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A Critical Evaluation of Vitamin D

Clinical Overview

Edited by Sivakumar Gowder

A CRITICAL EVALUATION OF VITAMIN D - CLINICAL OVERVIEW

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Meet the editor

Dr. Sivakumar Gowder received his academic training and carried out his research in institutions of high academic ranking in India and the US (University of Madras, Chennai, India; All India Institute of Medical Sciences, New Delhi, India; UT Southwestern Medical Center, Dallas, TX, USA; LSH Health Sciences Center, Shreveport, LA, USA and University of Pittsburgh School of Medicine, Pittsburgh, PA, USA). Before working for Qassim University, he worked as a faculty member at the Medical Universities in West Indies. Sivakumar has won prizes and awards in different levels of his academic career. He has developed his own research methods and techniques relevant to his research disciplines and has published several journal articles and book chapters. Dr. Gowder has also edited many books. Currently, he serves as an author and editor of books, editor in chief for an international journal, editorial member and reviewer for journals, fellow and advisory board member of international organizations and external examiner of doctoral thesis work for international universities. He has also served as an invited speaker and chairperson for international conferences.

Contents

Preface

Vitamin D, a fat-soluble vitamin also known as the “sunshine vitamin," is derived mostly from sun exposure and food. For normal activation, it has to undergo two hydroxylation reactions. Vitamin D affects more than 2000 genes in the body. A serum concentration of 25(OH) D indicates the ideal level of vitamin D in our bodies. The primary function of vitamin D is to regulate calcium and phosphorous absorption. Vitamin D deficiency leads to several diseases. From the therapeutic point of view, vitamin D helps in the treatment of many diseases. Lifestyle has an impact on our bodies' systems of getting sufficient amounts of vitamin D. In this context, due to industrialization and also changes in the environmental factors, any further study or work on vitamin D would be helpful for our society. I believe it offers a more than ample opportunity for me to present this book, *A Critical Evaluation of Vitamin D - Clinical Overview*, to the audience.

The book targets the principles, mechanisms and clinical significance of vitamin D. It covers four sections: “Vitamin D in Cardiovascular and Renal Diseases", “Vitamin D in Age and Neurological Diseases", “Vitamin D and Cancer" and “Therapeutic Measurements of Vitamin D". Each of these sections is interwoven with the theoretical aspects and experimental techniques of basic and clinical sciences. This book will be a significant source to students, scientists, physicians, healthcare professionals and also other members of this society who are interested in exploring the role of vitamin D in human life.

In the first section, the authors have disclosed the association of vitamin D deficiency in the pathogenesis and complications of cardiovascular disease—beneficial effects of vitamin D in renal patients, including end-stage renal patients and kidney transplant recipients and the pleiotropic effects of vitamin D in patients with kidney disease. In the second section, the authors have disclosed the prevalence of vitamin D deficiency and the associated risk factors among the geriatric population and the binding effects of vitamin D to amyloidogenic peptides in the brain. In the third section, we can notice information on the relationship between the vitamin D levels in the blood and the histological type and grade of colorectal tumors—the role of vitamin D and its receptor gene polymorphisms in the development of breast cancer and the topical or systemic application of vitamin D or its analogs to treat skin lesions for patients with neurofibromatosis type 1. In the last section, the authors reviewed the interrelationship between vitamin D status and the two well-known prothrombotic states, antiphospholipid syndrome and metabolic syndrome—therapeutic and prophylactic potential of vitamin D and the application of nanosystems for the encapsulation of vitamin D for different applications, such as food and pharmaceutical industries.

I appreciate the support of our higher authorities. I extend my gratitude toward my late mother and late father and my brothers for introducing me to higher education. I am contin-

uously indebted to my wife Anitha for her emotional and technical support throughout this project. The smiles of my daughter, Humsiha, encouraged me to finish this task in an easy way. I must acknowledge the interest and commitment from the Publishing Processing Manager of InTech Mr. Edi Lipovic, whose patience and focus were a fantastic support in this project. Finally, I express deep and sincere appreciation to all the authors for their valuable contributions and scholarly cooperation for the timely completion of this book.

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Section 1

Vitamin D in Cardiovascular and Renal Diseases

Chapter 1

Vitamin D and Cardiovascular Diseases

Claudia Lama von Buchwald and Seth I. Sokol

Additional information is available at the end of the chapter

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Abstract

Vitamin D deficiency is highly prevalent worldwide and has been implicated in the pathogenesis and complications of cardiovascular disease (CVD). Defining this relationship has been challenging, and the clinical application of vitamin D screening and supplementation for CVD risk prevention and modification remain uncertain. The available evidence includes large observational studies and smaller randomized trials mostly evaluating surrogate endpoints and scarcely directed at CV outcomes as a primary endpoint. Methodological heterogeneity is present among most of these trials. Clarification of the clinical application of this relationship through ongoing large randomized trials should have important implications for public health.

Keywords: vitamin D deficiency, cardiovascular disease, endothelial function, hypertension, vitamin D

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death in the developed world and is projected to be the leading cause of morbidity and mortality in developing countries by 2020 [1]. In the United States, one in three adults lives with CVD resulting in disability and losses of billions of dollars each year [2]. CVD is a multifactorial disease that embodies a complex interplay between genetic predisposition, environmental factors, and risk factors that tend to be more prevalent in certain ethnic groups and those with lower socioeconomic status. Despite substantial gains in CVD prevention, a significant amount of risk remains despite adequate control and modification of traditional risk factors. Identification of novel risk factors that are easily modifiable has been eagerly sought over the past decade.

The discovery of the vitamin D receptor (VDR) in multiple cell types, including cardiomyocytes and vascular cells [3, 4], has led to increasing interest in vitamin D's role in human health, including cardiovascular, beyond its well-known role in bone health. Deficient vitamin D levels (<20 ng/ml) have been independently linked to increased morbidity and mortality [5–7].

Experimental evidence has linked vitamin D to regulation of multiple pathways involved in the pathogenesis of CVD. Several ecological and epidemiological studies have suggested a relationship between CVD and vitamin D status, as CVD events are higher in the winter, a period when vitamin D levels are lowest [8, 9]. Additionally, certain populations with poor cutaneous production of vitamin D and subsequently lower plasma levels, such as African Americans, tend to be at greater risk for hypertension and cardiovascular disease [3, 8, 10, 11]. These lines of evidence do not prove causality but support a hypothesis for further study.

Randomized controlled trials have mostly been based on surrogate or secondary endpoints for CV risk reduction [12]. Study methodology has been heterogeneous and results are often conflicting. To date, large well-powered randomized trials of vitamin D featuring CV outcomes as a primary endpoint are still ongoing [13, 14]. In the absence of results from these trials, regular supplementation cannot be recommended for cardiovascular risk modulation. Despite the lack of recommendations, use of vitamin D supplements for this purpose has risen dramatically.

The following chapter will provide an overview on the biologic plausibility and current evidence linking vitamin D to CV health and disease. But first, a brief review of the prevalence and definition of vitamin D deficiency and description of vitamin D synthesis and metabolism is necessary.

2. Vitamin D deficiency

Vitamin D deficiency is prevalent in 30–50% of adults in developed countries [10, 15], and it is estimated that more than 1 billion individuals worldwide are vitamin insufficient or deficient [3, 10, 16, 17]. Vitamin D deficiency is prevalent in every segment of the US population but remains under recognized and under treated [15, 18].

Serum levels of 25(OH)D <20 ng/ml indicate deficiency, and levels >30 ng/ml are considered optimal for bone health (**Table 1**) [15, 19]. Vitamin D levels of 30–40 ng/ml are associated with maximal parathyroid hormone suppression [10, 16, 19, 20]. No consensus has been reached on the optimum level of 25(OH)D for purported benefits beyond skeletal health [3, 10, 16, 17, 21]. A recent study suggests a 25(OH)D threshold of 11–14 ng/ml below which signifies increased CVD risk [22].

Levels in the range of 21–29 ng/ml are considered insufficient. Using this definition, the majority of the US population would be labeled as vitamin D insufficient.

A decline in mean serum vitamin D levels in the US population was detected when comparing data from the National Health and Nutrition Examination Survey (NHANES). The NHANES

survey from 1988 to 1994 (n = 18,883) showed a mean 25(OH)D level of 30 ng/ml as compared to a mean 25(OH)D level of 24 ng/ml in the 2001–2004 (n = 13,369) survey [23, 24]. This difference may have been explained by different assays used during the more current survey as compared to prior surveys, but there still remained a small but significant reduction after accounting for these differences [25]. The decline was likely related to behavioral factors most notably sun avoidance and obesity.

	25(OH)D concentrations (ng/ml)	Symptoms and biochemical consequences
Deficiency	10–20	Severe hypoparathyroidism
–	–	Calcium malabsorption
–	–	Rickets
–	–	Osteomalacia
–	–	Myopathy
Insufficiency	21–29	Elevated PTH
–	–	Low intestinal calcium absorption
–	–	Reduced bone mineral density
–	–	Subclinical myopathy
Sufficiency	>30	–
Toxicity	>150	Hypercalcemia
–	–	Increased intestinal absorption

Based on Lee et al. [15] and Zittermann et al. [7]

Table 1. 25(OH)D concentration and its effects [3, 7, 15].

Risk factors for developing vitamin D deficiency include limited cutaneous synthesis due to inadequate sun exposure (sunscreen use, institutionalized or homebound patients) and low dietary intake [3]. Other risk factors include age >65, smoking, air pollution, dark skin pigmentation, obesity (resulting from storage in adipose tissue), kidney and/or liver disease, disorders affecting fat absorption (e.g., celiac disease, Crohn's disease, ulcerative colitis, some types of bariatric surgery), and end organ insensitivity to $1,25(OH)_2D$ [3, 9, 17].

3. Vitamin D metabolism

Vitamin D is a prohormone. Its active form, 1a 25-dihydroxyvitamin D ($1,25(OH)_2D$), plays an essential role influencing various metabolic pathways [7, 21, 26–30].

Skin synthesis from sunlight exposure (wavelength, 290–315 nm) contributes to 80–90% of vitamin D production in humans under natural conditions (D_3—cholecalciferol) [17]. UV-B irradiation of skin triggers photolysis of 7-dehydrocholesterol (provitamin D_3) in the plasma

membrane of human skin keratinocytes [3], which is then rapidly converted to vitamin D_3 by the skin's temperature (**Figure 1**) [7].

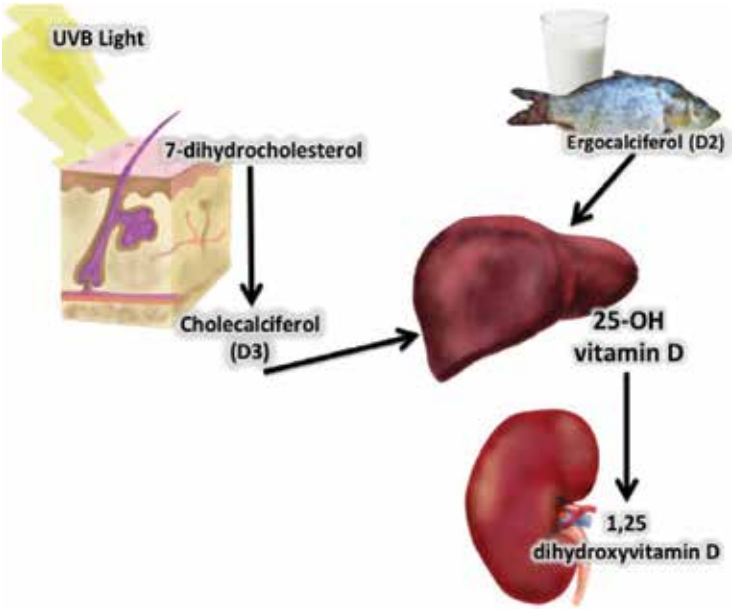

Figure 1. Vitamin D synthesis and metabolism [17].

The dietary supply of vitamin D (D_2—ergocalciferol) contributes 10–20% to the total amount of vitamin D in the body [31].

D_3 from the skin and D_2 from the diet undergo two sequential hydroxylations: 25 hydroxylation in the liver followed by 1,25-dihydroxylation in the kidney by 1-alpha hydroxylase (CYP27B1) (**Figure 1**). The major circulating metabolite of vitamin D is 25(OH)D, which should be measured clinically to assess vitamin D status, reflecting both intake and endogenous production [15, 31, 32]. $1,25(OH)_2D_3$ is the biologically active form. The hydroxylation of 25(OH)D to its biologically active form is under control of parathyroid hormone (PTH) [7, 15].

The majority of 25(OH)D and $1,25(OH)_2D_3$ in the circulation is bound to vitamin D-binding protein (DBP) (80–90%) and albumin (1–20%), while a small fraction is free. Production and levels are regulated by a feedback loop that includes serum PTH, calcium, and phosphate [3, 10, 16, 32].

Most of the known biological effects of $1,25(OH)_2D_3$ are mediated through the vitamin D_3 receptor (VDR), a member of the superfamily of nuclear hormone receptors, which mediates transcriptional gene regulation [3, 7, 33–35].

$1,25(OH)_2D_3$ enters the cell and interacts with its nuclear VDR. It then forms a heterodimeric complex with the retinoic acid X receptor. Once the receptor complex binds to vitamin D-

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responsive elements, a variety of transcriptional factors bind to it, resulting in gene expression [17] (**Figure 2**).

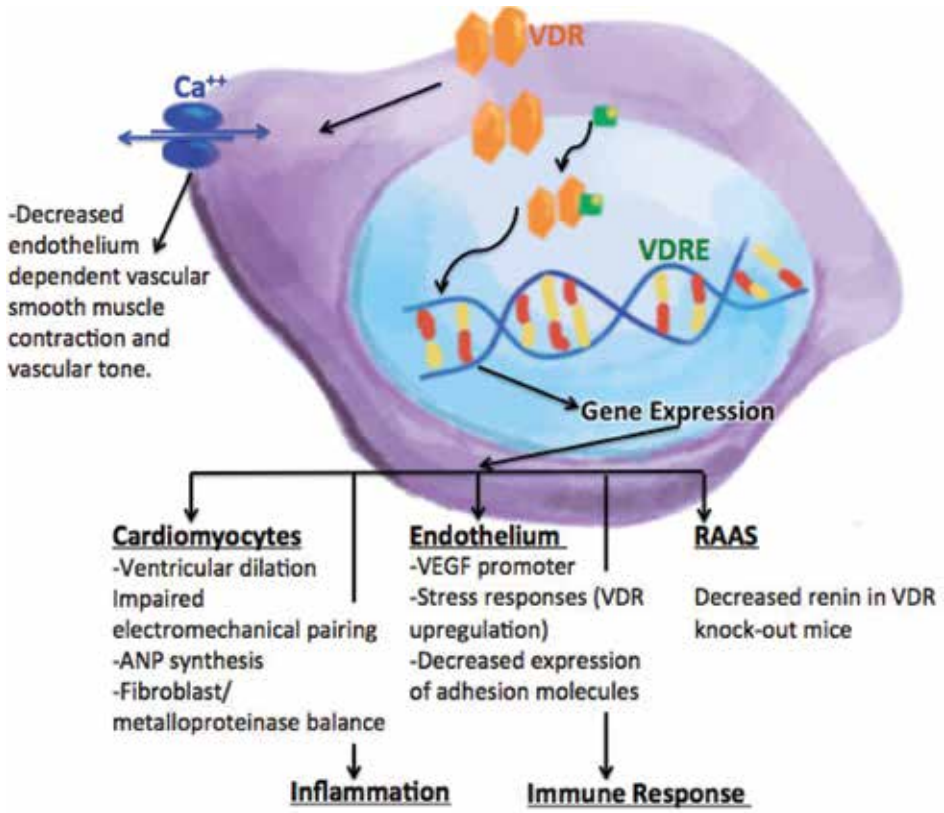

Figure 2. Cellular effects of vitamin D.

