30
	GG	52	53.59 (27.70)	26	61.43	46.95
	AA	9	39.60 (17.25)	29	50.90	41.50
VDR; rs2228570	AG	58	52.91 (28.44)	38	69.05	47.00
	GG	95	52.90 (25.45)	30	63.60	47.70

IQR = interquartile range, SD = standard deviation, percentile 50% and 75%

Table 34: Vitamin D concentrations (nmol/l) according to SNPs genotypes in mild intermittent/persistent asthmatics

SNP	Genotype	n	Vitamin D mean (SD)	IQR	75%	50%
	GG	57	85.46 (25.47)	28	70.65	53.30
DBP; rs4588	GT	17	57.96 (27.98)	46	79.95	56.80
	TT	4	46.47 (24.19)	45	67.25	48.80
	AA	14	58.62 (24.90)	39	74.73	61.55
DBP; rs7041	AC	34	59.62 (27.50)	30	72.48	52.95
	CC	30	55.19 (25.22)	37	70.68	54.90
	AA	26	60.79 (28.00)	32	72.60	57.70
VDR; rs731236	AG	41	56.49 (25.90)	34	69.80	53.30
	GG	11	55.15 (20.24)	30	73.10	50.20
	AA	24	56.69 (26.50)	40	74.30	57.60
VDR; rs7975232	AC	43	58.31 (28.05)	33	70.10	53.30
	CC	11	57.79 (13.89)	26	73.90	57.50
	AA	15	55.23 (24.40)	31	72.40	56.50
CYP2R1; rs12794714	AG	32	50.79 (21.90)	33	65.13	49.95
	GG	31	66.13 (28.30)	40	85.20	62.10
	AA	12	55.04 (22.00)	40	74.30	53.35
VDR; rs1544410	AG	41	54.61 (26.70)	33	67.20	52.80
	GG	25	64.16 (25.60)	25	72.80	57.90
	AA	2	71.45 (59.00)	--	--	--
VDR; rs2228570	AG	27	57.73 (17.90)	25	70.30	53.30
	GG	49	57.18 (28.40)	40	72.70	56.50

IQR = interquartile range, SD = standard deviation, percentile 50% and 75%

Table 35: Vitamin D concentrations (nmol/l) according to SNPs genotypes in moderate/severe asthmatics

SNP	Genotype	n	Vitamin D mean (SD)	IQR	75%	50%
	GG	34	59.10 (27.36)	39	76.55	53.50
DBP; rs4588	GT	18	45.76 (21.77)	34	62.80	39.85
	TT	1	--	--	--	--
	AA	12	44.00 (17.88)	31	59.90	43.95
DBP; rs7041	AC	26	55.45 (25.70)	39	73.63	53.50
	CC	15	58.78 (31.90)	42	75.60	53.50
	AA	26	58.90 (26.80)	52	72.68	53.75
VDR; rs731236	AG	21	49.67 (27.60)	35	62.40	38.60
	GG	6	46.65 (15.32)	27	60.35	42.70
	AA	13	46.58 (23.80)	26	59.50	37.80
VDR; rs7975232	AC	27	54.90 (25.40)	32	66.80	43.50
	CC	13	59.08 (30.87)	49	80.50	43.50
	AA	13	58.60 (27.04)	24	67.00	53.50
CYP2R1; rs12794714	AG	23	52.30 (26.25)	30	62.50	47.90
	GG	17	52.44 (26.81)	49	77.50	55.10
	AA	6	46.65 (15.32)	27	60.00	42.70
VDR; rs1544410	AG	23	50.90 (28.40)	34	62.50	47.90
	GG	24	58.57 (26.18)	32	71.60	43.75
	AA	2	33.15 (06.81)	--	--	33.15
VDR; rs2228570	AG	18	60.59 (29.00)	45	83.45	55.90
	GG	33	51.49 (24.50)	34	65.25	51.00

IQR = interquartile range, SD = standard deviation, percentile 50% and 75%

Chapter 4: Discussion

This study addresses the genetic and immunological relationship between asthma and vitamin D, a link that is still not clear. Worldwide, several studies have investigated the association between asthma and vitamin D levels as well as between asthma and polymorphism in genes involved vitamin D metabolism. In UAE, as in many other countries, the prevalence of both asthma and vitamin D deficiency have witnessed a steady increase over time, and asthma is expected to affect more people by 2025. This study is the first in the UAE that investigates the association between asthma and single nucleotide polymorphisms (SNPs) in vitamin D metabolic genes and vitamin D levels in adult Emiratis. Genetic association studies play significant roles in understanding complex diseases like asthma.

