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Vitamin D Deficiency in the Palestinian Population

By

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by
Mohammed Awad Abusarhan

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ABSTRACT

Objectives: Vitamin D plays an essential role in human health as it influences immune function, cell proliferation, differentiation and apoptosis. Vitamin D Deficiency (VDD) is a global pandemic with higher prevalence rates among Palestinians. Programs for supplying Vitamin D (VD) were carried out by the Ministry of Health and UNICEF but the issue did not resolve. In this study, we aim to (a) investigate the genetic variants of VD genes among the Palestinian population; and (b) investigate the efficacy of calcitriol vs D3 supplementation.

Methods: For genetic assessment, alleles on SNPs rs10741657 (CYP121) and rs7041 (GC) were investigated in 69 Palestinians. For calcitriol efficacy, 106 individuals joined in phase 1 for 1 month, and 92 individuals joined in phase 2 for 3 months.

Results: 94.2% of the 69 individuals assayed had reduced CYP2R1 and/or GC enzyme activity with a higher risk for developing VDD. Calcitriol efficacy in elevating VD levels is significantly higher than D3.

Keywords: Calcitriol, CYP2R1, D3 Supplementation.

الملخص

الهدف: فيتامين د له دور مهم في صحة الانسان حيث تؤثر وظائفه في مناعة الجسم، تكاثر الخلايا وتمايزها وموتها. يعد نقص فيتامين د وباءً عالمياً (أصيب به أكثر من مليار شخص) وتزيد نسب الإصابة لدى السكان الفلسطينيين. نظمت وزارة الصحة الفلسطينية بالتعاون مع اليونيسيف برامج لدعم استهلاك فيتامين د ولكن المشكلة نقص فيتامين د ظلت مستمرة. تهدف هذه الدراسة الى: أ) دراسة المتغيرات الوراثية لفيتامين د لدى الفلسطينيين، ب) دراسة فعالية دواء الكالسيتريول مقارنة بحبوب فيتامين د (D3) في رفع مستوى فيتامين د في الجسم.

منهج الدراسة: لتقييم المتغيرات الوراثية، تم دراسة المتغير rs10741657 من الجين CYP121 والمتغير rs7041 من الجين GC في 69 شخص فلسطيني. لدراسة فعالية الكالسيتريول، شاركت مجموعة مكونة من 106 اشخاص في المرحلة الأولى لمدة شهر وقسّمت الى ثلاث مجموعات أصغر (مجموعة الكالسيتريول، مجموعة D3، مجموعة المقارنة control)، ومجموعة مكونة من 92 في المرحلة الثانية لمدة شهرين تم تقسيمها الى مجموعتين (مجموعة الكالسيتريول، مجموعة D3).

النتائج: 94.2% من ال 69 شخص كان لديهم متغيرات وراثية تؤدي إلى نشاط منخفض إما لجين CYP2R1 او لجين GC أو كليهما معاً، مع احتمال أعلى للإصابة بنقص فيتامين د. الكالسيتريول اظهر فعالية أكبر من D3 في رفع مستوى فيتامين د في الجسم.

DECLARATION

I declare that the Master Thesis entitled "Vitamin D Deficiency in the Palestinian Population" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

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Dedication

To all students of knowledge who are working to make this world a better place

To my family, friends, and colleagues

To my teachers

Acknowledgment

I am sincerely grateful to my supervisor Dr. Areej Al-Khatib for her guidance and patience. Also, I am grateful to Prof. Mazin Qumsiyeh for his unlimited support.

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Chapter 1: Introduction

Vitamin D (VD) has many important biological functions, including control of cell proliferation, regulation of differentiation, inhibition of tumor growth and induction of apoptosis (Norman, 2012; Sassi et al., 2018). Thus, it is not surprising that Vitamin D Deficiency (VDD) is a global pandemic with significant health repercussions (Amrein et al., 2020; Holick, 2008). VDD could have environmental and genetic causes (Norman, 2012). In our region there seems to be fairly high incidence of VDD (Lips et al., 2019). The genetic factors that lead to VDD could include all aspects of metabolism, catabolism, transport, or receptor binding with many variations and polymorphisms in many genes reported associated with VDD (Bahrami et al., 2018; Jiang et al., 2019). Of these genes, CYP2R1 and GC genes are of special values as they have been repeatedly found to be associated with VDD outcomes (Slater et al., 2017).

The distribution of VDD varies globally (Van Schoor & Lips, 2017). In Palestine, the incidence rate of VDD is alarming (Manenti et al., 2016). Even though supplementation programs were carried out by Palestinian Ministry of Health and UNRWA, the rates are still above the average (Chaudhry et al., 2018).

In this study, we first investigated the genotypes of CYP2R1 & GC genes in the Palestinian population using NGS sequencing. Based on these findings, we also launched a small clinical trial to see if the active form of VD could be more efficient in maintaining VD levels in the Palestinian population.

Chapter 2: Literature Review

2.1 Metabolism of VD

The major source for VD is from exposure to sunlight but also can be acquired from diet and dietary supplements (Holick, 2007). Dietary sources include fatty fish, eggs, fortified milk and cod

liver oil. The human body uses ultraviolet B (UVB) radiation from sunlight to synthesize a significant portion of VD requirements.

The major two forms of VD are Vitamin D_2 (VD_2) and Vitamin D_3 (VD_3) (*Figure 1*). VD without a subscript refers to either VD_2 or VD_3 or both. Both UVB intensity and skin pigmentation level contribute to the rate of VD_3 formation (Holick et al., 1980). Melanin pigments can absorb UVB rays, therefore, people with dark skin may have lower 25(OH)D levels (Libon et al., 2013). A variety of endogenous factors and environmental influences can alter the skin's production of VD, including skin pigmentation, sunscreen use, clothing, latitude, season, time of day, and aging (Holick, 2004).

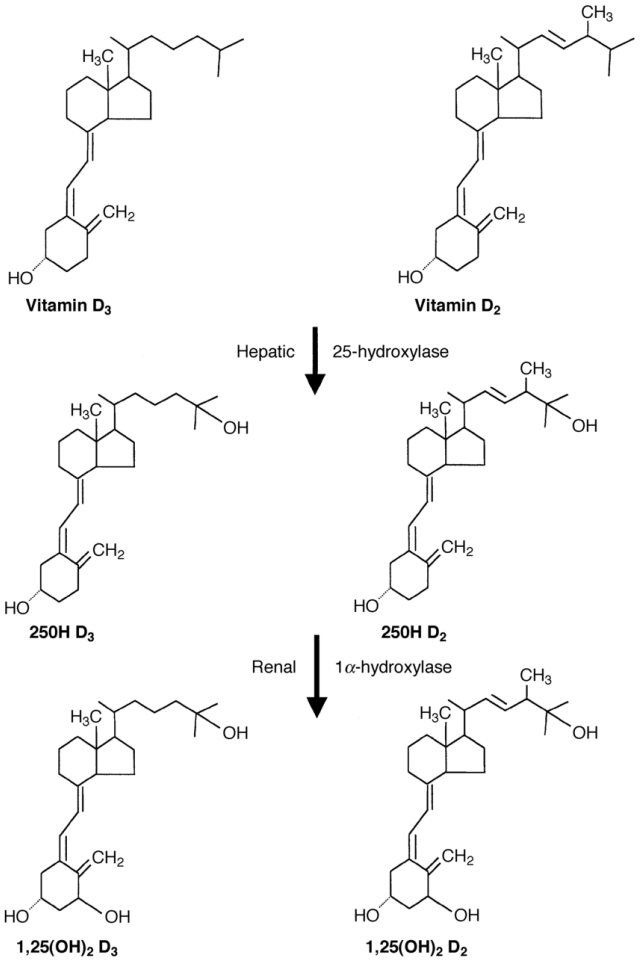

Figure 1 Metabolic activation of VD (Zerwekh, 2004).