Over 200 genes are regulated by $1,25(OH)_2D_3$. These include genes directly or indirectly responsible for renin and insulin production [15, 36], cytokine release [34], and vascular smooth muscle cell (VSMC) and cardiomyocyte proliferation [37].

$1,25(OH)_2$ is also involved in non-genomic mediated intracellular signaling demonstrating immunomodulatory, antiproliferative, and prodifferentiative activities in experimental settings [17, 31, 38].

4. Biologic plausibility

Although biologically plausible, the characterization of vitamin D deficiency as a primary risk factor for CVD is challenging because of the complexity and number of interplaying pathways vitamin D is involved with.

The vitamin D receptor is nearly ubiquitous. It has been found in many cells including vascular smooth muscle cells (VSMC), endothelial cells, cardiac myocytes, and juxtaglomerular and most immune cells, all of which have been implicated in the pathogenesis and progression of CVD [3, 6, 10, 15–17, 32, 37–41].

Activated CD4+ and CD8+ T cells, B cells, neutrophils, macrophages, and dendritic cells all possess the capacity to convert $25(OH)D_3$ into its active form $1,25(OH)D_3$. Moreover, it is known that the rate-limiting enzyme in this pathway, 1,25 hydroxylase, is also present in activated macrophages [41, 42]. VSMC [37] and endothelial cells also express their own 1,25 hydroxylase, suggesting that these cells contain an autocrine mechanism to modulate the effects of vitamin D on the vasculature [43].

Vitamin D has direct and indirect cardiovascular effects. In a direct manner, $1,25(OH)_2$ enhances proliferation of vascular smooth muscle cells and expression of vascular endothelial growth factor via the VDR and CYP27B1 expression in VSMCs and endothelial cells [37]. It also plays an important role in inflammation and thrombosis. Inverse associations between vitamin D deficiency and thrombogenicity, vascular inflammation, and vascular calcification have been demonstrated [7, 38]. Cardiac and smooth muscle contractility is controlled partly by intracellular handling of calcium that depends on extracellular calcium which is regulated by vitamin D. $1,25(OH)D_3$ has an inhibitory effect on hypertrophy and proliferation of VSMCs *in vitro* and in cultured cardiac myocytes, ultimately inducing apoptosis [37]. (**Figure 2**) The lack of VDR signaling results in chronically low nitric oxide production, caused by defective NO synthase.

Indirectly, the expression of renin *in vivo* is strongly regulated by vitamin D, and an inverse relationship between vitamin D levels and renin expression has been demonstrated experimentally [6, 27, 39, 40, 44, 45]. $1,25(OH)_2$ binds to the renin promoter region and inhibits renin transcription, thus reducing plasma renin activity [27, 40]. VDR knockout mice were proven to have increased levels of renin and angiotensin II and therefore higher prevalence of hypertension [27, 28, 40, 44, 45]. Thus, vitamin D may indirectly regulate blood pressure and affect cardiac hypertrophy through this mechanism.

Another indirect effect of vitamin D on CVD involves the production of matrix metalloproteinase 2 and 9, which promote insulin uptake beta-cell function and suppress pro-inflammatory cytokine release while increasing anti-inflammatory cytokine levels (IL-10) [34, 46]. These mechanisms help delay the inflammatory pathways implicated in coronary artery disease, by maintaining glycemic control and hindering secondary hyperparathyroidism and the formation of vascular calcification [33].

Vitamin D deficiency may also indirectly act deleteriously by inducing hyperparathyroidism. Parathyroid hormone (PTH) controls calcium homeostasis through specific receptors that are also present within vessel walls and the myocardium. PTH may promote the release of inflammatory cytokines, modulate vascular remodeling and lead to impaired glucose metabolism [47]. Several studies have demonstrated an association between high PTH levels and hypertension, myocardial dysfunction and vascular disease. In addition, hyperparathyroidism is also associated with increased mortality [6, 47, 48].

Lastly, increased biosynthesis and hyperlipidemia have also been associated to vitamin D deficiency. This is thought to result from decreased transcriptional activity of the VDR leading to the downregulation of insulin-induced gene-2 (Insig-2) expression. This ultimately results in increased 3-hydroxy-3-methylglutaryl-coenzyme reductase expression [49].

5. The clinical evidence

Vitamin D deficiency is a common finding in patients with CVD [15]. An inverse association between suboptimal 25(OH)D_3 and poor outcomes in CV health has been demonstrated by multiple trials. Most of these studies are observational, hindering the establishment of a causal relationship.

Significant differences across studies such as varying definitions vitamin D deficiency, lack of seasonal adjustment, and properly defined CV outcomes also hamper our ability to make valid and consistent conclusions. Additional questions arise from use of a single baseline measurement of vitamin D (which may not be an accurate indicator of vitamin D status overall), underestimating or poorly understanding the role of high PTH on CVD, and the confounding use of other disease-modulating drugs such as calcium and statins in both active and placebo groups.

6. Observational data

Several large-scale observational studies have been completed over the past decades.

The NHANES III national cohort registry analyzed 15,088 subjects using a cross-sectional design and found that. 25(OH)D levels were inversely associated with hypertension, diabetes mellitus, hypertriglyceridemia, and obesity [5, 35].

Similar conclusions were obtained in the prospective analysis of 41,504 patients from The Intermountain Heart Collaborative Study Group, in which serum 25(OH)D levels <30 ng/ml were associated with highly significant increases in the prevalence of diabetes, hypertension, hyperlipidemia, and peripheral vascular disease. Serum 25(OH)D levels were also highly associated with coronary artery disease, myocardial infarction, heart failure, stroke, and incident death [18].

In the Health Professionals Follow-up Study, men deficient in 25(OH)D (<15 ng/ml) were at increased risk for myocardial infarction compared with those considered to be vitamin D sufficient (>30 ng/ml) RR, 2.09; 95% CI, 1.24–3.54; $P = 0.02$ for trend) even after risk factor adjustment [50]. It could be hypothesized that this increased risk may be explained by a pro-inflammatory state induced by vitamin D deficiency.

In contrast, other prospective studies have had discordant results. The MIDSPAN family study followed 2338 subjects prospectively for a median of 14.4 years. Plasma levels of 25(OH)D

<15 ng/ml were not associated with a risk of cardiovascular diseases, but did relate to all-cause mortality. There was an association between 25(OH)D levels and incidence of type 2 diabetes, but there was no evidence that vitamin D supplementation improved outcomes in these subjects [51]. Follow-up of 3135 patients from the Osteoporotic Fractures in Men (MrOS) study and included in the MrOS Sleep Study failed to establish a significant association between circulating 25(OH) vitamin D and risk of CVD events [52].

Aside from actual CVD events and mortality, other endpoints using surrogate markers have been studied. In a prospective Austrian cohort of 3258 patients referred for coronary angiography and followed up for 7.7 years, low 25(OH)D levels correlated inversely with markers of inflammation (C-reactive protein and interleukin-6), oxidative burden (serum phospholipid and glutathione), and cell adhesion (vascular cell adhesion molecule-1 and intercellular adhesion molecule-1) [6].

A myriad of observational data relates low vitamin D status to an increased prevalence of hypertension. In the Third National Health and Nutrition Examination Survey (NHANES-III), systolic blood pressure (BP) had a significant inverse correlation to 25(OH)D levels. Mean systolic and diastolic BP were 3.0 and 1.6 mm Hg ($P < 0.05$) lower for participants in the highest quintile compared with the lowest, after adjusting for potential confounders. Age-adjusted systolic BP was significantly lower in individuals with vitamin D sufficiency [5].

A prospective analysis among 1211 non-hypertensive US men found an inverse relationship between vitamin D levels and development of hypertension over a 15-year follow-up period. VDR BsmI and FokI polymorphisms were also associated with increased risk of hypertension [53].

Additionally, a more recent study involving 746 patients failed to demonstrate significant relationship between serum vitamin D levels and the severity and extent of coronary artery disease [54].

Aside from the link between developing hypertension and low vitamin D levels, the Framingham Offspring Study suggested that low serum vitamin D levels may augment the risk associated with existing hypertension to dramatically raise the risk of future cardiovascular events [55].

7. Randomized controlled trials

Many randomized interventional studies have focused on improving surrogate endpoints rather than hard CV outcomes. Those focusing primarily on CV outcomes are sparse. Most of the available studies have varied methodologically in defining baseline vitamin D status, dose used, and definition and ascertainment of outcomes.

These flaws are seen in studies that evaluate all-cause mortality and those evaluating CVD outcomes.

A meta-analysis of randomized placebo control trials with varying levels of vitamin D using mortality as a secondary endpoint found a significant 8% reduction in mortality in individuals

receiving vitamin D. This study was limited by the inability to evaluate cause-specific mortality [56].

In another meta-analysis, individuals who took vitamin D at daily doses ranging from 300 to 2000 IU (average dose 528 IU) for an average of 5.7 years had a 7% lower risk of death (from all causes) than those who did not.

The relatively low dose of vitamin D and the short treatment period may have led to an underestimation of its effect. It was noted that the clinical evolution of chronic conditions may take longer to be influenced by vitamin D supplementation; hence, very long-term follow-up would be required to observe the full effect [57].

In the CVD arena, a double-blinded, placebo-controlled, randomized trial in the United Kingdom, including 2686 patients between the ages of 65 and 85, showed no benefits on CVD outcomes in the group that received 100,000 IU of supplemental vitamin D_3 every 4 months (833 IU daily) for 5 years [58]. A systematic review of 14 prospective studies and 18 randomized trials examining supplementation with vitamin D, calcium, or both and subsequent cardiovascular events concluded that vitamin D supplementation might reduce the risk of CVD. Separate analysis of the eight randomized trials found a non-significant reduction in CVD risk with vitamin D supplementation [30].

Results from the Women's Health Initiative (WHI) suggests that postmenopausal women receiving 400 IU/day of oral vitamin D_3 combined with calcium 1000 mg/day had no reduction in their risk of CHD events or stroke. In a subanalysis of the WHI, calcium and vitamin D_3 supplementations were not found to improve blood pressure or coronary artery calcium score. Furthermore, after 7 years of follow-up, there was no decrease in incident hypertension or prevention of the metabolic syndrome, diabetes, or decreases in cerebrovascular risk [59].

8. Hypertension

Contrary to observational evidence, randomized controlled trials have failed to demonstrate significant changes in blood pressure in individuals with prehypertension or stage I hypertension and vitamin D deficiency after supplementation [60–62].

In a trial involving 283 blacks (median age, 51 years) randomized into a four-arm, double-blind trial for 3 months of placebo, 1000, 2000, or 4000 UI/day of vitamin D, no effect was found on diastolic blood pressure, but there was a slight effect in lowering systolic blood pressure [63].

In VitDISH, a double-blind, placebo-controlled randomized trial, including 159 patients, vitamin D supplementation did not improve blood pressure or markers of vascular health in older patients with isolated systolic hypertension [64]. A study including patients with resistant hypertension who received vitamin D_3 supplementation for 6 months also showed similar results. Effects on left ventricular hypertrophy were also negligible, although the short-term follow-up may have been a limitation in assessing this outcome variable [65].

9. Diabetes

A meta-analysis of 11 prospective studies involving 3612 cases and 55,713 non-case participants suggested a strong inverse association between serum 25(OH)D concentration and incidence of type 2 diabetes. Results suggested that optimal levels may reduce the risk of future diabetes by 41% [66].

Other contrasting meta-analysis of 15 trials did not find sufficient evidence to recommend vitamin D supplementation for improving glycemia or insulin resistance in obese patients with diabetes, normal fasting glucose levels, or impaired glucose tolerance [67].

10. Endothelial dysfunction

Vitamin D deficiency has been associated with endothelial dysfunction as measured by flow-mediated dilation (FMD) and reactive hyperemia peripheral arterial tonometry (RH-PAT) [68].

A small study involving 23 asymptomatic subjects demonstrated that subjects with significant vitamin D deficiency had impaired brachial artery FMD, which improved after vitamin D replacement therapy. Recently, a stepwise change in FMD according to vitamin D status was demonstrated and an inverse association between serum 25(OH)D levels and vascular inflammatory markers was observed [33].

A prospective placebo-controlled pilot study evaluated the effects of vitamin D repletion on endothelial function and inflammation in subjects with both vitamin D deficiency and CAD. The study was conducted over a 12-week period in 90 subjects. RH-PAT was used to estimate endothelial function. No significant differences between groups were found in reactive hyperemia index, blood pressure, and levels of hs-CRP, IL-6, IL-12, interferon-gamma (INF-gamma), and CXCL-10 [68].

Similar results were obtained on a larger scale, the Prospective Study of the Vasculature in Uppsala Seniors (PIVUS), that studied 852 men and found no significant relationship between vitamin D levels and endothelium-dependent vasodilation, flow-mediated vasodilation, and reflectance index. However, serum 25(OH)D level showed a negative correlation with SYNTAX score (angiographic grading tool to determine severity of coronary disease) and high-sensitivity C-reactive protein (hsCRP) level. Logistic regression analysis identified 25(OH)D as an independent factor related to high SYNTAX scores. Patients whose vitamin D levels were in the lowest 25(OH)D category (<20 ng/ml) were more often in the high SYNTAX scores group, with their incidence about twofold higher than those in the highest 25(OH)D category (>30 ng/ml) [69].

In cross-sectional analyses, low 25(OH)D (<20 ng/ml) was not associated with stiffer arteries after adjustment for cardiovascular disease risk factors ($P > 0.4$). PTH >65 pg/ml was associated with stiffer arteries after adjustment for cardiovascular disease risk factors, other than systolic blood pressure [70].

Black normotensive teenagers who received 2000 IU/d of vitamin D_3 were compared with those who received 400 IU/d for 16 weeks in an RCT. Teenagers who received 400 IU/d of vitamin D_3 increased their levels of 25(OH)D from 13.6 ± 4.2 to 23.9 ± 7.2 ng/ml and showed no reduction in arterial stiffness. In contrast, teenagers who received 2000 IU/d of vitamin D_3 increased their mean levels of 25(OH)D from 13.2 ± 3.4 to 34.2 ± 12.1 ng/ml and significantly lowered their arterial wall stiffness. This is supported by the observation that serum 25(OH)D levels <30 ng/ml were strongly associated with hypertension, elevated blood glucose, and metabolic syndrome in adolescents [63].

11. Recommended daily allowances and supplementation

The Institute of Medicine (IOM) concluded that there is sufficient evidence to support a role for vitamin D in maintaining skeletal health, but a lack of evidence to support beneficial effects on non-bone-related health outcomes [19]. The Endocrine Society also does not recommend screening for vitamin D deficiency in individuals who are not at risk for vitamin D deficiency [16].