Seven SNPs in three genes (*GC*; rs4588, *GC*; rs7041, *VDR*; rs731236, *VDR*; rs7975232, *VDR*; rs1544410, *VDR*; rs2228570 and *CYP2R1*; rs12794714) have been studied. No significant difference was observed in the seven SNPs between mild asthmatics and controls. Most of the asthmatics in this study were classified as having mild intermittent or mild persistent asthma, and this may explain that no difference was found in genotypes or allele distributions of the seven SNPs between all asthmatics and controls. In agreement with a study reported that the features of severe asthma are different from mild-to-moderate asthma (Group, 2003).

Our findings suggest that *VDR*; rs7975232 CC homozygous recessive genotype is associated with a higher risk (2.7 times) of moderate/severe asthma and there was a significant difference in the genotype distribution between moderate/severe asthmatics when compared with controls. The distributions of allele C and genotype CC of *VDR*; rs7975232 polymorphism showed a significantly higher

prevalence in the moderate/severe asthmatics compared to controls in dominant, codominant, recessive and additive models and the C allele in moderate/severe asthmatics was 50% compared to 38% in controls. The difference in *VDR*; rs7975232 polymorphism between moderate/severe asthmatics and controls is in agreement with a previous report that in childhood and adult asthma C allele in *VDR*; rs7975232 is highly associated with increased asthma risk (Raby et al., 2004). Moreover, in a case-control study, Saadi and collaborators reported a significant association between *VDR*; rs7975232 polymorphism with high risk of asthma, and the allele C was highly dominant in asthmatic patients and associated with increasing the risk of asthma (Saadi et al. 2009) where the CC genotype was over-represented in asthmatics (55%) compared to controls (47%). The increased risk of asthma is supported by two meta-analyses (Han et al., 2016; Zhao et al., 2017).

On the other hand, case-control studies from Serbian, Tunisian, Santiago, Cypriot and Iranian did not find any statistical association of *VDR*; rs7975232 genotype CC or allele C with risk of asthma (Despotovic et al., 2017; Einisman et al., 2015; Maalmi et al., 2013; Papadopoulou et al., 2015; Poon et al., 2004). The lack of association in the above studies may be attributed to ethnic difference, small sample size or variation in the method employed for SNPs genotyping. The Meta-analyses studies have the advantage over individual studies because a meta-analysis includes many ethnic groups and the combined sample size is large enough to avoid lack of power that many primary studies may suffer.

The two other *VDR* mutations (rs731236 and rs1544410) were marginally associated with risk of asthma severity. The *VDR*; rs731236 polymorphism allele (A) showed a marginal association with lower asthma risk. In a study by Raby and

coworkers the minor allele (A) of *VDR*; rs731236 showed protective effects against asthma (Raby et al., 2004). In contrast to a study reported that both the *VDR*; rs731236 homozygous AA and heterozygous AG genotypes showed significant association with increased risk of asthma compared with homozygous genotype GG (Maalmi et al., 2013). In a case-control study from Cypriot, increase the risk of asthma was conferred by *VDR*; rs731236 homozygous minor genotype GG (Papadopoulou et al., 2015). In a meta-analysis study, *VDR*; rs731236 AA genotype showed a statistically significant association with an increased risk of asthma (Han et al., 2016). However, other studies failed to find any association between *VDR*; rs731236 genotypes and asthma (Despotovic et al., 2017; Zhao et al., 2017).

In this study, the SNP *VDR*; rs1544410 GG genotype has a marginal association with a lower risk of asthma severity. In contrast, a meta-analysis study in childhood concluded the *VDR*; rs1544410 polymorphism marginally associated with increased risk of childhood asthma (Zhao et al., 2017). A survey in Tunisian children by (Maalmi et al., 2013) reported that *VDR*; rs1544410 is significantly associated with asthma. Other studies showed no association between *VDR*; rs1544410 genotypes and asthma (Despotovic et al., 2017; Einisman et al., 2015; Papadopoulou et al., 2015; Saadi et al., 2009). Several of the studies mentioned above were done in childhood asthma settings and in a different ethnic group, therefore, their findings may not comparable to this study.