Upon exposure to sunlight, 7-dehydrocholesterol is converted to VD_3 (cholecalciferol) under the influence of UV light while VD_2 (ergocalciferol) is derived from the plant sterol ergosterol. Both VD_2 and VD_3 are then metabolized in the liver and the kidneys to produce the active form, calcitriol. VD_3 is converted in the liver to 25-hydroxycholecalciferol [$25(OH)D_3$] while VD_2 is converted to 25-hydroxyergocalciferol [$25(OH)D_2$] (Norman, 2012). These two metabolites, collectively called 25-hydroxyvitamin D [25(OH)D], are measured in serum to determine a person's VD status. 25(OH)D is then metabolized in the kidneys to the active form 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], also known as calcitriol.

In the liver, VD is metabolized by 25-hydroxylases (CYP2R1 and CYP27A1) to 25(OH)D while in the kidney 25(OH)D is metabolized by 1-α hydroxylase (CYP27B1) to $1,25(OH)_2D$. However, 25(OH)D and 1,25(OH)D can also be metabolized in the kidneys by CYP24A1 to $24,25(OH)_2D$ and $1,24,25(OH)_3D$, respectively. $1,24,25(OH)_3D$ is the inactive form of calcitriol and the first step in a catabolic process. Hence, CYP24A1 acts as a regulator of calcitriol levels (*Figure 2*) as it produces biologically inactive biliary excreted calcitroic acid (Lehmann & Meurer, 2010).

Calcitriol metabolism is a tightly regulated process (Figure 3). In the kidney, CYP27B1 is stimulated by PTH and inhibited by FGF23 (fibroblast growth factor 23), high calcium (Ca), and phosphate (P). The regulation of CYP24A1 is just the opposite of CYP27B1 (Meyer & Pike, 2020). Moreover, calcitriol regulates its own production directly through a negative feedback and by inhibiting PTH production, stimulating FGF23 production, and inducing CYP24A1 (Figure 2). PTH is secreted by the parathyroid gland in response to low serum calcium levels while FGF23 is secreted by osteoblasts and osteocytes in response to both high serum phosphate and calcitriol levels.

Upon the production of calcitriol, it enters the circulation, binds to VDBP, and delivered to target tissues such as intestine, bone, and kidney to maintain calcium and phosphate homeostasis (Heaney, 2008). In target tissues, calcitriol binds to VDR which induces both genomic and non-genomic pathways of calcitriol (Figure 3) (Jeon & Shin, 2018). In the genomic pathway, calcitriol binds to cytosolic VDR, which promotes phosphorylation of VDR, heterodimerizaion with retinoid-X receptor (RXR), and then nuclear translocation of the complex (Pike & Meyer, 2014).

The calcitriol–VDR–RXR complex binds to VD response element (VDRE) in the promoter region of its target genes and recruits transcriptional coactivators or co-repressors to regulate mRNA expression of target genes. In the non-genomic pathway, calcitriol binds to membrane bound VDR, which is identified as 1,25D-membrane-associated, rapid response steroid-binding protein (1,25D-MARRS); this interaction then induces acute changes in cell signaling pathways, including calcium and mitogen-activated protein kinase (MAPK) signaling, through direct protein–protein interaction with intracellular signaling molecules involved in certain phenotypic functions (Jeon & Shin, 2018).

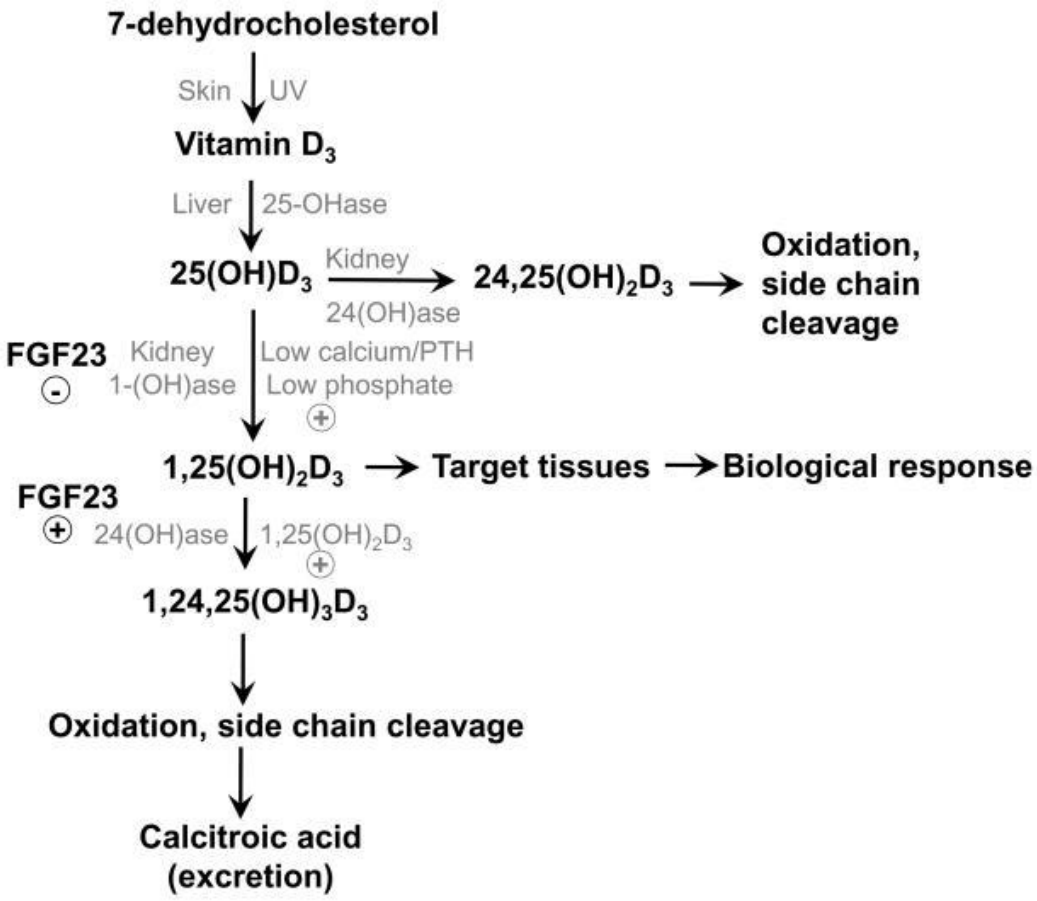

Figure 2 The metabolic pathway for vitamin D (Christakos et al., 2012)

Though the classical pathway for VD metabolism goes through liver and kidney, it can also be fully synthesized in the skin as the keratinocytes have the machinery enzymes to convert VD_3 to

its active form calcitriol (Bikle, 2011). Calcitriol is also synthesized locally by CYP27B1 present in most extra renal tissues, including many cancer cells, where it acts in a paracrine manner (Bikle, 2011).