The recommended dietary intake (RDI) of vitamin D is 400 IU/d for 0–12 months, 600 IU/d for ages 1–70 years, as well as for pregnant and lactating women, and 800 IU/d for ages 71 years and older [16, 20, 71, 72]. Measurement of 25(OH)D serum level is the best indicator for overall vitamin D status in the clinical setting, since it has a longer half-life (10–27 days after administration) [21].

Vitamin D can be found in foods such as oily fish (salmon, sardines, and mackerel—400 UI/3.5 oz), cod liver oil (400 IU/tsp), egg yolk (20 IU), fortified milk, orange juice, cereals, cheese, and mushrooms. (100 IU/8oz) [3, 10, 15–17].

In terms of supplements, for every 100 IU of vitamin D ingested, blood 25(OH) level increases by around 1 ng/ml (2.5 nmol/l).

Vitamin D supplementation has dose-dependent side effects, which are fairly rare, such as hypercalcemia, hypercalciuria, renal calcification, and increased bone resorption. Significant increase in triglyceride has also been described [62].

12. Future directions

Several large-scale randomized trials of moderate-to-high dose vitamin D supplementation for cardiovascular disease prevention are currently being conducted. The Vitamin D and OmegA-3 TriaL (VITAL) is a randomized, double-blind, placebo-controlled clinical trial among more than 20,000 US men and women above age 50, testing 2000 IU/day of oral vitamin D_3 and omega 3 fatty acid supplements in a 2 × 2 factorial design, with cardiovascular disease and cancer as primary prespecified outcomes. Results are expected in 2017 [13]. Another large

randomized trial of CVD prevention, the VIDA trial, is evaluating a higher dose of vitamin D (100,000 IU a month) over 3.3 years and expects results in late 2016 [14].

Evaluating whether common polymorphisms in the VDR receptor modifies the association between 25(OH)D concentrations and individual CVD risk has been proposed. A recent trial evaluating two previously studied VDR polymorphisms failed to reveal a significant role to this end; however, further study may be warranted [73].

13. Conclusions

Vitamin D deficiency has increasingly been implicated in the pathogenesis and complications of cardiovascular disease (CVD). Evidence supporting this relationship and the use of supplementation to prevent or treat CVD is inconclusive and divergent.

Clarification of the clinical application of this relationship through ongoing large randomized trials should have important implications for public health.

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Chapter 2

Vitamin D and Renal Disease

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Additional information is available at the end of the chapter

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Abstract

The metabolism of vitamin D (VD) is severely impaired in chronic kidney disease (CKD). Uremia is not only associated with the reduction of its active form 1,25-dihydroxyvitamin D but also in the reduction of all VD metabolites. CKD-associated abnormalities in VD are part of the CKD-related mineral-bone disease. However, VD has beneficial effect on the kidneys due to its pleiotropic effects, namely, antiproteinuric effect and renin-angiotensin-aldosterone system suppression, thus making the relationship between VD and the kidney even more complicated. The aim of our chapter is to reveal the changes in vitamin D axis in CKD, to outline the possible beneficial effects of vitamin D in renal patients, including end-stage renal patients and kidney transplant recipients, and to address the current opinions concerning treatment with cholecalciferol, calcitriol, and vitamin D analogs.

Keywords: vitamin D, chronic kidney disease, mineral bone disease, kidney transplantation, pleiotropic effects

1. Introduction: vitamin D and calcium-phosphorus metabolism in the healthy kidney

The kidney plays a pivotal role in vitamin D (VD) metabolism. In the proximal tubules the enzyme 1α hydroxylase (CYP27B1) transforms 25-hydroxyvitamin D into the active metabolite 1,25-hydroxyvitamin D (**Figure 1**). 25-hydroxyvitamin D (25VD) is absorbed in the proximal tubule cells via megalin-dependent pathway. The absorption, however, is severely impaired in nephrotic syndrome [1].

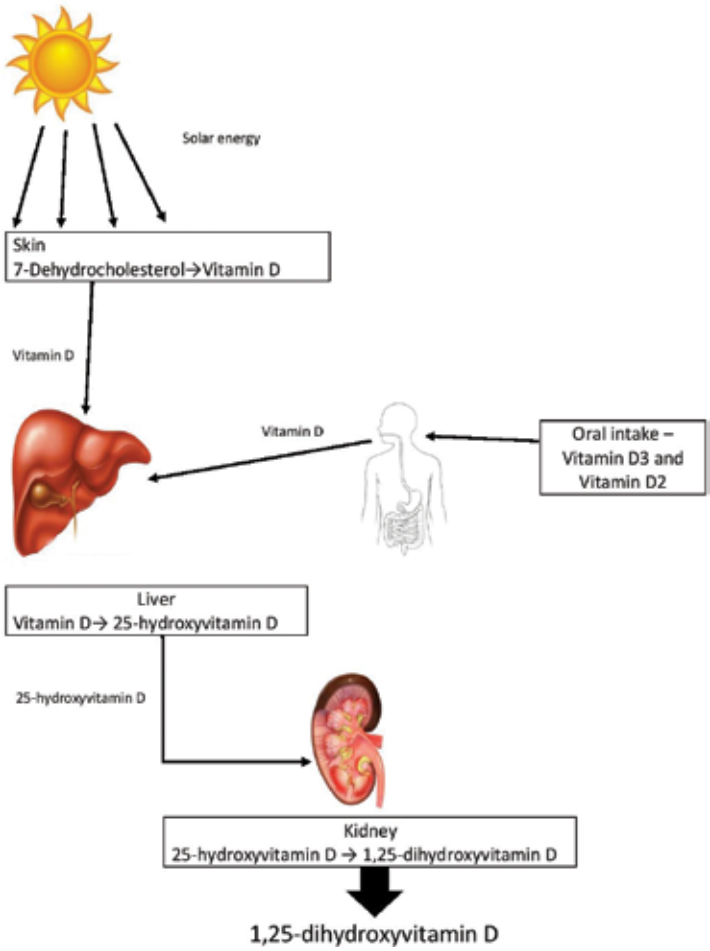

Figure 1. Vitamin D synthesis.

CYP27B1 activity is influenced by different factors. Parathyroid hormone (PTH), prolactin, human growth hormone, low serum calcium, and phosphorus increase CYP27B1 activity, whereas 1,25dihydroxyvitamin D, thyroid hormones, metabolic acidosis, and fibroblast growth factor-23 (FGF-23) suppress its activity [2–4]. The proximal tubules are the major site for activation of vitamin D (VD). However, nonrenal CYP27B1, transforming 25VD into 1,25VD, was detected in other tissues—skin (basal keratinocytes, hair follicles), lymph nodes (granulomata), colon (epithelial cells and parasympathetic ganglia), pancreas (islets), adrenal medulla, brain (cerebellum and cerebral cortex), and placenta (decidual and trophoblastic cells) [5]. Together with the widely distributed vitamin D receptor (VDR) in human body, these data are the basis of the suggested pleiotropic effects of VD. Of utmost importance for the nephrologist are the renoprotective properties of vitamin D, which are based on renin-angiotensin-aldosterone system suppression, nucleotide factor-kB downregulation, Wnt/β-catenin pathway suppression, and upregulation of slit diaphragm protein synthesis [6–8].

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The kidney is crucial in maintaining calcium-phosphorus metabolism. Apart from activation of VD, the kidneys increase calcium and phosphorus reabsorption in the tubules under the influence of 1,25-dihydroxyvitamin D (1,25VD). Furthermore, 1,25VD is involved in osteoclast activation and differentiation, as well as osteoblast activation thus taking part in bone remodeling. In addition, the proximal tubules are the target of major phosphatonins, such as FGF-23 (by α-klotho-dependent mechanism) and PTH [9]. The basic interactions of the kidney in the mineral bone metabolism are shown in **Figure 2**.

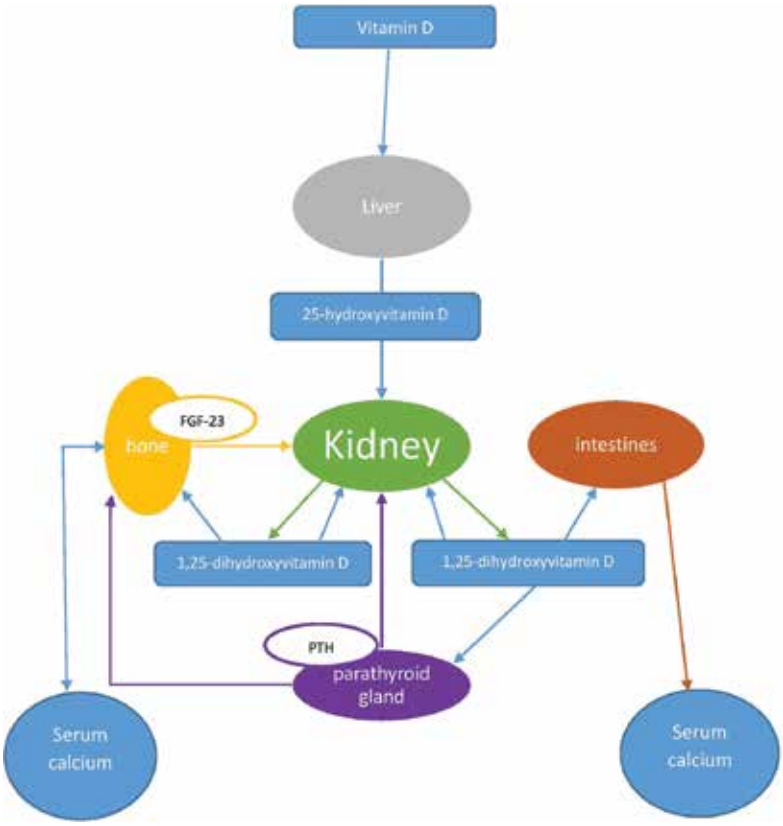

Figure 2. Role of the kidney in calcium-phosphorus metabolism.

A particular attention should be paid to FGF-23 and klotho pathways, as their discovery have changed significantly our knowledge of bone health and changes in calcium-phosphorus metabolism in chronic kidney disease (CKD). Fibroblast growth factor-23 is an osteoblast-/osteocyte-secreted hormone with primary physiological effects on the kidney and the parathyroid gland. FGF-23 stimulates phosphaturia by downregulating luminal expression of sodium-phosphate cotransporters in the proximal tubule and reduces systemic levels of 1,25VD by inhibiting renal 1-α hydroxylase and stimulating the catabolic 24-hydroxylase [10, 11] (**Figure 3**).

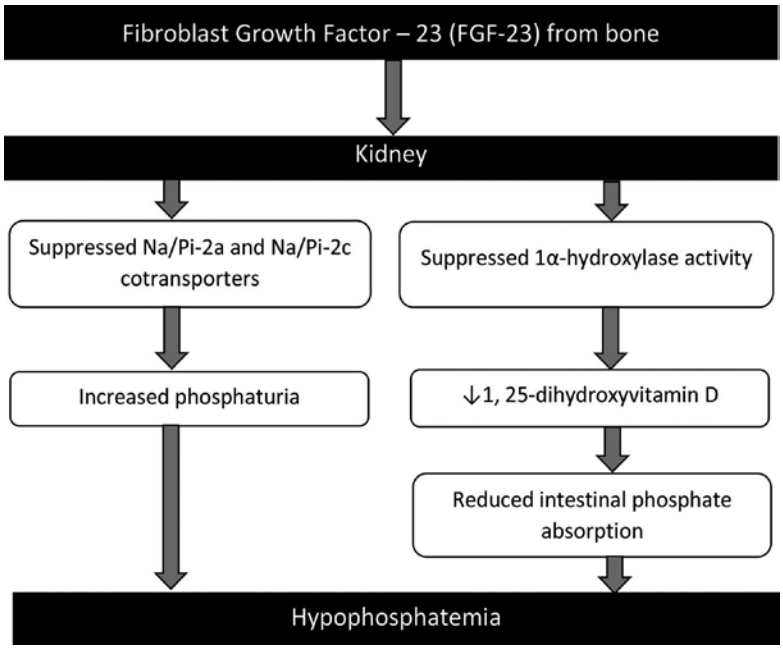

Figure 3. Role of FGF-23 in phosphate homeostasis.

In healthy subjects, FGF-23 suppresses PTH secretion [12]. In addition, extrarenal effects have been described on cardiovascular system and brain [13]. Alfa-klotho is a protein cofactor for FGF-23 signaling, as it forms complexes with FGF-23 receptor, thus increasing its affinity for the hormone [14]. A soluble klotho was also detected, functioning as humoral factor. Soluble klotho downregulates insulin-like growth factor I, thus exerting antiaging properties [15]. It also potentiates 1,25VD-associated renal calcium absorption [16]. Furthermore, soluble klotho causes hypophosphatemia and phosphaturia independently of FGF-23 and is regarded as an early marker of CKD [17, 18].

In summary, the kidney is closely linked to the VD axis and calcium-phosphorus homeostasis. Early changes in renal function are associated with significant changes in VD metabolism. We shall start with VD pathology in patients with renal disease and at the end of our review the topic vitamin D metabolism after kidney transplantation will be discussed.

2. Vitamin D metabolism in kidney disease: pathophysiology

2.1. Chronic kidney disease: definition

According to the widely accepted definition by the international foundation for Kidney Disease/Improving Global Outcomes (KDIGO), chronic kidney disease (CKD) is defined as abnormalities of kidney structure or function, present for more than 3 months, with implications for health [19] (**Table 1**).

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Markers of kidney damage (one or more)	Albuminuria (AER ≥ 30 mg/24 hours; ACR ≥ 30 mg/g [≥3 mg/mmol])
	Urine sediment abnormalities
	Electrolyte and other abnormalities due to tubular disorders
	Abnormalities detected by histology
	Structural abnormalities detected by imaging
	History of kidney transplantation
Decreased GFR	GFR < 60 ml/min/1.73 m^2 (GFR categories G3a–G5)

Abbreviations: CKD, chronic kidney disease; GFR, glomerular filtration rate; AER, albumin excretion rate; ACR, albumin:creatinine ratio.

Table 1. Criteria for CKD (either of the following for more than 3 months) [19].

CKD is a global health problem, affecting up to 10% of the population [20]. As the glomerular filtration rate (GFR) declines, especially below 60 ml/min/1.73 m^2, the ability of the kidney to excrete phosphate is diminished, leading to disruption of calcium-phosphorus homeostasis, pathological changes in hormone levels (PTH, FGF-23), and decrease in the level of VD metabolites. Subsequently, changes in bone morphology and extraskeletal calcifications occur. The changes in biochemical indicators, bone morphology, and extraskeletal calcium deposits are defined as chronic kidney disease-mineral bone disorder (CKD-MBD), **Table 2**. This is a new definition that clearly states the difference from renal ostheodystrophy, taking into consideration a broader problem in CKD patients [21].

A systemic disorder of mineral and bone metabolism due to CKD manifested by either one or a combination of the following:

- Abnormalities of calcium, phosphorus, PTH, or vitamin D metabolism.
- Abnormalities in bone turnover, mineralization, volume, linear growth, or strength.
- Vascular or other soft tissue calcification.

Definition of renal osteodystrophy

- Renal osteodystrophy is an alteration of bone morphology in patients with CKD.
- It is one measure of the skeletal component of the systemic disorder of CKD-MBD that is quantifiable by histomorphometry of bone biopsy.

Table 2. KDIGO classification of CKD-MBD and renal ostheodystrophy [21].