The three *VDR* SNPs (rs731236, rs7975232, and rs1544410) ACG haplotype was significantly associated with asthma severity (p-value = 0.045), and there is strong linkage disequilibrium between these three SNPs in moderate/severe asthmatics.

The *VDR*; rs2228570 polymorphism is functional and has an impact on VDR protein synthesis. It is a missense mutation that affects a start codon that produces more active and smaller size protein with 424 amino acids (Tizaoui et al., 2014). In this study, *VDR*; rs2228570 polymorphism has no association with asthma severity. This lack of association is in agreement with previous studies (Raby et al., 2004; Saadi et al., 2009). However, other studies found an association between *VDR*; rs2228570 genotypes and asthma (Han et al., 2016; Ismail et al., 2013; Maalmi et al., 2013).

In our study, two non-synonymous SNPs (*GC*; rs7041 and *GC*; rs4588) in vitamin D binding protein gene showed no association with asthma severity. The lack of association is in agreement with a study carried out on Tunisian adults (Lahmar et al., 2018) and in contrast with a survey on Chinese Han adults (Li et al., 2011).

The *CYP2R1* rs12794714 polymorphism is a synonymous mutation in exon 12. There was no statistically significant association between *CYP2R1* rs12794714 genotypes and risk of asthma severity. The lack of association is in agreement with (Li et al., 2011) and contrast with (Lahmar et al., 2018).

The reason behind the conflicting results in the genetic association studies may be due to the difference in allele frequency between different ethnicity (Elkum et al., 2014). In this study, the allelic frequency of *VDR*; rs731236 (61%) and *VDR*; rs1544410 (42%) polymorphisms in controls are in agreement with those reported from UAE in adult Emiratis (61%) for *VDR*; rs731236 and (43%) for *VDR*; rs1544410 (Al Safar et al., 2018). Minor Allele Frequency (MAF) is different between populations, for example, MAF of *VDR*; rs731236 is 43% in Caucasian whereas, it is 8% in Asian, such difference refers to the random time that the polymorphic mutation started before it becomes true polymorphism within a particular ethnic group. The

random introduction of mutations may explain the difference in SNPs frequency between the different ethnic groups. The three *VDR* (rs731236, rs7975232, and rs1544410) polymorphisms are nonfunctional with strong LD, but this LD is weak in some ethnic groups (Uitterlinden et al., 2002; Whitfield et al., 2001). The difference in the population composition may explain why the results of LD were different from other studies. Moreover, gene-environment interactions may affect the association studies, as asthma generally is attributed to both genetic and environmental factors. Study design including (methodology, criteria for selecting the participants, participant age and sex, sample size, the difference in statistical data analysis) all these might explain the discrepancies between genetic association studies.

Intronic synonymous polymorphisms *VDR*; rs7975232 may affect protein folding and structure as well as messenger mRNA splicing and stability and may change cellular response to specific medication (Hunt, Sauna, Ambudkar, Gottesman, & Kimchi-Sarfaty, 2009). Accordingly, more studies on synonymous polymorphisms are required to evaluate the association between synonymous polymorphisms and complex diseases such as asthma. A study by Chen and coworkers found that non-synonymous and synonymous SNPs have similar effect size for disease association (Chen et al., 2010).

In this study, a strong LD was observed at 3` end of *VDR* gene between rs731236, rs7975232, and rs1544410 polymorphisms, which are located in one haplotype block with LD (D` > 0.8), so they are not in the random association. We can hypothesize that they may transmit together between generations with low evidence of recombination. This LD between the three SNPs is in agreement with the study by (Despotovic et al., 2017).

In this study, the LD between *VDR*; rs731236 and *VDR*; rs1544410 was stronger in moderate/severe asthmatics. The above findings may indicate that the LD between SNPs was selected by asthma in particular ethnicity. The role of asthma as selection force was supported by Wray, who showed that the haplotype ACG (*VDR*; rs731236, *VDR*; rs7975232, and *VDR*; rs1544410) was more frequent in moderate/severe asthmatics compared to controls, and is associated with asthma risk (Wray, 2005).

In MAF analysis all tested SNPs conformed to HWE in controls, mild intermittent/persistent asthmatics and moderate/severe asthmatics with HWP-value >0.05 and the MAF was > 16% for all alleles. The HWE conformation indicates that there were no genotyping or population structure problems.