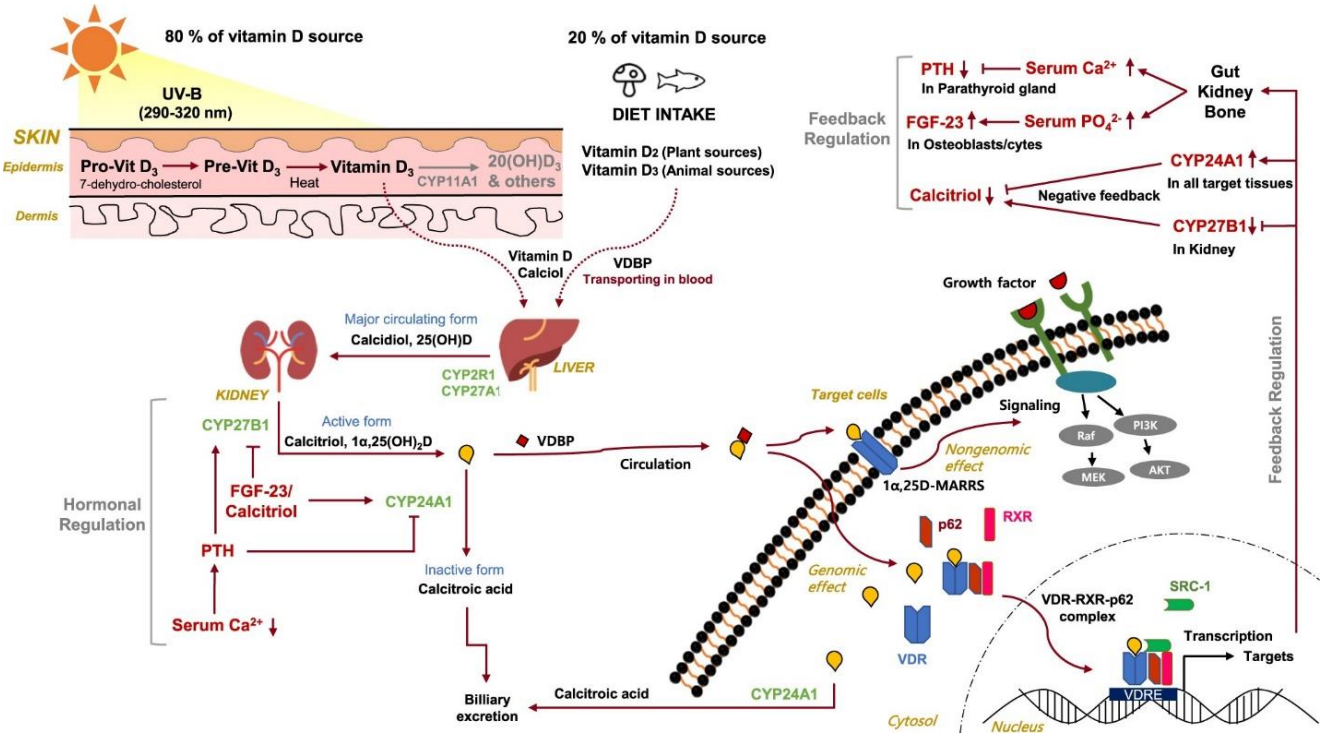

Figure 3 Overview of Vitamin D metabolism (Jeon & Shin, 2018).

2.2 Genetics of VD

VD metabolism is controlled by many genes with many allelic variations associated with different phenotypes (Jiang et al., 2019). These genes include but are not limited to CYP2R1, CYP24A1, CYP27B1, GC, VDR, DHCR1, SEC23A, and AMDHD1. Among those genes are CYP2R1 and CYP27B1, the genes that are responsible for VD hydroxylation in the liver and the kidney (Henry, 2011), and GC (group-specific component), the gene that encodes VD binding protein. Many of these genes have SNPs associated with serum levels of 25(OH)D such as rs10741657 (CYP2R1) and rs7041 (GC). The SNPs variations have also been associated with the efficacy of VD supplementation (Barry et al., 2014). Understanding the mechanisms underlying these allelic differences in relation to environmental factors as well as response to therapy could aid the

selection of optimal therapy for VDD prevention and treatment. Earlier studies of VD genetics were based on candidate gene approach but GWAS have been used recently to explore VD genetic variations (Ahn et al., 2010; Sapkota et al., 2016; Wang et al., 2010).

Variations in VD genes contribute towards the susceptibility of certain populations to VDD (Elkum et al., 2014). Lafi *et al.* (2015) identified an association between the following SNPs: rs7041 and rs4588 of GC and rs10741657 of CYP2R1, and VD serum levels in a Jordanian population.

2.2.1 CYP2R1

CYP2R1 is responsible for the hydroxylation of VD to 25(OH)D in the liver. The gene is located on chromosome 11p15.2 and covers 14.29 kb on the reverse strand. SNPs of CYP2R1 (rs10741657, rs10766197, rs12794714) are associated with 25(OH)D serum levels (Nissen et al., 2014). The G allele of rs10741657 is associated with lower VD (Wang et al., 2010) while carriers of two A alleles have normal VD levels. This SNP is also associated with the prognosis of Non-Small Cell Lung Carcinoma (Kong et al., 2020).

2.2.2 CYP27B1

CYP27B1 encodes 1a-hydroxylase which converts 25(OH)D to its active form $1,25(OH)_2D$. It is located on chromosome 12, at 12q13.1-q13.3, spanning 6.66 kb on the reverse strand. The SNP rs10877012 (C/A) that resides at position 1260 of CYP27B1 was widely explored for the association with 25(OH)D (Orton et al., 2008).

2.2.3 Group –specific Component (GC)

The group-specific component (GC) is the major VD-binding protein in plasma (Daiger et al., 1975). Variants in GC gene are associated with breast cancer risk (Chen et al., 2019), asthma (Fawzy et al., 2019), and VDD (Lauridsen et al., 2005). GC gene sequence was investigated early in 1992, and three phenotypes, GC1F, GC1S, and GC2, were identified (Braun et al., 1992). In SNP rs7041, the TT and TG genotypes are associated with lower VD serum levels than the wildtype GG (Lafi et al., 2015).

2.3 VD functions

VD has a broad range of functions in human body. Its classical function is to maintain normal levels of calcium and phosphorus in blood (DeLuca, 2004). Though VD is a prohormone in itself and not a vitamin, it affects the functions of many other vitamins, hormones and pathways (Sassi et al., 2018). It is known to regulate parathyroid growth and parathyroid hormone production; it plays a role in the islet cells of the pancreas, has a significant effect on the immune system, and can help in suppression of certain autoimmune diseases and certain cancers. Anticancer properties of VD have been proposed, however, the metabolism and functions of VD are dysregulated in many types of cancer which urges the need for more studies on the activation of VD signaling for prevention and treatment of many types of cancer (Jeon & Shin, 2018). Hence, the reduction in VD levels develops risks for type I diabetes, rheumatoid arthritis, Crohn's disease, multiple sclerosis, heart disease, stroke, infectious diseases among others (Huang et al., 2017; Kheiri et al., 2018). Vitamin D is important for bone, for its essential role in promoting intestinal calcium absorption and mineralization of bone matrix (Stechschulte et al., 2009).

Recent reports indicates the role of VDD in developing risks for type I diabetes, rheumatoid arthritis, Crohn's disease, multiple sclerosis, heart disease, stroke, infectious diseases among others (Kheiri et al. 2018; Huang et al. 2017).

Recent data had proven the involvement of VD in reproductive systems in humans. In women, VD status was associated with in vitro fertilization (IVF) outcome, features of polycystic ovarian syndrome (PCOS) and endometriosis, while in men, VD status has been associated with semen quality and sperm count, motility and morphology (Anagnostis et al., 2013).

VD functions have much to do with the immune system. The interest in VD as an immunoregulator started when VD Receptors where detected in human peripheral blood in 1983 (Provvedini et al., 1983). The first study to show the role of VD in suppressing the proliferation of the infectious pathogen *Mycobacterium tuberculosis* was published in 1986 (Rook et al., 1986). Since then, VD has been linked to several immune system-mediated diseases such as inflammatory bowel diseases, ulcerative colitis and Crohn's disease (Cantorna et al., 2004). It has been well established that VD

has fundamental roles in both innate and adaptive immunities as dendritic cells, Th1 and Th2 cells are found to express VDRs (Cantorna, 2011).