2.2. Changes in vitamin D and its metabolites

Changes in vitamin D metabolism are detected in the early stages of CKD in patients with GFR below 60 ml/min/1.73 m^2 [22]. Furthermore, the expression of the vitamin D receptor in CKD patients is suppressed [23]. These abnormalities are part of the biochemical component of CKD-related mineral bone disease, together with changes in PTH, bone alkaline phosphatase, serum levels of calcium, and phosphate.

2.2.1. Change in 1,25-dihydroxyvitamin D

The classical theory stated that the fall in the active VD metabolite is due to the initial kidney damage, thus leading to reduced calcium and phosphorus intestinal absorption and rise in PTH. With the discovery of FGF-23 and alfa-klotho axis however new explanation of the biochemical abnormalities appeared. Kidney damage leads to reduced ability of the tubules to eliminate phosphorus. This leads to rise in FGF-23 level in order to keep the phosphate level within normal limits. The rise of FGF-23, however, is the initial signal for suppressing renal 1-α hydroxylase and reducing 1,25VD. In addition, it leads to increased catabolism due to activation of 24-hydroxilase. FGF-23 starts to rise in patients GFR below 60 ml/min/1.73 m^2, keeping phosphate serum levels within normal limits well below this cut-off value [24].

To sum up, changes in hormones (PTH and FGF-23) and 1,25VD occur in the early stages of CKD, whereas deviations in calcium and phosphate are characteristic for the advanced CKD cases.

2.2.2. 25-Hydroxyvitamin D (25VD)

25-Hydroxyvitamin D is generally accepted marker for assessing vitamin D status due to its stable serum level and long half-life. Though there is no clear consensus on the definition of VD insufficiency, most of the studies define VD deficiency as 25VD level below 25 nmol/l, whereas insufficiency is defined as 25VD level between 25 and 80 nmol/l. Unfortunately, no clear definition for optimal 25VD level exists though some researchers define it as VD associated with normal PTH value in the general population or VD value above which there is no decrease in PTH [25–27]. Suboptimal levels are widely spread in CKD with prevalence peaking up to 92% in patients on hemodialysis [28]. Several factors can explain the low 25VD level in CKD (**Table 3**).

Poor VD status has been associated with a lot of complications and diseases, apart from its link to the calcium-phosphate homeostasis. Higher mortality was detected in the general population and in CKD patients with low 25VD [28]. Poor 25VD was also associated with higher risk for cancer, diabetes mellitus, hypertension, and depression in humans [29]. VDR was detected in malignant cells too. Activation of VDR in these cells was found to block the cell cycle or cause cell apoptosis [30]. Increased sun exposition had inverse correlation with prevalence of several malignancies [31]. Vitamin D increases insulin secretion and improves insulin resistance in diabetes. In addition, insulin receptor synthesis is improved, as well as systemic inflammation is reduced, which probably explains the positive effect of VD in animal models

and human studies. Vitamin D supplementation in early infancy/or prior to birth was found effective in reducing the prevalence of diabetes type 1 [32]. Several mechanisms have been proposed for the influence of VD on blood pressure—suppression of renin-angiotensin system, calcium ion influx control in smooth muscle cells of the vessels, and improved activity of nitric oxide (NO). Indeed, several cross-sectional studies show that poorer VD status is associated with higher blood pressure values and higher prevalence of hypertension [33]. Several studies indicate that vitamin D insufficiency is linked with higher incidence of depression, without any data for the severity of the disease. There are several possible mechanisms for this relationship—VD may play important role in brain signaling and neuroimmunomodulation, as brain VDR were detected; in addition, vitamin D takes part in serotonin synthesis [34].

Factor	Mechanism
Advanced patient's age	Reduced skin synthesis of cholecalciferol
Dietary restrictions in CKD	Reduced oral intake
Uremia	Reduced skin synthesis of cholecalciferol
Proteinuria	Increased urine loss
Higher prevalence of African race in CKD	Reduced skin synthesis of cholecalciferol
Higher prevalence of obesity	Reduced bioavailability of 25VD

Abbreviations: 25VD, 25-hydroxyvitamin D; CKD, chronic kidney disease.

Table 3. Determinants for lower 25-hydroxyvitamin D levels in CKD.

Further studies are needed to clarify the potential extraskeletal effects of VD in CKD, including larger randomized controlled trials (RTC). The clinical implications of impaired VD status and the possible treatment options in renal patients will be discussed later in this chapter.

2.2.3. The vitamin D receptor in CKD

1,25-Dihydroxyvitamin D mediates its effects via the vitamin D receptor (VDR). It is a nuclear peptide, belonging to a superfamily of nucleotide receptors, like the receptors for retinoic acid and the thyroid hormones. As 1,25VD is the active VD metabolite, VDR has almost 1000 times higher affinity for it than for other VD metabolites. However, the receptor can be activated by 25VD too in cases of toxic VD levels above 370 nmol/l. VDR is expressed in almost all the tissues in human body, with highest expression, however, in intestines, parathyroid gland, and bones. Once 1,25VD binds to VDR, the complex forms a heterodimer with the receptor for retinoid X (RXR) within the nucleus. The 1,25VD-VDR-RXR complex binds to vitamin D reacting elements, activating or suppressing genes.

Activation of VDR leads to increased calcium intestinal absorption, suppression of PTH synthesis in parathyroid gland, and modulation of osteoblast and osteoclast activity. However, due to its wide distribution, it is believed that VDR plays a more complicated role in human health, apart from controlling mineral homeostasis. Furthermore, VDR can be located in the

cellular membrane, thus placing the VDR-VD axis not only in the middle of genomic effects but also in activation of rapid transmembrane pathways [35].

In uremia, significant changes in VDR function occur. Low levels of 1,25VD lead to downregulation of VDR expression [36]. In addition, in areas of nodular growth in the parathyroid gland reduced VDR content was detected [37]. In CKD, there is a significant decrease in VDR-RXR binding to vitamin D reacting elements, as well as reduced RXR content in the parathyroid glands of uremic animal models, explaining increased PTH levels without the presence of hypocalcemia and hyperphosphatemia [38]. Hypocalcemia in advanced renal failure increases the parathyroid levels of calreticulin, a cytosolic protein that binds the DNA-binding domain of nuclear receptors, thus blocking VDR-mediated transactivation [39]. Higher levels of inflammatory cytokines were found to be associated with impaired binding of VDR-RXR to vitamin D reacting elements, contributing to vitamin D resistance in patients on hemodialysis [40]. Finally, hypocalcemia in CKD suppresses the calcium-sensing receptor (CaSR) in the parathyroid glands, which in turn downregulates parathyroid VDR expression. Stimulating CaSR by increasing extracellular calcium or by using calcimimetics upregulated VDR expression in rat models [41].

3. Vitamin D axis in renal disease: clinical implications

3.1. CKD-MBD: biochemical abnormalities

Monitoring biochemical indicators in CKD should start at CKD stage 3 (GFR below 60 ml/min). The most important indicators are serum calcium, phosphate, PTH, and alkaline phosphatase. 25VD should be tested too, with further testing should be considered according to initial levels and the need for supplementation. Generally, laboratory trends are more important than single values and monitoring should be tailored to CKD stage and presence of active treatment. The suggested KDIGO frequencies for monitoring calcium, phosphate, 25VD, and PTH are shown in **Table 4**.

Indicator	CKD stage 3	CKD stage 4	CKD stage 5 and 5D
Calcium and phosphate	6–12 months	3–6 months	1–3 months
PTH and alkaline phosphatase	Baseline	6–12 months	3–6 months
25-Hydroxyvitamin D	Baseline	Baseline	baseline

Table 4. Suggested frequency for monitoring biochemical abnormalities in renal disease [19, 21].

3.2. CKD-related mineral bone disease (CKD-MBD)

As it has already been mentioned, pathological changes in VD metabolism are present in the early CKD stages. Thus, the majority of renal patients have CKD-related bone disease, significantly higher fracture risk and higher morbidity and mortality compared to the

general population are detected in patients with GFR < 60 ml/min/1.73 m^2. Bone pathology is one of the most extensively studied complications in CKD. Often CKD-related bone changes overlap with age-related and postmenopausal osteoporosis, making the picture even more complicated.

3.2.1. Bone histology in CKD

Bone histology in CKD represents a broad spectrum of pathological changes, which are classified according to bone turnover, mineralization, and bone volume (TMV classification) [42].

Bone turnover (T) is a parameter, corresponding to bone formation rate (BFR). BFR can be abnormally low, normal, or very high. BFR is best assessed via bone biopsy and tetracycline labeling. Other measurements that can be used for estimating BFR are osteoblastic surface, the number of active osteoblasts, and the osteoid surface, but none is as accurate as tetracycline testing [43].

Mineralization (M) is the second parameter. Normally, the osteoblasts lay down new collagen and direct mineralization of the matrix. This process is impaired in CKD, leading to thickened osteoid. Mineralization is measured by osteoid maturation time and mineralization lag time. The osteoid maturation time is the osteoid width divided by the distance between labels per day. The mineralization lag time is the osteoid maturation time adjusted for the percentage of osteoid surface that has a tetracycline label [21, 43]. Mineralization is classified as normal and abnormal. A typical example of abnormal mineralization is osteomalacia (OM), where increased osteoid volume, increased osteoid maturation time, or increased mineralization lag time are detected.

Bone volume (V) sums up bone formation and resorption rates. It is generally accepted that bone volume is expressed as bone volume per tissue volume and ranges between low and high. There are different ways to measure bone volume—dual-energy X-ray absorptiometry (DEXA) and quantitative computed tomography (QCT). These two methods, however, have disadvantages in cases with low mineralization, as they reflect primarily bone density. In addition, there are differences in the microstructure in different diseases. In idiopathic osteoporosis, both cortical and cancellous bone volumes decrease, whereas in hemodialysis patients the cortical compartment is reduced, but the cancellous one is increased [44]. Bone volume is classified as low, normal, and high [42].

Evidently, the above-mentioned parameters are linked between each other and can be most accurately assessed via bone biopsy. The procedure, however, has its disadvantages. First, its invasive character is associated with pain, bleeding, and infection. The more serious problem is the representativeness of the obtained sample. Finally, few centers can use the bone-specific histomorphological staining techniques. Hence, though recommended by the KDIGO group, bone biopsy is not routinely performed in everyday renal practice. There are several indications for bone biopsy [42] (**Table 5**).

• inconsistencies among biochemical parameters
• unexplained skeletal fracture or bone pain
• severe progressive vascular calcification
• unexplained hypercalcemia and hypophosphatemia
• suspicion of overload or toxicity from aluminum
• before parathyroidectomy if there has been significant exposure to aluminum in the past or if the results of biochemical determinations are not consistent with advanced secondary or tertiary hyperparathyroidism
• before beginning treatment with bisphosphonates

Table 5. Suggested indications for bone biopsy.

Once the individual parameters for TMV are obtained, personal graph can be formed.

Osteomalacia is currently described as a low-turnover bone with abnormal mineralization. The bone volume may be low to medium, depending on the severity and duration of the process and other factors that affect bone. Adynamic bone disease (AD) is currently defined as low-turnover bone with normal mineralization. Bone volume can be at the lower end of the spectrum, but in some patients with normal mineralization and low turnover it will be normal. Mild HPT (mild hyperparathyroid-related bone disease) and osteitis fibrosa (or advanced hyperparathyroid-related bone disease) are currently used as distinct categories, but in actuality represent a range of abnormalities along a continuum of medium to high turnover, and any bone volume depending on the duration of the disease process. Mixed uremic osteodystrophy is variably defined internationally, but generally it represents with combination of the above-mentioned biopsy findings. For example, it can present as high-turnover, normal bone volume, with abnormal mineralization [42].

In summary, the TMV classification system more precisely describes the range of pathologic abnormalities that can occur in patients with CKD. However, due to the difficulties in performing bone biopsy, the older clinical classification is still used, based on bone turnover—high-turnover mineral bone disease (HTMBD) and low-turnover mineral bone disease (LTMBD). The KDIGO work group currently does not support the use of DEXA measurement of bone density and bone-derived turnover markers of collagen synthesis and breakdown in GFR below 60 ml/min/1.73 m^2 as these parameters do not predict the type of renal osteodystrophy. In contrast, in patients with CKD stages 3–5D, measurements of serum PTH or bone-specific alkaline phosphatase can be used to evaluate bone disease because markedly high or low values predict underlying bone turnover [21].

3.2.2. High-turnover mineral bone disease (HTMBD)

The typical histological substrates of HTMBD are osteitis fibrosa and mild HPT-related bone disease. The prevalence of HTMBD has remained stable over the last years, ranging between 40 and 50% in CKD patients [45].

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Generally, HTMBD is asymptomatic and is preceded by laboratory and X-ray changes. Clinical presentation is usually not typical and consists of nonspecific pain in the lower back, hips, and legs, aggravated by weight bearing. Bone fractures and bone deformities are also common. In addition, symptoms associated with impaired calcium levels (hypocalcemia or hypercalcemia) are present. Extraskeletal calcifications are commonly detected, as well as pruritus. Rarely calciphylaxis (calcific uremic arteriolopathy) is detected. Increased mortality is present.

Laboratory changes comprise of low calcium and high phosphate levels (in advanced CKD stages), elevated bone-specific alkaline phosphatase (BAP), and PTH. Calcium levels can be elevated due to calcium oral supplementation or vitamin D overdose. BAP and PTH are currently the most reliable noninvasive markers for bone turnover.

Routine radiology imaging is insensitive for the type of osteodystrophy and is performed only when symptoms appear. However, certain findings are described, which are not specific for CKD-related bone disease [46].

Table 6 summarizes the clinical, laboratory, and radiology findings in HTMBD.

Clinical presentation of HTMBD
Arthralgia
Bone pain
Myalgia
Bone fractures
Muscle weakness, spasms, tetany, paresthesia, convulsions (hypocalcemia)
Vomiting, nausea, hypertension (hypercalcemia)
Pruritus
Calciphylaxis
Laboratory findings
Serum calcium – N in early stages; ↓/N/↑ in advanced HTMBD
Serum phosphate – N in early stages, N to very high in advanced stages
BAP – N in early stages; ↑ in advanced HTMBD
PTH – N/↑ in early stages; ↑↑↑ in advanced HTMBD
Radiology findings
Subperiosteal erosions—hands, clavicles, and pelvis
Vertebral osteosclerosis
Brown tumors
Extraskeletal calcifications
Abbreviations: BAP, bone-specific alkaline phosphatase; PTH, parathyroid hormone; N, normal; ↓, decreased; ↑, increased values.

Table 6. Clinical presentation and laboratory/radiology findings in high turnover mineral bone disease (HTMBD).

3.2.3. Low-turnover mineral bone disease (LTMBD)

LTMBD encompasses two entities in bone pathomorphology in CKD—osteomalacia and adynamic bone disease. Osteomalacia presents with low turnover and abnormal mineralization, increased osteoid matrix, and is more frequently associated with aluminum toxicity. AD is characterized with low turnover and bone acellularity. In AD even the matrix formation is reduced, thus the mineralization seems unchanged [21]. OM in CKD prevalence is decreasing significantly since 1995 and currently is bordering at 0%. However, AD is getting more common than all other subtypes of renal osteodystrophy, with prevalence peaking to 40% [44].