No statistically significant association between vitamin D levels and risk of asthma severity (57.6% of asthmatic patients were vitamin D non-deficient; 44.5% of controls were vitamin D non-deficient). Our finding is supported by several studies that reported no association between serum Vitamin D levels and asthma phenotypes (Einisman et al., 2015; Jolliffe et al., 2018).

The lack of association is in contrast with studies reporting that Vitamin D levels were associated with asthma, and vitamin D levels were lower in asthmatics than in controls (Korn et al., 2013; Lahmar et al., 2018; Shahin, El-lawah, Amin, & El-Tawil, 2017; Tamašauskienė, Gasiūnienė, Lavinskienė, Sakalauskas, & Šitkauskienė, 2015). The difference between our study and the other studies may be explained by the fact that we merged the five severe asthmatics with moderate asthmatics in one group. Mahboub and colleagues showed that 70% of the asthmatic patients were under control in UAE (Hassan Mahboub et al., 2010) and this may

explain why we have very few severe cases in this study. Moreover, 56.6% of Vitamin D non-deficient asthmatics were under vitamin D supplementation. Thus, vitamin D supplementation invalidates the analyses on the association between asthma and vitamin D levels.

The limited number of males 24.2% among asthmatics versus 3.7% among controls made it difficult to compare between females and males. It was reported that adult-onset asthma is more common in females due to sex hormones (Melgert, Ray, Hylkema, Timens, & Postma, 2007; Ridolo et al., 2018) this might explain why we have more females asthmatics than males.

Among the limitations of this study were the rather small sample size, the discrepancy in the number of males and females, the difference in the age range between asthmatic cases and controls.

Chapter 5: Conclusion

Our study is the first in the UAE that examined the association between asthma and the polymorphisms in vitamin D metabolic genes as well as vitamin D levels. Our results demonstrated that both *VDR* rs7975232 genotypes and alleles are significantly associated with asthma severity and increase the risk of moderate/severe asthma (2.7 times for *VDR*; rs7975232 CC genotype; and 1.74 times for *VDR*; rs7975232 C allele). Moreover, the ACG haplotype of three *VDR* SNPs (rs731236, rs7975232, and rs1544410) showed a significant association with asthma severity. *VDR*; rs7975232 polymorphism may be considered as a biomarker for asthma severity and might play a role in the management of asthma in adult Emirati patients.

Mild asthma showed different genotyping and allelic frequency from moderate/severe asthma. The *GC*; rs4588, *GC*; rs7041, *VDR*; rs2228570 and *CYP2R1*; rs12794714 polymorphisms did not display any statistical association with risk of asthma severity. No difference was found in vitamin D levels between moderate/severe, mild intermittent/persistent asthmatics and controls. The strongest Linkage Disequilibrium (LD) was between *VDR*; rs731236 and *VDR*; rs1544410 polymorphisms and is more evident in moderate/severe asthmatics compared with controls.

A large sample sized study on the association of vitamin D and its metabolic genes polymorphisms with asthma is required to draw a sound conclusion. It is important to study more polymorphisms in the *VDR* gene and their relation with asthma, as well as the genes near the *VDR* gene and how the other genes are linked to the *VDR* gene. Moreover, to do more studies on the haplotype map of the *VDR* gene,

to have a better understanding of the extent of the association between *VDR* gene polymorphisms and asthma.

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Appendix

The SNPs sequences from NCBI:

https://www.ncbi.nlm.nih.gov/snp

VDR; rs731236

A/G

AATAGAAGGA GGGAA*GC*TGA CGTGGTCTGG *GC*TACAGAGG TAGAGTGTTG CCAGGAATGG
CCTTTTGGAG GAAGACCTTT TAA*GC*TGTTA TCCAAAGGAT CAGTAAGAGT CTG*GC*AAAGA
TA*GC*AGA*GC*A GAGTTCCAAG CAGAGGGA*GC* ACAGATGTGA AG*GC*TGGTGG CCAGAGA*GC*A
TG*GCGC*ATCG GGAC*GC*TGAG GGATGGACAG A*GC*ATGGACA GGGA*GC*AAGG CCAG*GC*AGGG
ACAGG*GC*CAG GT*GCGC*CCAT GGAAGGACCT AGGTCTGGAT CCTAAAT*GC*A CGGAGAAGTC
ACTGGAGG*GC* TTTGGG*GC*CA G*GC*AGTGGTA TCACCGGTCA *GC*AGTCATAG AGGGGTG*GC*C
TAGGGGGT*GC* T*GC*CGTTGAG TGTCTGTGTG GGTGGGGGGT GGTGGGATTG A*GC*AGTGAGG
G*GC*CCA*GC*TG AGA*GC*TCCTG T*GC*CTTCTTC TCTATCCCCG T*GC*CCACAGA TCGTCCTGGG
GT*GC*AGGACG CC*GCGC*TGAT