2.4 VDD

2.4.1 Phenotype

Vitamin D Deficiency (VDD) has been reported in all phases of life throughout the world. VDD increases risk for type I diabetes, rheumatoid arthritis, Crohn's disease, multiple sclerosis, heart disease, stroke, infectious diseases, as well as increased risk of dying of many deadly cancers including colon, prostate, and breast (Holick, 2007). Sufficient intake of VD is required to avoid risks for developing osteoporosis, hypertension, cancer, and several autoimmune diseases (Pearce & Cheetham, 2010). Single nucleotide polymorphisms (SNPs) in the Vitamin D Receptor (VDR) and vitamin D3 synthesis and degradation pathways have been implicated in affecting the risk of cancer development (Slattery, 2007).

The first documented linkage between VD and cancer was made in the 40s of the last century. Since then, VD has been linked to at least 18 different types of cancers. For example, people with VD levels below 30 ng/mL had twice the risk for colon cancer (Garland et al., 1989) while the incidence is doubles if VD is less than 20 ng/mL (Tangrea et al., 1997). Also, the risk of breast cancer for women who are regularly exposed to sunlight and consumes adequate amounts of VD is significantly lower (Nimitphong & Holick, 2013). In a large scale study that included 19,000 men, those with VD levels below 16 ng/mL had a 70% higher incidence rate of prostate cancer than those with levels above 16 ng/mL (Ahonen et al., 2000).

There is a consistent association in the published literature between higher BMI and PTH and lower VD levels (Vanlint, 2013) which is partially attributed to decreased availability of D3 from cutaneous and dietary sources because of its deposition in body fat compartments (Wortsman et al., 2000).

2.4.2 Epidemiology

VDD is widely recognized as a global pandemic with over one billion records from all over the world (Hilger et al., 2014; Holick, 2008; Wortsman et al., 2000). Prevalence rates are various among countries with certain regions, including Eastern Europe and the Middle East, have higher prevalence rates (Figure 4). In Europe, VDD is widespread with variable rates among countries, however, a large-scale study (>53,000 individuals) showed a 13% prevalence rate (Cashman et al., 2016). Nevertheless, regional reports indicate higher prevalence of VDD. For example, in Jordan, VD levels <30 ng/ml is 22.2% (Batieha et al., 2011), however, another study reported a 89.7% rate of VD <30 ng/ml levels (El-Khateeb et al., 2019). In Turkey, the rates of VD <30 ng/ml were 88.7% (Hekimsoy et al., 2010). In Saudi Arabia, populations at higher risk showed 50%-80% prevalence rate for VDD (Mohammed et al., 1993).

VDD is most common in pregnant women, children, and elderly. However, it's also prevalent in adults, especially in the Middle East and Asia regions (Van Schoor & Lips, 2017). VDD prevalence tends to be higher during winter periods and among dark-skinned ethnic groups (Cashman et al., 2016).

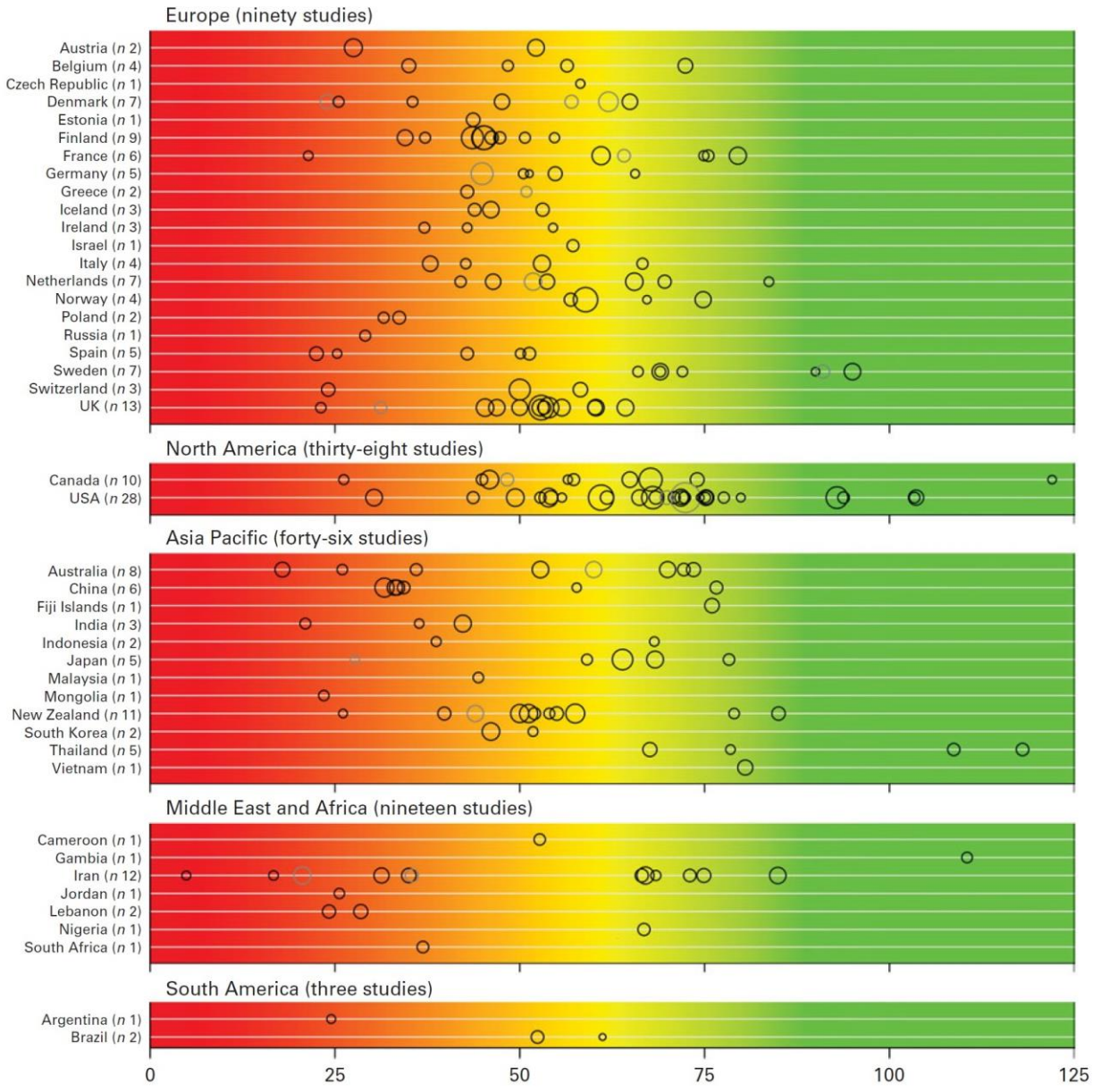

Figure 4 Mean (black circles)/median (grey circles) 25-hydroxyvitamin D (25(OH)D) values, by geographical region and country (Hilger et al., 2014).

2.4.3 Vitamin D Deficiency in Palestine

Vitamin D Deficiency (VDD) in Palestine is a prolonged problem (Manenti et al., 2016). The latest national-scale study in Palestine reports a 60.7% prevalence rate of VDD (Chaudhry et al., 2018). In a study on postmenopausal women in Palestine, 85.9% of the study sample had VDD (Kharroubi et al., 2017). Also, in a cross-sectional study on 163 hemodialysis patients, only 12.9% had sufficient levels of VD (>30 ng/ml; Nazzal et al., 2021). Other smaller unpublished studies show similar high incidence of VDD among Palestinians (Daibes et al., 2021; Saabneh et al., 2021).

A 2004 study in Denmark found that children of Palestinian origin have higher privilege of VDD than Danish children of same age (0-9 years-old) (Glerup et al., 2004). In another study by same group, VDD was found to be higher among women of Palestinian origin, even when compared to Muslim women of Danish origin that use same clothing style (Glerup et al., 2000).