AD increases in importance not only due to its higher incidence, but also because of its clinical role in CKD. AD is associated with cardiovascular calcification, increased mortality, and higher fracture risk compared to HTMBD [46]. The major factors contributing for AD development are increased use of calcium-containing phosphate binders, excessive use of calcitriol/vitamin D analogs (VDAs), and excessive PTH suppression. Increased patients' age, diabetes mellitus, and peritoneal dialysis as renal replacement therapy were found to be important contributors too. All possible factors leading to AD are listed in **Table 7**.

Iatrogenic factors
• Excessive vitamin D treatment
• Excessive calcium binders use
• Excessive PTH suppression
• High calcium concentration in dialysate fluids
• Peritoneal dialysis
• Aluminum treatment
Other factors
• Diabetes mellitus
• Age
• Hypogonadism
• Malnutrition
• Low thyroid hormone levels
• Altered growth factors and cytokines
• Vitamin D receptor polymorphisms

Table 7. Factors for adynamic bone disease in CKD.

Similarly to HTMBD, the symptoms in AD are nonspecific. In most of the cases the disease is asymptomatic, pain in the bones, fractures, and bone deformities are one of the most common

symptoms. In addition, signs of hypercalcemia, extraskeletal calcifications, and pruritus are also present. In cases of aluminum-associated OM, anemia and dementia can be detected [47].

Bone imaging detects fractures, looser zones, deformities, osteoporosis, and osteopenia. Laboratory findings are the key to the differential diagnosis between HTMBD and LTMBD. In LTMBD, lower PTH and bone-specific AP are present, as well as higher calcium and low phosphate levels. However, high PTH in biopsy-proven AD can be detected; therefore, PTH levels cannot be regarded as the best marker of differentiation between LTMBD and HTMBD [48]. Yet, PTH and PAP are significantly lower in LTMBD and a downward trend in these parameters indicates development of AD [45]. The clinical, laboratory, and radiologic findings in LTMBD are summarized in **Table 8**.

Clinical presentation of LTMBD
Bone fractures
Arthralgia
Bone pain
Vomiting, nausea, hypertension (hypercalcemia)
Calciphylaxis
Pruritus
Aluminum toxicity—anemia, dementia
Laboratory in LTMBD
Serum calcium—early stages N/↑; advanced stages - ↑↑
Serum phosphate—early stages N/↓; advanced - ↓/↑
PTH—early stages N/↓; advanced - ↓
BAP—early stages N/↓; advanced - ↓
Radiology in LTMBD
Fractures
Looser zones
Bone deformities
Osteopenia and osteoporosis

Abbreviations: PTH, parathyroid hormone; BAP, bone-specific alkaline phosphatase; N, normal; ↓, decreased; ↑, increased.

Table 8. Clinical presentation, laboratory, and radiologic findings in low turnover mineral bone disease (LTMBD).

3.2.4. Soft tissue and vascular calcifications

Soft tissue calcification is the third component of the diagnosis of CKD-related mineral bone disease and is more prevalent in CKD patients compared to the general population [49]. The most dangerous locations of extraskeletal calcification are the vasculature and the heart, increasing the risk for cardiovascular event. In renal disease, the pathogenesis of the deposits is not only passive deposition of calcium and phosphate, but also involves active cellular osteogenic transformation [50].

3.3. Pleiotropic effects in CKD patients

Poorer VD status is associated with a broad spectrum of nonskeletal clinical effects, probably due to the widely spread VDR and the presence of nonrenal 1-α hydroxylase. As already mentioned, VD is linked to renin-angiotensin aldosterone system suppression, renal protection, antiproteinuric effects, improved diabetes control, and reduced cancer risk. Pleiotropic effects were detected in CKD patients too—treatment with VD and vitamin D analogs in patients with renal disease led to reduced proteinuria; similar findings were reported in patients with diabetic nephropathy with a relatively low risk for hypercalcemia [51, 52]. However, the studies dealing with CKD patients are relatively few, compared to those reporting VD pleiotropy in the general population. Furthermore, no clear-cut data is present what VD treatment dose and target levels are needed to achieve the extraskeletal effects.

4. Vitamin D treatment in renal disease

The major directions in treating CKD-MBD are reducing phosphate levels, controlling PTH, and treatment of bone changes with bisphosphonates and other medications. Vitamin D preparations are used mainly in suppression of secondary hyperparathyroidism.

4.1. Vitamin D preparations

There are three types of vitamin D preparations used in CKD patients: native cholecalciferol/ergocalciferol, which is the form of vitamin D prior to hydroxylation in the liver and kidneys; calcitriol, which is the active form of vitamin D (dihydroxyvitamin D, 1,25VD) and vitamin D analogs. Vitamin D analogs are artificially synthesized molecules, aiming at reducing the side effects of calcitriol—hypercalcemia and hyperphosphatemia—while preserving its ability to suppress PTH. By changing the original structure of calcitriol modified affinity for VDR and vitamin D responding elements in the nucleus is created [53]. The most widely used analogs are doxercalciferol, paricalcitol, alfacalcidol, falecalcitriol, and 22-oxacalcitriol (maxacalcitol).

4.1.1. Cholecalciferol/ergocalciferol (nutritional vitamin D)

Cholecalciferol is the parent vitamin D, synthesized in the skin, known also as vitamin D3. Ergocalciferol is known as vitamin D2, and is detected in certain vegetable foods, whereas

vitamin D is found in fish oils and other foods of animal origin. Both vitamin D3 and vitamin D2 have equal biological activity.

Low 25VD levels are widely detected in CKD. In patients with CKD stages 3–5 not on dialysis supplementation with cholecalciferol/ergocalciferol is suggested as initial treatment of secondary hyperparathyroidism, as well as calcium supplementation and controlling phosphate levels [21]. Different dosing regimens have been suggested. In a study by Kooienga et al., 800 IU cholecalciferol with calcium supplementation was found effective in improving vitamin status and lowering PTH levels in elderly women with different GFR categories. However, it was impossible to differentiate the effect of vitamin D from that of calcium [54]. In another study, vitamin D2 supplementation according to the National Kidney Foundation - Kidney Disease Outcomes Quality Initiative (NKF-KDOQI) protocol effectively suppressed PTH and improved 25VD level [55].

4.1.2. Calcitriol and vitamin D analogs (VDAs)

Treatment with calcitriol and VDAs is preserved for more advanced stages of secondary hyperparathyroidism, in cases with optimal 25VD level (above 75 nmol/l) with progressively rising or persistently high PTH and in patients on dialysis [21, 56]. The KDIGO group on CKD-MBD assessed the effect of the treatment on these four groups of indicators: patient-centered indicators—mortality, morbidity, and cardiovascular/cerebrovascular events; vascular calcifications; bone histology; and biochemical endpoints—PTH, calcium, phosphate, and BAP levels. In order to present the issue more clearly, we will present the data for predialysis patients and patients on dialysis.

4.1.2.1. Patients with CKD stages 3–5, not on dialysis

Patient-centered endpoints. There are several studies suffering from serious methodological limitations. Thus, no clear-cut can be made for this group of indicators.

Vascular calcifications. No study assessed the effect of cacitriol/VDA on vascular calcifications.

Bone morphology. Nordal and Dahl reported of improved histology in patients with osteitis fibrosa when treated with calcitriol. However, adynamic bone disease was not discussed in the paper, which is a major limitation of the study [57]. Hamdy et al. reported of improved findings in patients with osteitis fibrosa in cases treated with alfacalcidol versus placebo. In the treated group higher incidence of AD was detected [58].

Biochemical endpoints. Treatment with calcitriol, doxercalciferol, paricalcitol, and alfacalcidol effectively reduced PTH levels in renal patients [57–60] versus placebo. Similar findings were reported for BAP [54, 55]. Calcium significantly increased in the treated group with alfacalcidol and calcitriol, whereas paricalcitol and doxercalciferol therapy was associated with upward trend for calcium and calcium-phosphorus product. No difference between active and placebo arms was detected for hyperphosphatemia in doxercalciferol and paricalcitol trials [59, 60].

4.1.2.2. Patients on dialysis

In this patients group, the suggested target level of PTH is 2–9 times the upper normal limit for the assay [21]. The KDIGO group recommends that calcitriol or VDA treatment initiation and monitoring should be based on PTH, calcium, and phosphate level.

Patient-centered endpoints. Currently, no randomized controlled trials (RCT) have assessed the benefit from calcitriol/VDA treatment on mortality and other patient-centered indicators in dialysis patients. There are several observational trials with conflicting results. Treatment with calcitriol/VDA led to lower mortality compared to patients without treatment; use of paricalcitol and doxercalciferol was found superior to calcitriol in hemodialysis patients, with no difference detected between the two types of VDA [61]. Another study also reported superiority of paricalcitol over calcitriol [62]. However, these findings were not confirmed by the Dialysis Outcomes and Practice Patterns Study (DOPPS) analysis in 2009 [63]. Definitely, RCT are needed to evaluate the effect of calcitriol/VDA treatment on mortality.

Vascular calcification. Currently, there are not sufficient trials performed with endpoint soft tissue calcification; therefore, no recommendations have been formed [21].

Bone morphology. In two interventional studies, treatment with calcitriol versus placebo was assessed both in adults and children. Calcitriol significantly improved bone morphology in cases with osteitis fibrosa, but was associated with lower bone turnover and increased risk for AD [64, 65].

Biochemical endpoints. Treatment with VDA and calcitriol significantly reduced PTH and BAP in dialysis patients [64]. VDA were equivalent or superior to calcitriol in reducing PTH, with lower incidence of hypercalcemia and hyperphosphatemia [66]. Comparing the route of administration, the reports are conflicting. A meta-analysis reported of superiority of intravenous over oral vitamin D treatment [67]. Once the higher doses of intravenous vitamin D were removed, there were no differences in PTH suppression [68].

4.2. Monitoring vitamin D therapy

The major indicators for the effect of treatment are PTH and BAP, whereas calcium and phosphate are used mainly for assessing the risk for adverse events.

4.2.1. Patients with CKD stages 3–5, not on dialysis

In this group of patients, the target PTH value is not known. As already mentioned, in cases of elevated PTH, calcium, phosphate, and 25VD levels should be corrected first. If these measures fail VDA/calcitriol may be initiated. In these cases, calcium and phosphate levels follow-up is indicated. According to NKF-KDOQI guidelines, serum levels of calcium and phosphorus should be monitored at least every month after initiation of therapy for the first 3 months, then every 3 months thereafter. Plasma PTH levels should be measured at least every 3 months for 6 months, and every 3 months thereafter [56]. If trends for hypercalcemia and hyperphosphatemia occur dose of VDA/calcitriol should be adjusted or stopped.

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4.2.2. Patients on dialysis

The target PTH values in dialysis patients range between 2 and 9 times the normal values [21]. In cases of rising or persistently high PTH, VDA/calcitriol treatment should be initiated. Treatment choice, however, should take calcium and phosphate level into consideration, as present hypercalcemia and hyperphosphatemia are contraindications for vitamin D sterol treatment. In cases where PTH level drops below the target value, or hypercalcemia/hyperphosphatemia develop, VDA/calcitriol treatment should be stopped or dose should be reduced. A possible frequency for testing calcium, phosphate, and PTH in these patients is suggested by NKF-KDOQI, serum levels of calcium and phosphorus are to be monitored at least every 2 weeks for 1 month and then monthly thereafter. Plasma PTH should be measured monthly for at least 3 months and then every 3 months once target levels of PTH are achieved [56].

4.2.3. Adverse effects from vitamin D treatment

The major problems in using vitamin D preparations (native vitamin D, calcitriol, VD analogs) are oversuppression of PTH, hypercalcemia, and hyperphosphatemia. These laboratory findings are the basis for increased AD in recent years. Therefore, regular control of PTH, serum calcium, and phosphorus levels is warranted as directed by the NKF-KDOQI guidelines. Other adverse possible events are associated with vitamin D toxicity (weakness, metallic taste, weight loss, muscle or bone pain, constipation, nausea, vomiting) and hypercalcemia (nausea, vomiting, loss of appetite, weight loss, constipation, increased thirst or urination, confusion). Other possible complications associated with VD analogs are chills and flu-like symptoms. However, these events are rare—none were detected in our everyday practice with native VD/calcitriol/VDAs.

4.3. Cost-effectiveness of VD treatment

Treatment with vitamin D analogs is an expensive issue, especially compared to the price of native vitamin D. Therefore, prior to VDAs and calcitriol, the first steps to be performed in the treatment of secondary hyperparathyroidism is correction of hyperphosphatemia (reduction of oral intake, calcium phosphate binders if applicable), hypocalcemia, and vitamin D insufficiency. If PTH is progressively rising, calcitriol/VDAs can be initiated [21].

5. Vitamin D metabolism after kidney transplantation

5.1. Impaired vitamin D metabolism after kidney transplantation: prevalence and pathophysiology

Suboptimal VD levels are commonly detected in kidney transplant recipients (KTRs), with prevalence of VD sufficiency below 20% [69]. Similar findings were detected in our institution in patients with duration of kidney transplantation (KTx) more than 6 months (n = 289), [70], **Figure 4**.

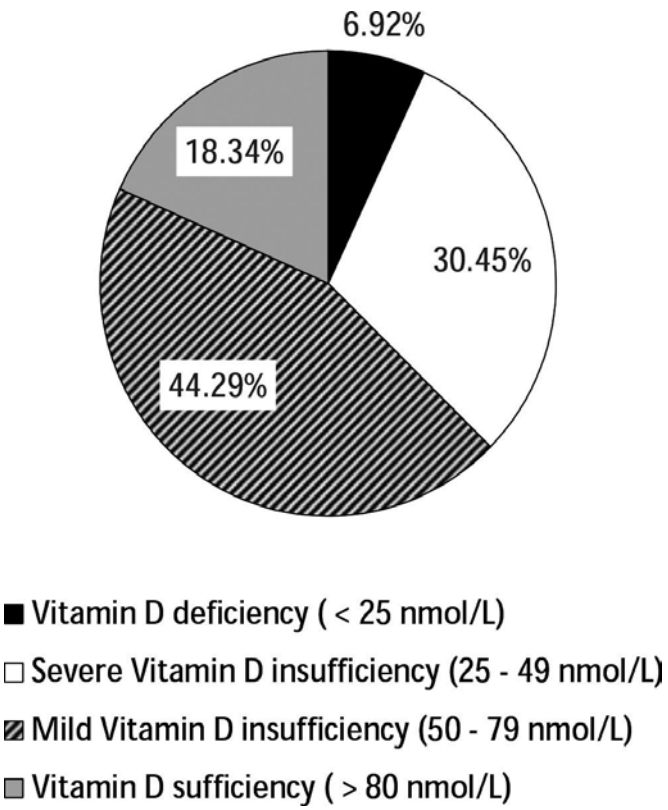

Figure 4. Prevalence of suboptimal 25-hydroxyvitamin D levels in Bulgarian kidney transplant recipients, *n* = 289. Filipov et al.

During winter-spring fall, during the annual nadir of 25VD, the share of VD sufficient KTRs follow-up in our center dropped to 2.59% [71].