Y

GAG*GC*CATCC AGGACC*GC*CT GTCCAACACA CT*GC*AGACGT ACATCC*GC*TG CC*GC*CACCCG
CCCCCGG*GC*A *GC*CACCT*GC*T CTAT*GC*CAAG ATGATCCAGA A*GC*TA*GC*CGA CCT*GCGC*A*GC*
CTCAATGAGG A*GC*ACTCCAA *GC*AGTACC*GC* T*GC*CTCTCCT TCCA*GC*CTGA GT*GC*A*GC*ATG
AA*GC*TAAC*GC* CCCTTGT*GC*T CGAAGTGTTT G*GC*AATGAGA TCTCCTGACT AGGACA*GC*CT
GTG*GC*GGT*GC* CTGGGTGGGG CT*GC*TCCTCC AGG*GC*CACGT *GC*AG*GC*CCG G*GC*TG*GC*GG
CTACTCA*GC*A *GC*CCTCCTCA CCCCGTCTGG GGTTCA*GC*CC CTCCTCT*GC*C ACCTCCCCTA
TCCACCCA*GC* CCATTCTCTC TCCTGTCCAA CCTAACCCCT TTCCT*GC*GGG CTTTTCCCCG
GTCCCTTGAG ACCTCA*GC*CA TGAGGAGTTG CTGTTTGTTT GACAAAGAAA CCCAAGTGGG
*GGC*AGAG*GGC* AGAG*GC*TGGA

VDR; rs7975232

A/C

CGGGGAAAAG CCC*GC*AGGAA AGGGGTTAGG TTGGACAGGA GAGAGAATGG *GC*TGGGTGGA
TAGGGGAGGT G*GC*AGAGGAG GG*GC*TGAACC CCAGACGGGG TGAGGAGG*GC* T*GC*TGAGTAG
CC*GC*CA*GC*CC CGG*GC*CTG*GC* ACGTG*GC*CCT GGAGGA*GC*AG CCCCACCCAG *GC*ACC*GC*CAC
AG*GC*TGTCCT AGTCAGGAGA TCTCATT*GC*C AAACACTTCG A*GC*ACAAGGG *GC*GTTA*GC*TT
CAT*GC*T*GC*AC TCAG*GC*TGGA AGGAGAG*GC*A *GC*GGTACT*GC* TTGGAGT*GC*T CCTCATTGAG
*GC*T*GCGC*AGG TCG*GC*TA*GC*T TCTGGATCAT CTTG*GC*ATAG A*GC*AGGTG*GC* T*GC*CCGGGGG
CGGGTG*GC*GG CA*GC*GGATGT ACGTCT*GC*AG TGTGTTGGAC AG*GC*GGTCCT GGATG*GC*CTC
AATCA*GCGC*G *GC*GTCCT*GC*A CCCCAGGACG ATCTGTGG*GC* ACGGGGATAG AGAAGAAG*GC*
ACAGGA*GC*TC TCA*GC*TGG*GC*

M

CCTCACT*GC*T CAATCCCACC ACCCCCCACC CACACAGACA CTCAACG*GC*A *GC*ACCCCCTA
G*GC*CACCCCT CTATGACT*GC* TGACCGGTGA TACCACT*GC*C TG*GC*CCCAAA *GC*CCTCCAGT
GACTTCTCCG T*GC*ATTTAGG ATCCAGACCT AGGTCCTTCC ATGG*GCGC*AC CTG*GC*CCTGT
CCCT*GC*CTGG CCTT*GC*TCCC TGTCCAT*GC*T CTGTCCATCC CTCA*GC*GTCC CGAT*GCGC*CA
T*GC*TCTCTGG CCACCA*GC*CT TCACATCTGT *GC*TCCCTCTG CTTGGAACTC T*GC*TCT*GC*TA
TCTTT*GC*CAG ACTCTTACTG ATCCTTTGGA TAACA*GC*TTA AAAGGTCTTC CTCCAAAAGG
CCATTCCTGG CAACACTCTA CCTCTGTA*GC* CCAGACCACG TCA*GC*TTCCC TCCTTCTATT
CTACCCTTCT GTA*GC*ATCAC TTACTTTATT TTTAAAACTC TT*GC*CTTGTT AGGAATTAAT
AATTAAATAA CATTTGTTTA