2.5 Laboratory Assays

VD status assessment is based on measuring serum 25(OH)D, the sum of serum 25(OH)D_2 and 25(OH)D_3 concentrations. However, substantial variability exists in 25(OH)D measurements when using different assay methodologies (L. Li et al., 2016) which leads to variations in results (Figure 5; Holick, 2009). This issue of standardization has led to the creation of the Vitamin D Standardization Program (Durazo-Arvizu et al., 2017).

Serum 25(OH)D is hydrophobic and exists in three structurally different forms (25(OH) D_3, 25(OH)D_2, and 3-epi-25(OH)D) and is tightly binding to VD binding protein (DBP) which makes the its measurement a difficult task (Carter, 2012). Different measuring methods exist for measuring 25(OH)D that includes: Liquid chromatography–mass spectrometry (LC-MS), automated chemiluminescence, radioimmunoassays, and ELISA (Arneson & Arneson, 2013).

VD has different metabolites with a very different efficacy, half-life, and risk of toxicity (Figure 6; Vieth, 2020). It is argued that serum total 25(OH)D concentration – the sum of the 25(OH)D_3 and 25(OH)D_2 concentrations – is the best biomarker to define VD status (Tsuprykov et al., 2018).

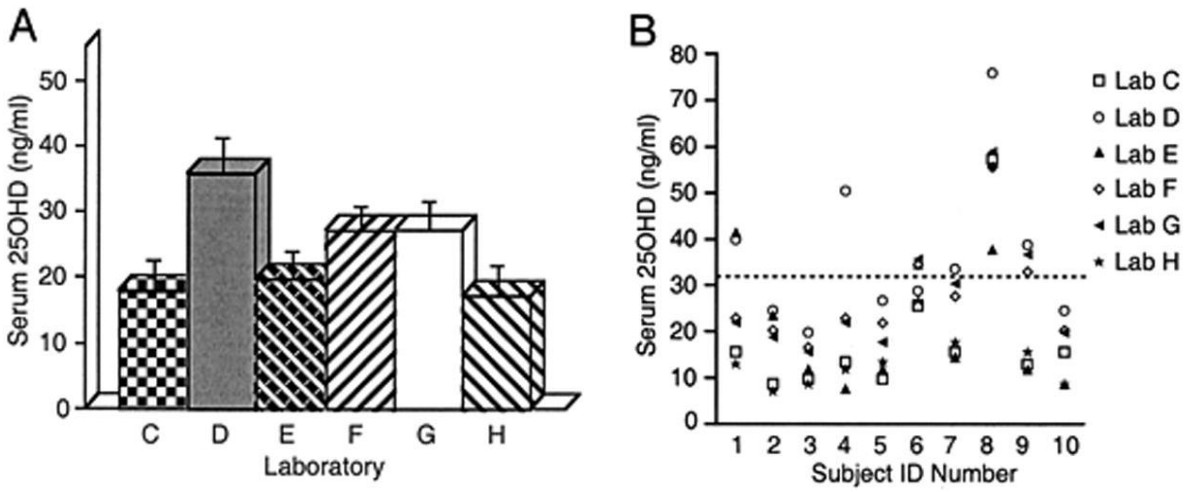

Figure 5 Examples of between-laboratory variability in 25(OH)D measurement (Holick, 2009).

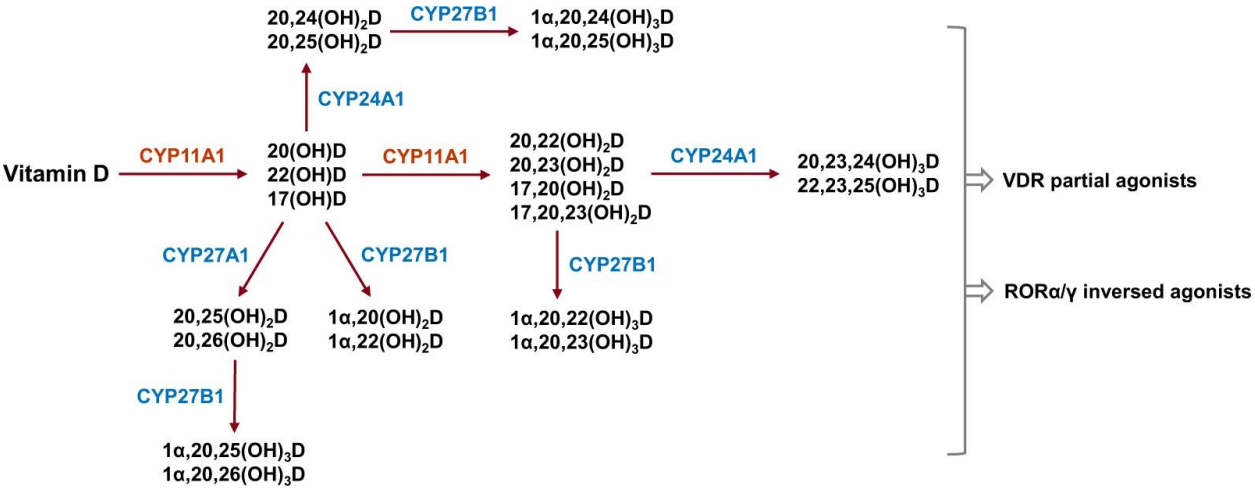

Figure 6 The metabolites generated from alternative vitamin D metabolic pathway (Jeon & Shin, 2018).

Chapter 3: Materials and Methods

3.1 Participants

Participants for the genetic assessment were random people with good health profiles who chose to checkup on their genetic profiles. They include 35 males and 34 females, from the West Bank, Palestine with an age range from 16 years old up to 75 years old. Limited additional data were collected on patient demographics for the mutation testing (see below). All participants signed informed consent.

For the clinical trials, eligible participants aged 18 years or older. Upon initial screening, only individuals with VD levels below 30 ng/ml were considered. A total of 106 individuals joined in phase 1 and 92 individuals joined in phase two (Table 1).

Table 1 Participants in the genetic assessment and clinical trials.

Genetic Assessment			
	Males	Females	Total
	35	34	69
Phase one			
	Males	Females	
Calcitriol	17	23	40
D3	13	23	36
Placebo	15	15	30
			106
Phase two			
	Males	Females	
Calcitriol	25	27	52
D3	15	25	40
			92

3.2 Study Design and Treatment

For the genetic assessments, the data of the 69 individuals were considered for analysis. Their genetic data was collected over the last 3 years via a commercial genetic profiling test provided by AnantLife (Toronto, Canada). Using the commercial kit, a saliva swap was collected from the individuals and preserved at 4 C before shipping to AnantLife for sequencing. The resulted report shows genotypes of informative SNPs of >100 genes related to detoxification, dietary, fitness, nutrition among other profiles (Annex 1 shows a sample report). Also, the report indicated the enzyme activity (increased, reduced) and risk for deficiency. On VD, the following two SNPs are reported: SNPs rs1074165 of CYP2R1 and rs7041 of GC. The percentage of each variant alone and combined with other variants were calculated.

For the clinical trials, the study done on two phases. Phase one aimed to investigate the efficacy of VD and calcitriol vs placebo while in phase two the efficacy of VD vs Calcitriol was investigated. Phase one lasted one month, after which phase two lasted for another two months. In phase one, the participants were divided into 3 groups: group 1 received Calcitriol drug, group 2 received D3, and group 3 are the control (Table 1).

For phase one participants, serum 25(OH)D, creatinine, urea, and Ca levels were measured at Pharmacare before starting the trials and after one month. The first group was supplied with daily 0.25 µg calcitriol (active ingredient: $1,25[OH]_2D_3$) provided by Pharmacare, the second group was supplied with daily 5,000 IU D3, where the third group didn't receive neither calcitriol nor D3.