The marked impairment of VD axis after KTx can be explained with transplantation-specific and CKD-related issues. The transplantation-related ones are sun exposure avoidance in order to minimize the risk for skin cancer, immunosuppressive treatment (steroids, calcineurin inhibitors), new onset diabetes after transplantation (NODAT), and higher incidence of obesity after KTx. The CKD-related causes for poor VD status were already outlined. The prevalence of CKD stage 3 and over in our department ranges between 49 and 58% over the last 4 years, which is in accordance with or even better than the results of other centers [72, 73]. **Table 9** summarizes the possible causes of VD insufficiency after KTx with the possible pathophysiological mechanisms involved.

5.2. Vitamin D after kidney transplantation: clinical implications

There are two basic aspects of vitamin D insufficiency after kidney transplantation—post-transplant mineral bone disease (PTx-MBD) and vitamin D pleiotropy.

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Cause	Mechanism
Increased CKD prevalence	Reduced skin synthesis, reduced protein intake
Reduced sun exposure	Reduced skin synthesis
Use of sun-protecting cosmetics	Reduced skin synthesis
Proteinuria	Increased urine loss
NODAT	Decreased intestinal resorption
Higher prevalence of obesity	Reduced bioavailability
Steroids	Increased catabolism
Other immunosuppressive agents – CNI, MMF	Increased catabolism/suppressed liver synthesis

Abbreviations: CNI, calcineurin inhibitors; MMF, mycophenolate mofetil; NODAT, new onset diabetes after transplantation.

Table 9. Causes for vitamin D insufficiency after kidney transplantation.

5.2.1. Posttransplant mineral bone disease

After Ktx, PTx-MBD develops at the background of CKD-MBD, and consist of the same three components—biochemical abnormalities, bone pathology, and vascular/soft tissue calcifications. Poor vitamin D status is one of the factors for developing PTx-MBD, together with immunosuppressive therapy, persistent hyperparathyroidism, malnutrition, persistent CKD, hypogonadism, metastatic cancer disease, smoking, duration of dialysis and transplantation, cumulative steroid dose, diabetes, etc.

Biochemical abnormalities. Calcium levels tend to rise and phosphate usually decreases below normal values due to elevated PTH. These parameters should be monitored weekly during the first month posttransplant. PTH starts to decrease after successful KTx, but persistent hyperparathyroidism can be present in up to 43% after the first year [74]. Similar findings (33.69%) were detected in our center, coupled with persistent hypercalcemia or hypophosphatemia. Urine levels of phosphate and calcium were not evaluated routinely in our patients. Vitamin D levels normalize later—around 18 months after KTx [75]. After the early posttransplant period (up to 3 months after transplantation), regular follow-up of the parameters should be based on kidney function and the trends of the values. KDIGO suggested frequency of testing for KTRs similar to the one for pretransplant CKD patients (Table 4). In our center, during the first month calcium and phosphate are monitored once weekly, with gradually decreasing the frequency until the suggested values are reached; 25-hydroxyvitamin D levels are monitored at least twice annually, taking into consideration its seasonal variations.

Bone. Rapid reduction in bone density is widely reported, with faster bone loss during the first months after successful KTx, though reduced bone density loss was reported years after the operation [76]. The factors contributing to posttransplant bone disease were already listed.

A major complication is increased fracture risk, associated with increased morbidity and mortality.

Vascular calcification. Assessing the development of soft tissue calcification is difficult due to the high prevalence of vascular calcification in advanced CKD. Only one study demonstrated possible slowing of calcification process after KTx [77]. Therefore, currently there is paucity in scientific data for this problem.

5.2.2. Vitamin D pleiotropy after kidney transplantation

With the advance of transplantology, short-term kidney survival has improved significantly over the last decades. However, long-term graft survival still remains a problem, hardly exceeding 70–80% survival at the 10th year after the operation. The major reasons for late graft loss are death of the patient due to cardiovascular disease (CVD), malignancy, and infection; also, calcineurin toxicity and chronic rejection are also other significant causes. The already mentioned pleiotropic effects of VD were described mostly in the general population or CKD patients. It can be expected that these properties can improve patient- and graft-targeted outcomes.

5.2.2.1. Vitamin D and diabetes mellitus after kidney transplantation (KTx)

New onset diabetes after transplantation (NODAT) is associated with higher morbidity and mortality after transplantation, and is linked to the use of steroids and calcineurin inhibitors after the operation. Many experimental and animal studies indicate that better vitamin D status is associated to improved insulin secretion and insulin resistance. Several human studies report of inverse correlation between 25VD level and diabetes prevalence [78, 79]. The data after solid organ transplantation are scarce. Our findings do not show any link between 25VD level and glycemic control [80], thus not supporting any association between VD status and glycemia after KT. No interventional studies assessed the effect of VD supplementation or use of calcitriol/VD analogs in solid organ transplantation patients on diabetes prevalence after the procedure [81].

5.2.2.2. Vitamin D and cardiovascular risk after KTx

The risk for cardiovascular disease (CVD) is increased after transplantation compared to the general population [82]. Low vitamin D levels were associated with arteriosclerosis and endothelial dysfunction in end-stage renal patients [83]. Other studies also reported of increased CVD incidence in poor VD status. A possible explanation may be that VD-receptor activation in the cardiomyocytes suppresses their proliferation. However, the studies after solid organ transplantation are lacking. Furthermore, higher doses of vitamin D may be associated with increased risk for vascular calcifications [84].

5.2.2.3. Vitamin D and malignancy after KTx

Malignancy is one of the major contributors to patient and graft loss after transplantation, especially in the long run. Several studies reported lower prevalence of different types of

neoplasia in subjects with better VD status [85, 86]. The findings were detected in renal transplant patients [87], though the results should be confirmed by prospective studies, as some reports indicate that high 25VD may increase the risk for prostate neoplasia [88].

5.2.2.4. Vitamin D and infection after KTx

Infections play a key role for mortality and morbidity after transplantation. Experimental studies have shown that macrophages express VD receptor and its activation leads to increased antimicrobial activity of the macrophages [89]. Human studies also report of beneficial effect of better VD status on infection prevalence. However, only one trial has demonstrated inverse correlation between 25VD and infection rate in lung transplant patients [90]. Vitamin D had no influence on urinary tract infection rate in Bulgarian KTRs followed-up in our center [91]. Therefore, further studies are needed to evaluate the association between VD and infection after transplantation.

5.2.2.5. Vitamin D and rejection after KTx

Rejection episodes are still a matter of concern and linked to reduced graft survival. On the other hand, long-term immunosuppression contributes to serious adverse events. Higher 25VD was related to lower rejection incidence in KTRs [92]. Experimental reports indicate that calcitriol suppresses T-cell activity and proliferation, as well as B-lymphocyte proliferation, IgG secretion, and major histocompatibility complex class II expression [93–95]. In two small prospective studies, calcitriol treatment was found to have significant immunomodulatory effect [96, 97]. However, the trials in transplant patients are small and single centered, and further research is needed.

5.2.2.6. Vitamin D and renoprotection after KTx

The effect of VD on renal protection has been described earlier in this chapter together with the possible mechanisms and experimental data supporting them. In the setting of renal transplantation, low VD was associated with higher proteinuria [98]. In addition, calcitriol therapy was found beneficial for renal graft function [99]. However, in a small prospective study by Courbebaisse et al. biopsy findings were compared between cholecalciferol-treated KTRs and KTRs without supplementation. The findings did not demonstrate significant difference in terms of interstitial fibrosis and tubular atrophy [100]. Due to the small number of trials and their small size conclusions cannot be drawn.

5.2.2.7. Vitamin D and mortality after KTx

Better VD status was associated with lower mortality in the general population and CKD patients, including patients on hemodialysis [98–100]. However, there are no data in terms of mortality in patients after renal transplantation.

In summary, the data for VD pleiotropy after renal transplantation are relatively scarce, originate from single-center studies, and usually with small number of patients. Therefore, further studies including prospective interventional ones are needed.

5.3. Vitamin D treatment after KTx

The influence of vitamin D treatment after KTx was mainly assessed for its effect on biochemical abnormalities in calcium phosphorus metabolism. The data about the effect on fracture risk, bone density, and pleiotropy are still insufficient. Guidelines are available only for the first posttransplant year. The data for the treatment after the first year are insufficient [21].

5.3.1. Native vitamin D (cholecalciferol/ergocalciferol)

Cholecalciferol supplementation effectively suppressed PTH in renal transplant patients [101]. A meta-analysis performed by KDIGO showed improved bone density in patients with cholecalciferol-/ercalciferol-treated KTRs versus KTRs without VD supplementation [21]. The suggested cholecalciferol dose corresponds with the recommended dose for the general population [21]. As no data are present for patient-targeted endpoints such as fracture risk, no guidelines are available for this issue. Similarly, no specific recommendations can be given for VD pleiotropy. However, there are two large randomized trials assessing cholecalciferol supplementation in KTRs and its effect on renal graft function, NODAT incidence, infection risk, cancer prevalence, and mortality after KTx [82].

5.3.2. Calcitriol/vitamin D analogs

Treatment with calcitriol/VDAs in renal transplant patients with CKD stages 3T–5T is based on the same principles as in patients with CKD stages 3-5 (GFR below 60 ml/min). The reason for accepting the same approach is the paucity of RCTs in KTRs treated with calcitriol/VDAs [21]. However, certain considerations should be taken into account. The most important one is the high prevalence of persistent hyperparathyroidism and hypercalcemia after KTx. If the PTH levels do not resolve parathyroidectomy should be considered in these cases. As already mentioned, further research is needed in terms of pleiotropic effects of calcitriol/VDA treatment in KTRs.

5.3.3. Treatment monitoring in transplant recipients

Similarly to pretransplant CKD stages, monitoring calcium, phosphate, PTH, bone AP, and 25-hydroxyvitamin D depends on the renal function, the trend in biochemical abnormalities, and the intervention performed. Still, certain frequencies are suggested (**Table 4**) after the first 3 months posttransplant.

6. Conclusion

Vitamin D metabolism is significantly impaired at different levels in the early stages of renal disease. The influence of the abnormalities spans beyond calcium-phosphorus metabolism, having impact on mortality, cardiovascular morbidity, cancer risk, renal protection, etc. Thus, VD metabolites have pivotal role in controlling a great number of intracellular pathways that are impaired in renal disease contributing to poorer patient outcomes.

However, there are still problems to be clarified: the dose of vitamin D supplementation, target levels of 25-hydroxyvitamin D and PTH in predialysis patients, possible biochemical abnormalities due to treatment (hypercalcemia, hyperphosphatemia), and vitamin D pleiotropy in CKD patients prior and after kidney transplantation. The great number of unsettled problems in this sphere and its great potential for improving patient outcomes guarantee that vitamin D will be a "hot topic" in the world of renal disease over the next years.

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Chapter 3

Pleiotropic Effects of Vitamin D in Kidney Disease

Chia-Chao Wu and Kuo-Cheng Lu

Additional information is available at the end of the chapter

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Abstract

Vitamin D is metabolized in the liver and kidneys and then converted to the active form, 1.25-dihydroxyvitamin D [1.25(OH)2D]. Chronic kidney disease patients usually lack both 25-hydroxyvitamin D [25(OH)D] and 1.25(OH)2D due to impaired renal function and 1α-hydroxylase deficiency. Chronic kidney disease patients have a high incidence of cardiovascular and infectious morbidities. Increasing evidence indicates a relationship between vitamin D deficiency and cardiovascular and infectious mortality risks. Vitamin D may have significant biological effects beyond its traditional roles on mineral and bone metabolism. Many extrarenal cells have the capability to produce local active 1.25(OH)2D in an intracrine or paracrine fashion. Vitamin D has a significant association with nonskeletal diseases, such as immunodeficiency, metabolic syndrome, insulin resistance, diabetes, hyperlipidemia, cardiovascular disease, proteinuria, and acute kidney injury. This article aims to review and summarize the pleiotropic effects of vitamin D in patients with kidney disease, particularly the immunological, metabolic, cardiovascular, and renal effects.

Keywords: vitamin D, pleiotropic effects, immunity, metabolic, cardiovascular, acute kidney injury, chronic kidney disease

1. Introduction

Most animals cannot synthesize all vitamins. Vitamin D is a lipid-soluble vitamin and the only vitamin that can be synthesized by humans. Evolutionally, vitamin D has been synthesized by a photochemical process in land vertebrates to satisfy the requirement for a calcified skeleton for more than 350 million years [1, 2]. Vitamin D is metabolized by 25-hydroxylase and 1α-hydroxylase in the liver and kidneys, respectively, and converted to the active form, 1.25-dihydroxyvitamin D [1.25(OH)2D] [3]. Recently, the extrarenal conversion

of 25-hydroxyvitamin D (25(OH)D or calcidiol) to 1.25(OH)2D (calcitriol) may play important biological roles beyond its traditional roles [4]. Chronic kidney disease (CKD) patients usually lack both 25(OH)D and 1.25(OH)2D due to impaired renal function and 1α-hydroxylase deficiency. CKD patients have a high incidence of cardiovascular and infectious morbidities. Increasing evidence indicates a relationship between vitamin D deficiency and cardiovascular and infectious mortality risks [5].

Vitamin D plays new roles through activation of the vitamin D receptor (VDR), which involves several pleiotropic effects. Immune systems are clearly impaired in CKD patients [6, 7]. In innate immunity, the conversion of 25(OH)D to 1.25(OH)2D within monocytes and macrophages may produce cathelicidin and β-defensin to enhance the disinfectant effects [8, 9]. Vitamin D also has an inhibitory effect on the adaptive immune system by regulating the function of antigen-presenting cells (APCs), T lymphocyte activation and proliferation, and cytokine secretion [10, 11]. Therefore, vitamin D plays an essential role in immunomodulation.

The metabolic syndromes and insulin resistance are increased in CKD patients [12]. Recently, an association between insulin resistance, diabetes mellitus (DM), and vitamin D deficiency has been proposed [13]. Low vitamin D levels are associated with hypertension (HTN) and endothelial dysfunction [14]. Vitamin D also has protective effects on improving proteinuria and progression of renal function in CKD patients [15, 16]. Vitamin D deficiency is a biomarker to predict acute kidney injury (AKI) and is independently associated with increased morbidity and mortality in critical illness [17]. This review focuses on the influence of vitamin D on immunological, metabolic, cardiovascular, and renal effects in patients with kidney disease.

2. Vitamin D and immune regulation

Vitamin D has been used to treat infections such as tuberculosis for more than 100 years [6, 18]. Epidemiological experiments have shown that vitamin D deficiency is closely related to autoimmune and infectious diseases [2, 19–21]. Immune cells carry VDR and 1α-hydroxylase, which produces the active metabolite 1.25(OH)2D through local synthesis and heightens immunomodulatory properties [8]. Increasing evidence indicates that vitamin D deficiency may cause dysregulation of the innate and adaptive immune systems and promote microinflammation [22].