VDR; rs2228570

A/G

GAGGGTTTCT CTCCACATGT AGGT*GC*TGAG *GC*TGAGGGAG GACTCTCATT TTCCCTTGGA

GGGG*GC*GTTG G*GC*AGGATAG AA*GC*CCCTGA CCTGGTTCAG GTCTGT*GC*CT GAG*GC*AGA*GC*
TAGT*GC*CAGT A*GC*ATGAATG GGTTCAT*GC*A TATGATCCTT ACACCCTGGA AGTAAAACAC
CTCTTCCAAT *GC*AGACA*GC*G GGG*GC*AT*GC*A GAGGTGAACC ACTAAACCCA AATTAACCTG
ACAGAT*GC*AA CATCTGAAAC CAG*GC*A*GC*TG ATTCCAA*GC*C AT*GC*TCTGAG CCA*GC*TATGT
AGG*GC*GAATC ATGTATGAGG *GC*TCCGAAGG CACTGT*GC*TC AG*GC*CTGG*GC* CCTGGGGAGA
T*GC*CCACCCT T*GC*TGA*GC*TC CCTGGTGGTG GGGGGTGGGG *GC*GGTGGGAT GAG*GC*TGGGG
GTGGGTG*GC*A CCAAGGAT*GC* CA*GC*TG*GC*CC TG*GC*ACTGAC TCTG*GC*TCTG ACCGTG*GC*CT
*GC*TT*GC*TGTT CTTACAGGGA

N

GGAG*GC*AATG *GC*G*GC*CA*GC*A CTTCCCT*GC*C TGACCCTGGA GACTTTGACC GGAACGT*GC*C
CCGGATCTGT GGGGTGTGTG GAGACCGA*GC* CACTG*GC*TTT CACTTCAATG CTATGACCTG
TGAAG*GC*T*GC* AAAG*GC*TTCT TCAGGTGA*GC* CCTCCTCCCA G*GC*TCTCCCC AGTGGAAAGG
GAGGGAGAAG AA*GC*AAGGTG TTTCCATGAA GGGA*GC*CCTT *GC*ATTTTTCA CATCTCCTTC
CTTACAATGT CCATGGAACA T*GC*G*GC*G*C*TC ACA*GC*CACAG GA*GC*AGGAGG GTCTTGGTGA
GTGGTATCTT CTTTTCCCTC CTCTCA*GC*TC CAGATGTTCC TCTGACTCTC TTGGAAATCG
CTTTCCTGAG GTT*GC*TGTGT GGGTCTCTGT CTTTCCATTA C*GC*CTGTAAC CCACA*GC*CTC
CTACACCAAC CCACGTGTCC ATCCTTCCAG AGTGAACCTC CTCCCTGTTG ATGATCACAG
CTTCCTCACC CAAGAGACAG

DBP; rs7041
A/C

TTACTCCTGT ATTGGTGTTT TCCAACATAG TGAGTTGAGA AGTAGATAAA TTGAGGGTGA
AATTATATAA ATTATGAGAG GAAAAAAAGG CATTAA*GC*TG GTATGAGGTC CTGTAAAGGA
AATATTCTTT AAGGAATTTG AAATTTAAAA CTGAAGAGAA GGTGAAAGGT TAGGATAAAA
TAGAAGAGGT ACTCTTCCAT TTTGAAATAA TGA*GC*AAATG AAAGAAGACT GGACTTCCAA
TTCA*GC*A*GC*G ATTTGTATGT TTATTTTTAT GATCTCGAAG AG*GC*ATGTTT CACTTTCTGA
TCTCAAATTG ACTATTCTAT ACCACAGGTA TAGAATTTTC TTGAGACAGG CAAGTATTTC
TATTTTCATT TTTATTGTAA AAGATCTGAA ATG*GC*TATTA TTTT*GC*ATTA GAAATTTGTA
TAAAATAAAT ACATGTAGTA AGACCTTACA TTTAAATGGT TTTTCAGACT G*GC*AGA*GC*GA
CTAAAA*GC*AA AATT*GC*CTGA