For phase two, 92 participants joined, 40 took D3 and 52 took calcitriol. Like phase one, serum 25(OH)D, creatinine, urea, and Ca levels were measured after two months of starting phase two (three months after starting phase one). Also, PTH, PO_4 and calcitriol were measured for individuals taking calcitriol.

For measuring serum 25(OH)D, Vitamin D3 kits from Euroimmun were used, while for measuring calcitriol, $1,25\ (OH)_2$ Vitamin D kits from DRG were used. Both kits are ELISA based.

Chapter 4: Results

4.1 Genetic Assessment

Out of the 69 individuals who tested for rs1074165 (CYP2R1) and rs7041 (GC), 65 individuals showed elevated risk for developing VDD (Table 2).

Table 2 Risk for VDD by gender.

Risk for VDD	F	M	Total
Elevated	32	33	65
Normal	2	2	4

94.2% of the 69 individuals assayed had reduced CYP2R1 and/or GC. Over half of the individuals showed reduced enzyme activity of both CYP2R1 and GC, while 22 individuals had reduced activity of CYP2R1 and only 6 individuals had reduced activity of GC (Table 3).

Table 3 Enzyme activity by gender.

Enzyme Activity	F	M	Total
Reduced CYP2R1 and GC	17	20	37 (53.6%)
Reduced CYP2R1	12	10	22 (31.9%)
Reduced GC	3	3	6 (8.7%)
Normal	2	2	4 (5.8%)
Total	34	35	69 (100%)

Four different genotypes of rs10741657 (CYP2R1) were present in the study population. The genotypes are AG, GG, AA, and TT with AG being the most dominant with 47.8% of cases while the TT genotype was lowest with 2.8% of cases (Table 4). For rs7041 (GC) gene, four genotypes were present also (TT, TG, GG, and CC) with the TT genotype being the most dominant at 52.2% of cases and the CC genotype was noted in only one case (lowest) (Table 5).

Table 4 Genotypes of CYP2R1.

CYP2R1 Genotypes	F	M	Total
AG	19	14	33 (47.8%)
GG	10	16	26 (37.7%)
AA	4	4	8 (11.6%)
TT	1	1	2 (2.9%)
Total	34	35	69 (100%)

Table 5 Genotypes of GC.

GC Genotypes	F	M	Total
TT	16	20	36 (52.2%)
TG	13	11	24 (34.8%)
GG	4	4	8 (11.6%)
CC	1	0	1 (1.4%)
Total	34	35	69 (100%)

The most common combination was AG genotype for CYP2R1 gene in combination with the TT genotype in the GC gene with 19 cases of 69 while the second most combination was GG (CYP2R1) and TT (GC) with 15 cases (Table 6).

Table 6 Combinations of Cyp2R1 and GC Genotypes.

CYP2R1	GC	# of cases	Percentage
AG	TT	19	27.5%
	TG	12	17.4%
	GG	1	1.4%
	CC	1	1.4%
AG Total		33	47.8%
GG	TT	15	21.7%
	TG	8	11.6%
	GG	3	4.3%
GG Total		26	37.7%
AA	TG	4	5.8%
	GG	3	4.3%

	TT	1	1.4%
AA Total		8	11.6%
TT	TT	1	1.4%
	GG	1	1.4%
TT Total		2	2.9%
Total		69	100.00%

The four cases that had normal enzyme activity of both CYP2R1 and GC had AA and TT genotypes for CYP2R1 and GG and TT genotypes for GC (Table 7).

Table 7 Genotypes of the normal cases.

CYP2R1	GC	Enzyme Activity	Risk for VDD
AA	GG	Normal	Normal
AA	GG	Normal	Normal
TT	GG	Normal	Normal
TT	TT	Normal	Normal

4.2 Clinical Trials

Phase 1 was done for one month with 106 participants divided into three groups: placebo, D3 treatment, and calcitriol treatments with the idea of testing efficiency for those with VDD of the two treatments against the placebo. The results of phase 1 shows that VD levels at point 1 in D3 and calcitriol groups are significantly higher than the placebo group (Table 8 and Figure 7).

Table 8 VD average at points 0 and 1 by group.

	Average of VD at point 0 (ng/ml)	Average of VD at point 1 (ng/ml)
Calcitriol	16.24	29.75
D3	17.88	39.02
Placebo	24.46	23.28

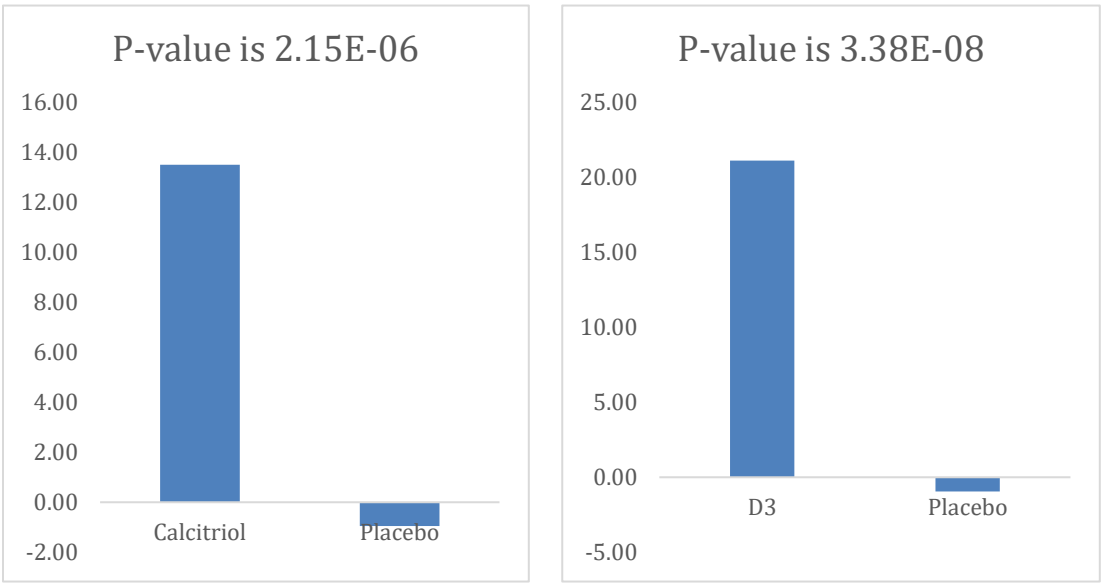

Figure 7 Average Change in VD by group.

Phase 2 was proposed to continue evaluation after removing the placebo and checking longer term treatment of D3 and Calcitriol for two additional months for 92 cases. The results for phase two were interrupted due to technical issues of patient and medication availability, the calcitriol levels were measures only in the calcitriol group and the results were compared to 25(OH)D levels in the D3 group (Figure 8).

Phase two results did not show a significant difference in VD changes in D3 and calcitriol groups. However, we measured difference in calcitriol levels vs VD in the calcitriol groups and the difference was significant compared to D3 group (Figure 8).