2.1. Vitamin D and innate immunity

Vitamin D can stimulate the differentiation of monocytes into mature phagocytic macrophages to enhance the effects against pathogens [23]. During infection, macrophages and monocytes are exposed to pathogen-associated molecular patterns (PAMPs), which may activate Toll-like receptor (TLR) 1/2 heterodimer and sequentially upregulate 1α-hydroxylase activity and VDR expression to produce 1.25(OH)2D [24]. 1.25(OH)2D inhibits the release of the proinflammatory cytokine tumor necrosis factor α (TNF-α), regulates the activity of nuclear factor κB (NF-κB), and suppresses the expressions of TLR2 and TLR4 in human mono-

cytes, which reduces cytokines release [25]. Lipopolysaccharide (LPS) can induce TLR4, interferon-γ (IFN-γ), and CD14 activity to increase 1α-hydroxylase expression. When serum 25(OH)D levels are above 30 ng/mL (75 nmol/L), 25(OH)D can convert to its active form, 1.25(OH)2D, via 1α-hydroxylase in macrophages in an intracrine or autocrine manner [23]. Consequently, 1.25(OH)2D enters the nucleus by binding VDR complexes with retinoid X receptor (RXR), which causes direct signaling on the transcription of cathelicidin and β-defensin 2 [24]. Both the above peptides can cleave microbial membranes and promote innate immunity in response to infectious agents. Hence, the macrophage's functions deteriorate, which decreases its antibacterial effect in vitamin D-deficient patients compared with people with adequate vitamin D [24, 26].

25(OH)D supplements increase induction of cathelicidin, which is associated with the capacity for killing *Mycobacterium tuberculosis* and promoting antibacterial activity [18, 24, 26]. Vitamin D binding to VDR can also upregulate the expression of β-defensin 4A (DEFB4A) through nucleotide-binding oligomerization domain 2 (NOD2) activation and NF-κB stimulation [23]. Autophagy is an important macrophages defense mechanism against intracellular pathogens by the elimination of materials, which acts as a dynamic recycling system that yields new components and energy for cellular renovation and homeostasis [18]. Antibacterial cathelicidin, β-defensin 4A, and maturation of autophagosomes cooperate to enhance bacterial killing, which is highly dependent on vitamin D status [27]. Therefore, in innate immunity, vitamin D promotes macrophages to produce cathelicidin and β-defensin 2 and enhances the capacity for autophagy via TLR activation.

2.2. Vitamin D and adaptive immunity

VDR are presented in activated T cells and B cells; therefore, vitamin D plays a functional role in modulating adaptive immunity [27]. 25(OH)D or 1.25(OH)2D suppresses the maturation of professional APCs and dendritic cells (DCs) by decreasing costimulatory marker expression and affecting the binding ability and expression of VDR, thereby reducing antigen presentation and regulating adaptive immune responses [28–31]. Furthermore, vitamin D can influence T cell function through endocrine, paracrine, and intracrine mechanisms. Vitamin D directly influences T-cell proliferation and cytokine production [27]. Vitamin D increases anti-inflammatory T-helper 2 (Th2) cytokine production and suppresses Th1 cytokines, which shifts from Th1 to Th2 axes [30, 32].

The suppression of DC maturation by 1.25(OH)2D has the potential to induce Treg cells, which exhibit anti-inflammatory effects [29–31, 33]. Vitamin D can significantly increase the percentage of Tregs through direct endocrine systemic calcitriol effects or intracrine conversion of 25(OH)D to 1.25(OH)2D by Tregs themselves, or indirectly through the APCs remaining in an immature status. Vitamin D also inhibits the development of Th17, which is associated with tissue damage, inflammation, and host-graft rejection in autoimmune diseases [34]. In humoral immunity, 1.25(OH)2D results in reduced proliferation and differentiation of B lymphocytes, and immunoglobulin production [35].

2.3. Vitamin D and immune dysfunction in CKD

CKD patients usually have obvious immune dysregulation, which may play a role in infection and contributes to an important cause of morbidity and mortality [36]. Vitamin D deficiency causes dysregulation of the innate and adaptive immune systems and promotes microinflammation. Low 1.25(OH)2D levels have been related to elevated mortality rates in CKD patients [36]. Consequently, CKD leads to a diminished response to infection and misapplied inflammatory response as in a state of immune dysregulation and sustained inflammation [6]. On the one hand, strong associations have been shown between the prevalence of vitamin D deficiency and susceptibility to infection [37], and on the other hand, vitamin D also has an antioxidative effect. Both immunomodulatory and antioxidative activities may contribute to immune dysfunction in CKD. It is difficult to clarify whether the immunomodulatory or antioxidative effect of vitamin D is more predominant during the process. However, the results of vitamin D supplementation trials did not always demonstrate consistent protective effects [38]. Prevention through vaccination remains the best strategy to minimize the adverse consequences associated with infections. Patients with CKD demonstrate inadequacies of immunity for generating a protective vaccine response. Vitamin D might influence immune responsiveness and its potential modulating role in vaccine immunogenicity [39]. Can we translate vitamin D immunomodulating effect on innate and adaptive immunity to vaccine response? According to current evidence, it is still premature to recommend vitamin D for practical therapeutic or preventive use to enhance vaccine response. More research and large trials are needed for further confirmation.

3. Roles of vitamin D in metabolic disturbance

3.1. Vitamin D and metabolic syndrome

Metabolic syndrome is a condition characterized by the presence of at least three of the following: abdominal obesity, increased blood pressure (BP), impaired glucose tolerance or diabetes, dyslipidemia (elevated levels of triglycerides), and low concentration of high-density proteins [40]. Metabolic syndrome is associated with an increased risk of renal injury, cardiovascular disease, type 2 diabetes, and all-cause mortality [41]. The relationship between metabolic syndrome and CKD is complex and bidirectional. Low 25(OH)D3 levels are associated with metabolic syndromes. A meta-analysis of observational studies showed a significantly inverse association between blood 25(OH)D levels and the risk of metabolic syndrome [42]. There is a 51% reduction in the prevalence of metabolic syndrome with a high level of vitamin D. Furthermore, another meta-analysis provided a dose-response relationship between the blood vitamin D concentration and metabolic syndrome risk. A 25 nmol/L increase in 25(OH) D levels was associated with a 13% decrease in the risk of metabolic syndrome. However, there was some heterogeneity among the studies. The association was somewhat stronger in the elderly populations with metabolic syndrome [43]. Although the observational (epidemiological) studies demonstrated significant associations between vitamin D and metabolic syndrome, their causal relationship is still undetermined. Further

studies, particularly longitudinal randomized clinical trials, are needed to determine whether vitamin D supplementation plays a role in the prevention of metabolic syndrome.

3.2. Vitamin D, insulin resistance, and DM

3.2.1. Vitamin D and insulin resistance

CKD patients experience impaired insulin secretion and enhanced insulin resistance [12, 44]. Vitamin D deficiency, secondary hyperparathyroidism, inflammation, and oxidative stress all can alter glucose metabolism and contribute to insulin resistance. Active vitamin D (1.25(OH)2D) may stimulate pancreatic insulin secretion directly through the interaction of the 1.25(OH)2D3-RXR-VDR complex, thus increasing insulin synthesis [45, 46]. Insulin secretion is a calcium-dependent process and vitamin D may indirectly increase the calcium concentration by alternating calcium flux within the β islet cells; therefore, it has adverse effects on β islet cells' secretary function. In addition, vitamin D and calcium regulated insulin sensitivity by stimulating the insulin receptor and activating peroxisome proliferative-activated receptor γ (PPAR-γ) [47]. Extrarenal 1α-hydroxylase leads to the local production of 1.25(OH)2D, which has a role in ensuring calcium influx into cells, and may be essential to the actions of insulin in skeletal muscle and adipocytes [48, 49].

Chronic inflammation is involved in the development of insulin resistance. Vitamin D has immunoregulatory effects by decreasing inflammatory responses to reduce insulin resistance and the risk of diabetes [13]. Therefore, parathyroid hormone (PTH) may negatively affect insulin sensitivity through altering body composition and inhibiting insulin signaling by reducing the number of glucose transporters to promote glucose uptake, suppress insulin release, and promote insulin resistance in adipocytes [50, 51].

However, there appears to be a need for randomized trials to evaluate the definite effects of vitamin D supplementations in insulin resistance and whether supplementations of vitamin D may be a suitable management strategy to ameliorate insulin resistance.

3.2.2. Vitamin D and type 2 DM

The association between vitamin D and type 2 DM has been explored recently [52]. There is an inverse association between vitamin D status and glycemic outcomes [13]. Insulin resistance increases the risk of type 2 DM. Lower vitamin D status is associated with higher risk of incident type 2 diabetes in observational studies; however, the effect of vitamin D supplementation on glycemic outcomes was not evident in some studies [48]. In a large cohort of middle-aged women, both vitamin D and calcium intakes were additive and inversely associated with risk of type 2 DM development. For both vitamin D and calcium, intakes from supplements rather than from diet were significantly associated with a lower risk of type 2 diabetes [53, 54]. Hence, a high intake of vitamin D and calcium was associated with a lower risk of type 2 diabetes. An inverse association was shown between serum 25(OH)D levels and prevalence of diabetes and its complications, and the improvement of symptoms after vitamin D supplementation. Underlying mechanisms may be associated with the role of vitamin D in immunity, β-cell

function, and insulin sensitivity [13]. Overall, the available data are currently insufficient to support the contention that type 2 diabetes can be improved by raising 25(OH)D concentrations. The confirmation of a potential beneficial effect of vitamin D on type 2 diabetes is needed in large trials.

3.3. Vitamin D and lipid metabolism

Lipid metabolism abnormalities with alterations in lipid profiles are commonly seen in CKD patients; therefore, the prevalence of dyslipidemia in CKD is much higher than that in the general population [55, 56]. Markedly reduced high-density lipoprotein quantity and function is the key dyslipidemia leading to persistent chronic inflammation, increased oxidative stress, and subsequent progression of cardiovascular disease in CKD. CKD also induces downregulation of lipoprotein lipase and very low-density lipoprotein (VLDL) receptor contributing to further hypertriglycemia and elevated VLDL levels. The vitamin D binding to VDR may affect bile acid synthesis and reduce cholesterol levels in hepatocytes and serum. Activation of the VDR by 1.25(OH)2D may suppress the expression of small heterodimer partner (SHP) and the activation of cholesterol 7-α-hydroxylase (CYP7A1) which is the rate-limiting enzyme in bile acid synthesis, and its expression controls serum cholesterol levels [57–59]. In addition, VDR activation downregulated farnesoid X receptor (FXR) and SHP expression to inhibit CYP7A1, which is responsible for lowering cholesterol [60, 61]. The vast majority of intervention studies did not show a significant effect of vitamin D on blood levels of serum cholesterol levels in CKD patients. However, there is evidence for a triglyceride-lowering effect of vitamin D in CKD patients, a group with elevated triglyceride levels. Thus, adequately designed primary prevention trials are needed to provide more evidence for the clinical application of vitamin D.

4. Roles of vitamin D in cardiovascular disease

4.1. Vitamin D and endothelial dysfunction

The vascular endothelial function of CKD patients is dysregulated. Calcium deposition in atherosclerotic plaques or vessel walls participates in the vascular calcification process, which causes major cardiovascular morbidity and mortality. Vitamin D has been associated with increased vascular calcification and evidence conversely supports a protective effect. Recent studies have demonstrated the relationship between vitamin D status and endothelial function. Vitamin D therapy can improve endothelial function. Oral vitamin D (cholecalciferol) improves endothelial vasomotor and secretory functions in CKD patients [62, 63]. In a clinical trial of patients with type 2 DM, who were vitamin D deficient, a one-time large dose of vitamin D improved flow-mediated brachial artery vasodilation and significantly decreased systolic BP compared with placebo [64]. In 42 subjects with vitamin D insufficiency, normalization of 25(OH)D at 6 months was associated with increases in reactive hyperemia index and subendocardial viability ratio, and a decrease in mean arterial pressure [14]. However, the available

data are currently insufficient to support the reverse endothelial dysfunction by administrating vitamin D in the general population.

An *in vitro* study indicated that vitamin D may attenuate the adverse effects (including increased NF-κB expression) of advanced glycation end products on endothelial cells [65]. Inflammatory processes can also increase ischemic mediators like intercellular adhesion molecule-1, which increases neutrophil-endothelial interactions [66]. Endothelial injury directly affects afferent arterioles and results in endothelin release and further vasoconstriction, which together cause microcirculatory dysfunction. In addition, vitamin D3 administration enhanced vascular regeneration by inducing stromal cell-derived factor 1 expression in the healthy population. Active vitamin D may increase Klotho secretion and upregulate the expression of osteopontin, a calcification inhibitor, to inhibit vascular calcification and improve vascular endothelial function [67]. Therefore, vitamin D3 may be viewed as a new approach for promoting vascular endothelial repair in the future.

4.2. Vitamin D and the renin-angiotensin-aldosterone system (RAAS)

There is an inverse correlation between changes in vitamin D and changes in plasma renin activity [68]. Individual with 25(OH)D deficiency had higher circulating angiotensin II (Ang II) levels and significantly blunted renal plasma flow responses to infused Ang II when compared with individuals with sufficient 25(OH)D levels. Low plasma 25(OH)D levels may result in the upregulation of the renin-angiotensin-aldosterone system (RAAS) in otherwise healthy humans [69]. Animal and clinical studies have provided important mechanistic clues regarding the crosstalk between RAAS and vitamin D, which affects BP and volume regulation [70]. VDR-knockout mice demonstrated increased renin gene expression in the kidneys and had enhanced RAAS signaling in the blood, which led to significant sodium retention, vascular resistance, and HTN [69]. Conversely, treatment with calcitriol reduced renal renin production independent of calcium and PTH. Calcitriol binds to the VDR and blocks the formation of CRE-CREB-CBP complexes in the promoter region of the renin gene, thus reducing its level of expression [71].

4.3. Vitamin D and hypertension

The observation that people living at higher altitudes have a higher incidence of essential HTN during the winter raised the hypothesis that vitamin D deficiency may contribute to essential HTN [72, 73]. There is an inverse relationship between serum 25(OH)D concentration and HTN incidence, with an odds ratio of 0.73 for the highest versus the lowest category of blood 25(OH)D. In patients with HTN exposed to sufficient sunlight, the 25(OH)D levels were upregulated and subsequently BP was normal [73]. Another study showed that native vitamin D supplementation may improve HTN in type 2 diabetic patients [74]. Pooled data from previous clinical trials have produced mixed results [53, 75]. Data from normotensive individuals showed a small, but statistically significant, effect on reduction in BP with vitamin D intervention. In contrast, a meta-analysis to evaluate the effect of vitamin D supplementation on BP showed no significant BP-lowering effect of vitamin D supplements [75]. Hence, an appropriately high dose of vitamin D can normalize or nearly normalize blood 25(OH)D levels

and significantly reduce BP in hypertensive cohorts with vitamin D deficiency. Treatment of vitamin D-deficient or vitamin D-insufficient normotensive individuals with vitamin D for short period results in minimal effects on BP. Subgroup analysis displayed a significant reduction in diastolic BP in participants who had preexisting cardiometabolic disease.

The mechanisms underlying vitamin D's effect on HTN have not been elucidated yet. Several biological mechanisms relating vitamin D deficiency and HTN have been proposed. First, low vitamin D levels have been associated with increased vascular stiffness, endothelial dysfunction, inflammatory cytokines, and higher coronary artery calcium scores [72]. Other possible mechanisms concerning vitamin D deficiency leading to HTN include vitamin D deficiency leading to increased renin expression, high PTH and low calcium levels, and increased sympathetic nervous activity. Vitamin D deficiency is also an epigenetic risk factor that favors increased vascular tone, which may not play an important role in the regulation of normal BP homeostasis, but serves as a trigger to contribute to the development of HTN in vulnerable middle-aged people.