K

*GC*CACACCCA CGGAACTG*GC* AAA*GC*TGGTT AACAA*GC*ACT CAGACTTT*GC* CTCCAACT*GC*
TGTTCCATAA ACTCACCTCC TCTTTACTGT GATTCAGAGG TAGGAAAATG TAACCCTCCA
CTTAACATGG CAGAATCTTT TAAGAACGTA T*GC*ACTCCAA TCTACTCATT TCTTTCCTGT
TATTGAGATG CCATTATGTG ACAG*GC*TTTT CCTGGTGTTA TTGTAACTTG *GC*TGTCTTTG
CAATGAAAGT AAGAAACATA ACTGATTTCA T*GC*TAT*GC*TC ATTTAAAA*GC* AAGTTGAGGT
AGTTGTAAAA CTTAGGAAAT TTTATACTTT TTTTAAGAAA CAAAAGAGTC TGTGAACACA
G*GC*AACAAAG TATGTGTAGT GTTTTACAAA TTGTGAGTTG TAACCTAGTA ACA*GC*CTATT
TAAATGAAAA AGAGAAGAAT AAGAATACAG TGAGAACATC AGAGTTCATT ACCTTCACAT
AGTGAAGGTA AATTGTATTT

DBP; rs4588
G/T

TTGGTGTTTT CCAACATAGT GAGTTGAGAA GTAGATAAAT TGAGGGTGAA ATTATATAAA
TTATGAGAGG AAAAAAAG*GC* ATTAA*GC*TGG TATGAGGTCC TGTAAAGGAA ATATTCTTTA
AGGAATTTGA AATTTAAAAC TGAAGAGAAG GTGAAAGGTT AGGATAAAAT AGAAGAGGTA
CTCTTCCATT TTGAAATAAT GA*GC*AAATGA AAGAAGACTG GACTTCCAAT TCA*GC*A*GC*GA
TTTGTATGTT TATTTTTATG ATCTCGAAGA G*GC*ATGTTTC ACTTTCTGAT CTCAAATTGA
CTATTCTATA CCACAGGTAT AGAATTTTCT TGAGACAG*GC* AAGTATTTCT ATTTTCATTT
TTATTGTAAA AGATCTGAAA TG*GC*TATTAT TTT*GC*ATTAG AAATTTGTAT AAAATAAATA
CATGTAGTAA GACCTTACAT TTAAATGGTT TTTCAGACTG *GC*AGA*GC*GAC TAAAA*GC*AAA
ATT*GC*CTGAT *GC*CACACCCA

H

GGAACTG*GC*A AA*GC*TGGTTA ACAA*GC*ACTC AGACTTT*GC*C TCCAACT*GC*T GTTCCATAAA
CTCACCTCCT CTTTACTGTG ATTCAGAGGT AGGAAAATGT AACCCTCCAC TTAACATG*GC*
AGAATCTTTT AAGAACGTAT *GC*ACTCCAAT CTACTCATTT CTTTCCTGTT ATTGAGAT*GC*
CATTATGTGA CAG*GC*TTTTC CTGGTGTTAT TGTAACTTGG CTGTCTTT*GC* AATGAAAGTA
AGAAACATAA CTGATTTCAT *GC*TAT*GC*TCA TTTAAAA*GC*A AGTTGAGGTA GTTGTAAAAC
TTAGGAAATT TTATACTTTT TTTAAGAAAC AAAAGAGTCT GTGAACACAG *GC*AACAAAGT
ATGTGTAGTG TTTTACAAAT TGTGAGTTGT AACCTAGTAA CA*GC*CTATTT AAATGAAAAA