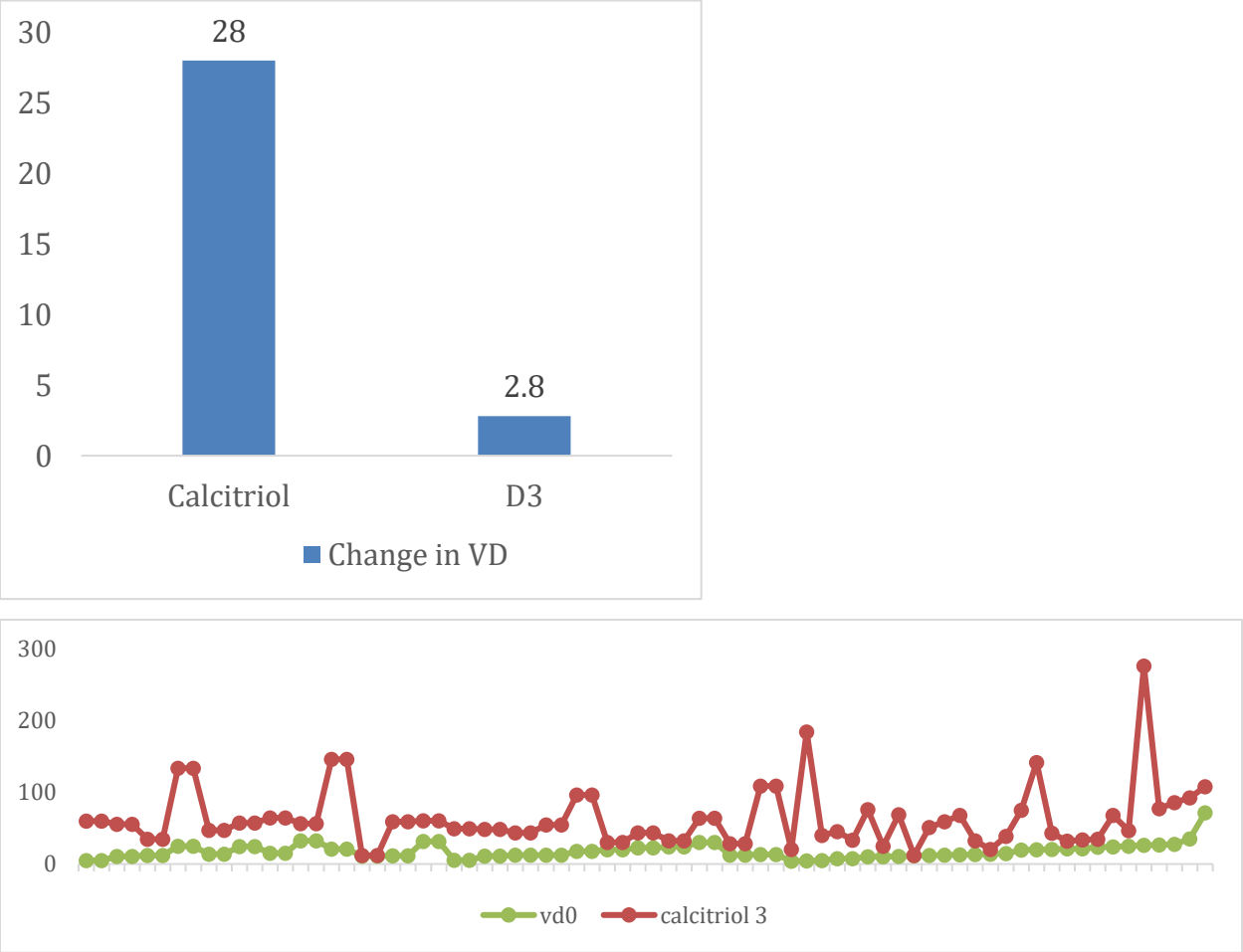

Figure 8 Change in Calcitriol vs VD.

Urea levels both at 1 month and three month levels were lower in the D3 treated cohort than in the calcitriol treated patients (Figure 11). However, Ca levels were higher than normal in 23% of the calcitriol group (Figure 9). To account for this, we measured PTH, and results were above the lower limit in the calcitriol group participants (Figure 10).

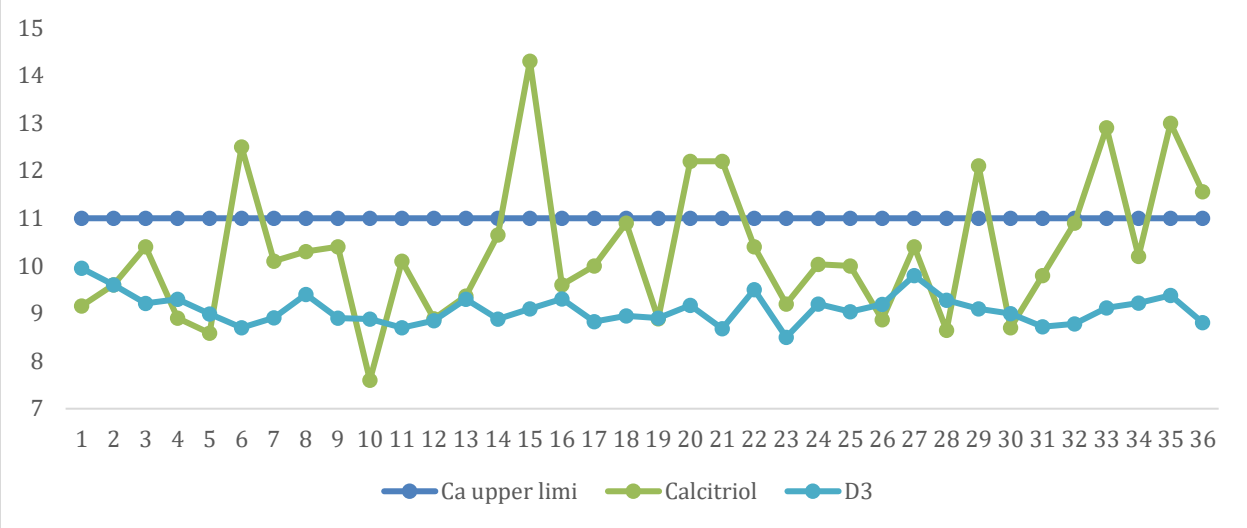

Figure 9 Ca levels in Calcitriol and D3 groups.

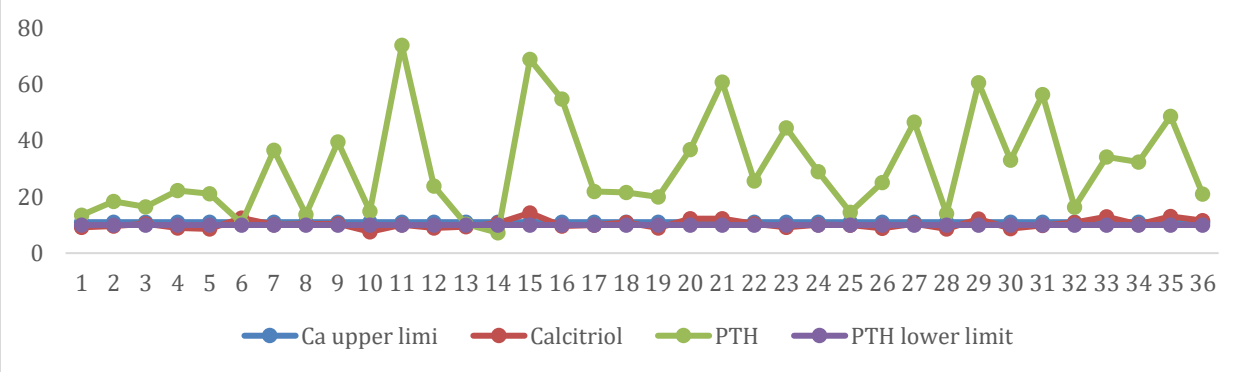

Figure 10 PTH levels in the calcitriol group

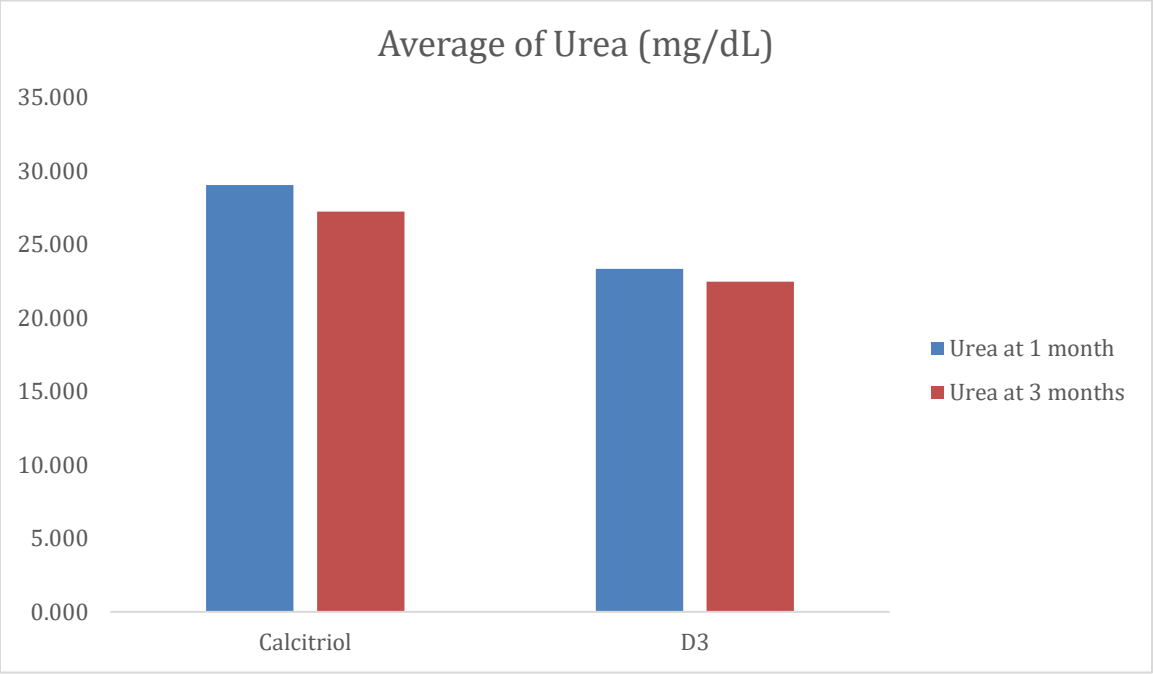

Figure 11 Urea levels for Phase 2 participants

Creatinine measurements were also lower in the D3 vs the Calcitriol group (Figure 12).

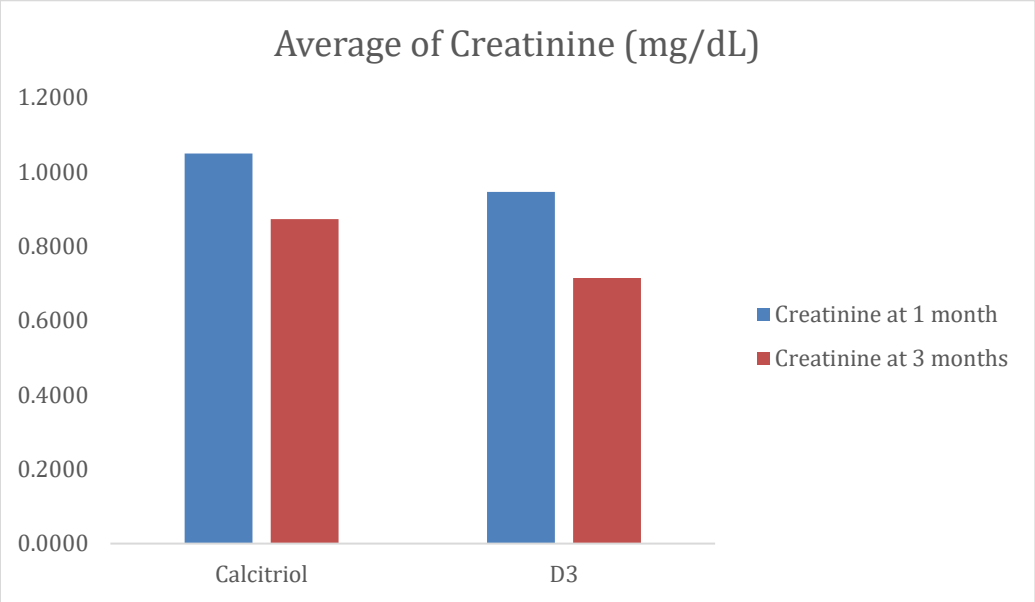

Figure 12 Creatinine levels for Phase 2 participants.

Chapter 5: Discussion

Alleles on SNPs rs10741657 (CYP121) and rs7041 (GC) were investigated in 69 Palestinians and revealed very high incident of VDD associated alleles in the Palestinian population with 65 of unsymptomatic random 69 individuals (94.2%) having elevated risk for developing VDD. The genotypes AG (CYP2R1) and TT (GC) were the most dominant. The results show that the T allele in GC is associated with reduced enzyme activity and higher risk for VDD which is in line with previous findings (Powe et al., 2013). Also, the D-25-hydroxylase (CYP2R1) enzyme that is responsible for the first hydroxylation of VD in the liver is found to have reduced activity in 85.5% of the study sample.

In a recent study in Jordan, 3 genotypes of both rs7041 (GG, TG, TT) and rs10743657 (GG, GA, AA) were documented (Lafi et al., 2015). It is worth noting that CC of rs7041 and TT of rs10743657 are present in our sample study but not in theirs. The genotypes frequencies for both rs7041 and rs10743657 are shown in Table 9.

Table 9 Genotypes frequencies comparison between Palestinian and Jordanian populations.

Rs7041 Genotypes	**Palestinians (this study)**	**Jordanians (Lafi et al. 2015)**	**rs10743657 Genotypes**	**Palestinians (this study)**	**Jordanians (Lafi et al. 2015)**
TT	52.2%	21%	AG	47.8%	47%
TG	34.8%	50%	GG	37.7%	41.8%
GG	11.6%	29%	AA	11.6%	11%
CC	1.4%	-	TT	2.9%	-

Different polymorphisms exist in the GC gene that result in different VD serum levels (Kamboh & Ferrell, 1986; Merchant et al., 2018). Hence, it would be critical to get relevant data on the Palestinian population for better understanding of VD genetic makeup and the underlying causes for VDD.

Higher consanguinity marriages among Palestinians can have a vital role in accumulating genetic mutations (Assaf & Khawaja, 2009; Bittles, 2001). It is possible that historical bottlenecks of the Palestinian population explain the high incidence of genetic predisposition for VDD and other recessive genetic disorders (Bittles, 2010; Qumsiyeh & Dasouki, 1997).

The data presented here and elsewhere disprove the claims that lower VD in the Arab region is mainly due to clothing styles and cultural backgrounds (El-Khateeb et al., 2019; Ray et al., 2009; Singh et al., 2019).

The majority of the literature on VD genetics is based on samples from Western populations (Batai et al., 2014) where it would be critical to get national genetic data from the Palestinian population as they may have their own genetic makeup (see for example Glerup et al., 2004).

It is worth the investigation of the enzyme 25-hydroxyvitamin D-24-hydroxylase (CYP24) that catabolizes both 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D into biologically inactive, water-soluble calcitroic acid. If CYP24 is overexpressed, this could result in VDD.

There is a common cautious when dealing with calcitriol as it's known to cause hypercalcemia (Li et al., 2015). However, there are no studies where calcitriol is given to normal individuals. The results presented here indicate that calcitriol could be an option for people with VDD, or at least for people who proved to have resistance for D3 supplementation. People undergoing calcitriol supplementation would be advised to monitor their calcium levels periodically. Our results showed that people who got hypercalcemia, quickly recovered their calcium levels to normal range when they stopped taking calcitriol.

Limitations of the study included the small number of the sample for both the genetic assessment and the clinical trials. The measurement methods for 25(OH)D and calcitriol could be more accurate if used HPLC/ LC-MS. The genetic assessment can be expanded to include other SNPs of CYP2R1 and GC and SNPs of other genes like VDR.

Chapter 6: References

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