GAGAAGAATA AGAATACAGT GAGAACATCA GAGTTCATTA CCTTCACATA GTGAAGGTAA
ATTGTATTTC ATATATCTGT

CYP2R1; rs12794714
A/G

CCT*GC*TATTA ACCATCTAGA ACTCAGAACG CCTTGATTTT CCGACAA*GC*C *GC*GT*GC*A*GC*T
TCCACAGA*GC* T*GC*GAG*GC*CG ACTC*GC*AAGA C*GC*CC*GC*ACC TGAGG*GC*ATG CGTCCACCCT
GGACCTGAAG TG*GC*G*GCGC*G *GC*TG*GC*GA*GC* CAAACG*GCGC* AG*GCGC*AGGA *GC*AAGA*GC*TC
GA*GC*GGTA*GC* GGGAAAATCA GGACTGGATC *GC*CTCGGA*GC* CTCG*GC*CCGG AGTGGGTCAC
CG*GC*AGGG*GC* GGG*GC*TCCCC CT*GC*AAGGGG *GC*ACG*GC*GTC GAGGACTTCT CCCTTCCAGA
CCCGGGAA*GC* GGGTGTCCCT CAAAGG*GC*AG CCG*GC*ACACG GAGAGGTCCC GACTAGTCGG
CCCAGGG*GC*G *GC*CATAAGTC CAACCAGGAA G*GC*CCTG*GC*C A*GC*CCGG*GC*C A*GC*CTG*GC*GG
CCCTCCCT*GC* CCGGG*GC*CCG TCGGG*GC*TGT ACCTCTCCGT ACACCTG*GC*T CT*GC*TTTCTC
ATGTAGACAT GGGGAA*GC*TC

R

GATGAG*GC*TG CCAGGGAATA GATGTT*GC*CG ATAAATG*GC*A *GC*CCCG*GC*GG CCCCGGGGGG
AA*GC*CCATCG *GC*C*GC*CTCTG CTTCA*GC*A*GC* TG*GC*GGACCC CTA*GCGC*GAA GA*GC*A*GC*AGG
AAGA*GCGCGC* C*GC*CGA*GCGC* C*GCCGCGCC*C TCTTCA*GC*TC TCCAAA*GC*TT CCACATCG*GC*
CCGA*GC*TGGA GGT*GC*GAACT CCACA*GC*A*GC* CCTGAGACCC AG*GC*ACTCCC TCCA*GC*CCTG
CCATACTCCC ATTG*GC*AGGA TACCCTCAGG TCCC*GC*CCTA *GC*TCCGTG*GC* CATTG*GC*TGA
CTGAGTGA*GC* *GC*TCAG*GC*CC CTTCGGGACA ACTACCTTCT AGTTTT*GCGC* G*GC*TGAGAGT
GGACTTT*GC*G GA*GC*GGGTGG CCGGT*GC*TAC CCTTTGGAGG TG*GCGC*G*GC*T CTAG*GC*GGAG
CAATGA*GC*CA GGAGTCCGTT TTCTAGAATG TTGACACAAG G*GC*ATTGACG AGGTGTCAGG
ACG*GC*CTCAC TCACTGG*GC*T

VDR; rs1544410
A/G

CGGGGAGTAT GAAGGACAAA GACCT*GC*TGA GG*GC*CA*GC*TG G*GC*AACCTGA AGGGAGACGT
A*GC*AAAAGGA GACACAGATA AGGAAATACC TACTTT*GC*TG GTTT*GC*AGAG CCCCTGTGGT
GTGTGGAC*GC* TGAGGT*GC*CC CTCACT*GC*CC TTA*GC*TCT*GC* CTT*GC*AGAGT GT*GC*AG*GC*GA
TTCGTAGGGG GGATTCTGAG GAACTAGATA A*GC*AGGGTTC CTGGG*GC*CAC AGACAG*GC*CT
GC

R

CATTCCCAAT ACTCAG*GC*TC T*GC*TCTT*GC*G TGAACTGG*GC* TCAACATTCC TGTTATTTGA
GGTTTCTT*GC* GG*GC*AGGGTA CAAAACTTTG GA*GC*CTGAGA GATGGTTCTG CCTATATAGT
TTACCTGATT GATTTTGGAG *GC*AATGT*GC*A GTGACCCTTG ACCTCTTCCG CTGGTTAGAG
GTGAGAAGAG GGAGAAAAGG CCGAAGAGGA AGTTATTGTG ACCTTGGGGA CATGATGTCG
GTGATGAGGT CCAAAGAGGG *GCGGC*CCT*GC* CTCA*GC*CTGT *GC*TAGTG*GC*C TGT*GC*CCAGG
GAT*GC*TTTCC TGGACTGGAG *GC*TCAAGGAA TGGAGATGGG CTCCTCTACC CCT*GC*CCA*GC*
CA*GC*CTTCTC TCATTCATTC ATCCACTTAG CAACAATTTA TTGA*GC*AC