4.4. Vitamin D and anemia

Anemia due to erythropoietin deficiency or resistance is the major cause of renal anemia in CKD. Chronic inflammation, iron imbalance, and increased hepcidin production also contribute to anemia in CKD patients [76]. Several factors, such as the use of phosphate binders and antacids, loss of blood during hemodialysis, and intake of erythropoiesis-stimulating agents (ESA) cause iron deficiency. Vitamin D deficiency may increase inflammatory cytokines production (interleukin-6, IFN-γ, TNF-α), which stimulate hepcidin production, thus inhibiting ferroportin activity and limiting iron usability [77, 78]. In addition, secondary hyperparathyroidism will directly inhibit erythroid progenitors, endogenous erythropoietin synthesis, and red blood cell survival as well as indirectly promote bone marrow fibrosis and hyperphosphatemia [76, 79]. All of these factors will lead to ESA hyporesponsiveness. Providing vitamin D or active vitamin D may promote anti-inflammation and erythroid proliferation to correct ESA resistance, improving anemia, and reduce ESA requirements [80, 81]. Therefore, vitamin D levels and ESA requirements exhibit an inverse relationship in CKD patients.

5. Roles of vitamin D in renal disease

5.1. Vitamin D and chronic kidney disease (CKD)

Vitamin D deficiency is a prominent feature of CKD. Vitamin D deficiency is related to albuminuria, CKD progression, and subsequent cardiovascular diseases [15, 16]. VDR is highly expressed in the kidney; therefore, the kidney can be considered a classic vitamin D target organ [82, 83]. Vitamin D has been prescribed for renal patients to prevent osteodystrophy and increased attention has focused on its renoprotective activity in recent decades. Molina et al. reported that vitamin D supplements may effectively reduce albuminuria at CKD stages 3–4 [84]. In the VITAL study, the administration of paricalcitol in addition to RAAS blockade further reduced albuminuria compared with RAAS blockade alone in patients with diabetic

nephropathy [85]. A meta-analysis study showed a higher risk for nephropathy in vitamin D–deficient patients with diabetes, but these association studies did not show causality. However, pooling the results of available clinical trials showed no significant change in proteinuria after vitamin D supplementation. More vitamin D research is needed for a more comprehensive and precise conclusion.

Activation of the VDR is essential in reducing proteinuria [85]. Traditionally, using RAAS blockers can reduce albuminuria [86]. 1.25(OH)2D3 is known as a RAS inhibitor by its negative regulatory effect on renin production to provide additional renoprotection [69]. The renoprotective effects of vitamin D can improve proteinuria, glomerulosclerosis, and interstitial infiltration and reduce renal oxidative stress [87]. Combined treatment with paricalcitol and losartan suppressed the induction of fibronectin, transforming growth factor β (TGF-β) and monocyte chemoattractant protein-1 (MCP-1), and reversed the decline of the slit diaphragm proteins nephrin, Neph-1, ZO-1, and alpha-actinin-4 [88]. VDR knockout in diabetic mice was associated with severe albuminuria and glomerulosclerosis [69]. Alternatively, vitamin D might slow the progression of diabetic nephropathy by improving insulin secretion, delaying destruction of β islet cells, affecting osteocalcin, and consequently assisting in glucose metabolism. TGF-β, MCP-1, hepatocyte growth factor, thrombospondin-1, and plasminogen activator inhibitor are other possible molecular targets of vitamin D action [87, 89, 90].

5.2. Vitamin D and acute kidney injury (AKI)

A significantly lower plasma 25(OH)D concentration was associated with low plasma cathelicidin level in patients with sepsis compared with healthy controls. A low 25(OH)D level was a biomarker to predict AKI and has a significant impact on length of stay, organ dysfunction, infection rates, and survival in critically ill patients [17, 91, 92]. Vitamin D deficiency was independently associated with increased morbidity and mortality as well as significantly associated with AKI with RIFLE-Injury and -Failure stages in intensive care units (ICU) [93]. The levels of bioavailable 25(OH)D were strongly and inversely associated with the severity of sepsis and inversely associated with hospital mortality. Because the levels of the major metabolite of vitamin D, 24R.25(OH)2D3, were not elevated in AKI, the reduced levels of 25(OH)D resulted from decreased production and not enhanced catabolism related to FGF23. The strong association between bioavailable 25(OH)D versus total 25(OH)D levels and severity of sepsis may be related to the selective uptake of bioavailable 25(OH)D by macrophages and nontraditional target organs [94].

The exact mechanism is unknown. 1.25(OH)D can modulate the levels of inflammatory cytokines and may play a role in LPS-induced immune activation of endothelial cells during Gram-negative bacterial infections. Renoprotective effects of vitamin D has been identified in several AKI animal models, including contrast-induced AKI, gentamicin-induced AKI, cisplatin-induced AKI, cyclosporine-induced AKI, ischemia-/reperfusion-induced AKI, and the obstructive nephropathy model [95–99]. The data from experimental AKI studies suggest that vitamin D analogs protect the kidney by targeting three major pathways: the local RAS, antioxidation, NF-κB and PPAR-γ pathways to suppress inflammatory, fibrotic, apoptotic, and proliferative factors [95, 100–102]. In contrast to the role of vitamin D in CKD patients, the role

of vitamin D in AKI is not as well defined. It is reasonable to hypothesize that the predisposition of vitamin D-deficient critically ill patients to AKI is related to the innate and adaptive immune response.

6. Conclusion

Vitamin D is a critical substance for bone and mineral regulation and is also a hormone with pleiotropic functions. Vitamin D exerts beneficial effects on immunomodulatory effects, alleviates metabolic syndrome, improves insulin resistance, maintains regular blood pressure, increases vascular endothelial cell function, and manages renal anemia (**Figure 1**). Vitamin D has protective effects on improving proteinuria and progression of renal function in CKD patients. Vitamin D deficiency is independently associated with increased morbidity and mortality in critical illness and a biomarker to predict AKI. Thus, more trials are needed to provide more evidence for clinical application of the pleiotropic influence of vitamin D on the immunological, metabolic, cardiovascular, and renal effects in patients with kidney disease.

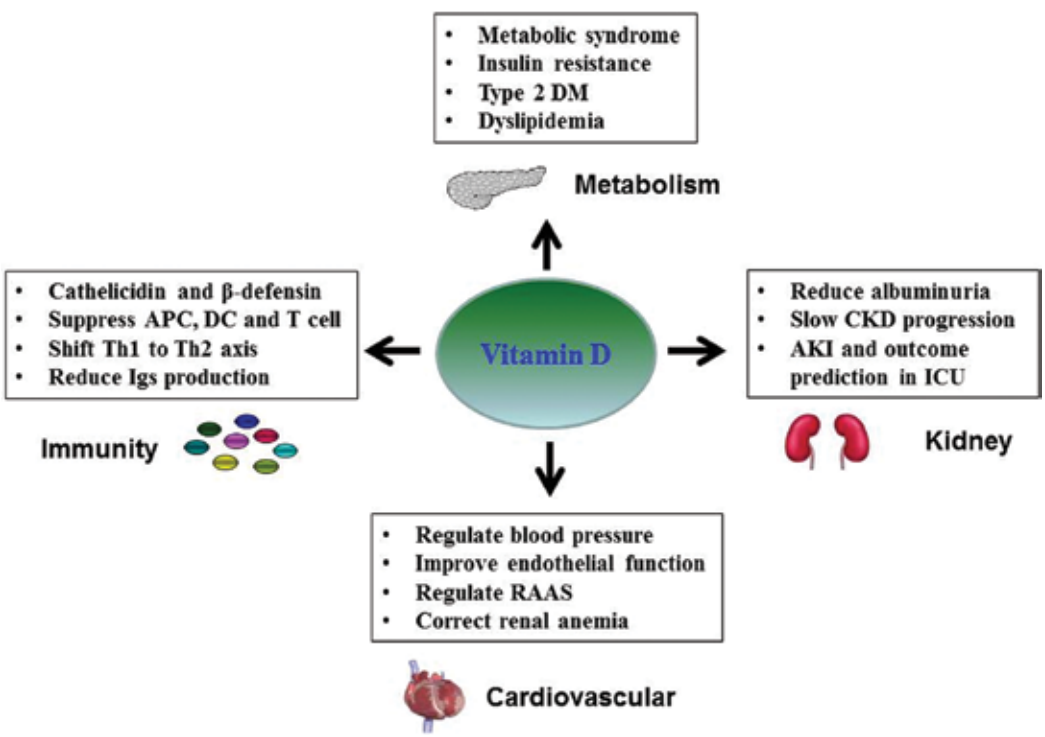

Figure 1. Pleiotropic effects of vitamin D in kidney disease. Vitamin D exerts beneficial effects on immunological, metabolic, cardiovascular, and renal effects in patients with kidney disease.

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Section 2

Vitamin D in Age and Neurological Diseases

Chapter 4

Vitamin Deficiency Among the Elderly Institutionalized Patients

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Additional information is available at the end of the chapter

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Abstract

Objectives: Deficiency in vitamin D (Vit D) is usually associated with elderly patients. This chapter investigates its prevalence amongst the elderly in long-term care in Qatar.

Methodology: The research material included patient chart reviews, electronic data and other evidence-based research papers collated between April 2010 and April 2012. Geriatric patients 65 years and above in healthcare facilities in Qatar were considered as the sample group in this study; its results were analysed and compared, in order of diagnosed Vit D deficiency severity.

Results: The total number of patients studied was 889; 66% were female and 34% male, with an average age of 75 ± 8.7 years. The Vit D serum level mean baseline utilized was 24.4 ± 13.5 ng/ml; 72% of patients had Vit D deficiency with 31 and 30% being mildly and moderately deficient, respectively, while approximately 11% were severely deficient. A positive link was identified between HDL-C and Vit D levels $r < 0.17$, $P < 0.001$; however, HbA1c levels showed a negative link with Vit D $r < 0.15$, $P < 0.009$.

Conclusions: Vit D deficiency was found to be substantially high (72%) among the elderly in Qatar. This low level of Vit D was associated with higher HbA1c and lower HDL-C levels.

Keywords: elderly, institutionalized patients, geriatrics, vitamin D deficiency, comorbidities

1. Introduction

Vitamin D (Vit D) plays an important role in normal physiological function and is essential for bone mineralization [1]. Recently, Vit D deficiency is under consideration due to the fact that it has been associated with cardiovascular disorders, malignancy, fractures and deaths [2–4]. Vit D deficiency represents an important public health concern which is commonly observed worldwide [5–7]. Vit D deficiency remains an underrecognized problem in the general populace and is poorly defined in elderly patients. This phenomenon results from reduced capacity of the skin to produce vitamin D, low skin exposure, skin pigmentation, sunscreen use, skin covering clothes and a diet low in fish and dairy products. In the elderly the reduced dermal synthesis of vitamin D is unlikely to be compensated by dietary intake of vitamin D.

In a geriatric population, Vit D deficiency has been associated with poor muscular, physical and cognitive physical performance as well as falls and fractures [8]. In a study of community dwelling persons, performed in the Chianti area in the centre of Italy which has a mild pleasant climate and sunlit rural areas, Vit D deficiency was found to be significantly high. Vitamin D levels (VDL) noticeably lessens with age in both males and females alike, but then the decline starts substantiality earlier and is sharper in females starting from the perimenopausal age. In males the decline in vitamin D levels becomes apparent 20 years later starting from their 70s, Vit D deficiency is significantly associated with aging and elderly patients who need hospitalization for longer periods and as a result more susceptible [9, 10].

Advanced age and low exposure to sunlight are the major factors associated with Vit D deficiency. Van der Wielen et al. [11] found that regardless of geographical location, free-living elderly (>70 years) living in 11 European countries are at substantial risk of inadequate Vit D status during winter and spring time and in the oldest and more obese subjects. In fact, 86% of these subjects with multiple risk factors were vitamin D deficient. Several studies have reported Vit D deficiency among different populations from the Middle East [12–15].

A report from Kuwait showed subclinical Vit D deficiency among veiled women [16]. Also, reports from Saudi Arabia demonstrated higher Vit D deficiency in Saudi women. The authors found female gender, sedentary lifestyle and low milk consumption to be independently associated with lower Vit D levels [17].

In a previous study from Qatar, El-Menyar et al. reported a high percentage (91%) of low Vit D level (<30 ng/ml) in adults (mean age: 49 ±12 years); they also found a strong association between low Vit D and hypertension [14]. Several studies addressed the association between low Vit D and high triglyceride (TG) levels, low levels of high density lipoprotein (HDL-C) and the quality of HDL [18]. Furthermore, the interference of 'Vit D' in cholesterol synthesis and potential synergistic action with statins has been reported [18].

Vit D also plays a role in insulin secretion and therefore is associated with type 2 diabetes mellitus (T2DM). Earlier studies suggested a significantly higher risk of T2DM in Vit D–deficient patients [19, 20]. In contrast, Hidayat et al. [21] observed no significant association between

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the incidence of T2DM and Vit D deficiency in an older population. Vit D insufficiency is frequently associated with abnormal bone metabolism including secondary hyperparathyroidism which leads to increase in bone turn over and bone loss particularly cortical bone. Patients with chronic kidney disease (CKD) have an exceptionally high rate of Vit D deficiency that is further exacerbated by their reduced ability to convert 25-(OH) Vit D into the active form: 1,25 dihydroxy-Vit D [22]. There are no studies in the elderly population in the Gulf region. Therefore, the present study was designed to assess the prevalence of Vit D deficiency and the associated risk factors among the geriatric population in Qatar.

2. Patients and methods

2.1. Significance for public health

Low vitamin D levels have been associated with causing a range of chronic conditions. A few studies have evaluated the prevalence of low vitamin D prominence in Middle Eastern countries like Qatar and its possible correlation with other causes of chronic disease.

Information available recognizes the high prevalence of vitamin D deficiency in Qatar and highlights the need to develop a nationwide illustrative study to evaluate further. Subsequently, the study may assist in the development of public health strategies for the prevention of diseases in Qatar.

2.2. Study setting

This study was conducted between April 2010 and April 2012 and involved data collected from elderly patients (65 years). Geriatric patients 65 years and above in healthcare facilities in Qatar were considered as the sample group; serum total 25-hydroxyvitamin D (25(OH) D) levels were measured, individual patient characteristics, treatment plans, treatment and results were analysed and compared in order of diagnosed Vit D deficiency severity. Patients who had not been screened for Vit D levels or who had incomplete data were excluded.

2.3. Measures

A data-extraction tool was developed that built in information relating to demographics, body mass index (BMI, calculated based on height and weight; kg/m^2) and blood examinations (full blood count, serum albumin, calcium, phosphorus, comorbidities, medications, and outcome).

An immunoanalyser (Liaison, Diasorin Inc.) was used for the measurement of Vit D. 'It is an automated direct competitive chemiluminescence immunoassay (CLIA) for quantitative determination of total 25-OH Vit D in serum or plasma. The imprecision at 56 and 19 ng/ml as measured by coefficient of variation was 8.7 and 13.2%, respectively [14]'.

The Diazyme's 25(OH) D assay is one of the fast track diagnostic methods with complete testing results in less than 2 hours. 'The test is user-friendly, and can be performed manually or

easily adapted for use on a wide range of fully automated microtiter plate readers, making it suitable for use in laboratories of all sizes and with all manner of testing needs'.

Vit D deficiency was defined as level less than 30 ng/ml which was further subdivided into mild (20–29 ng/ml), moderate (10–19 ng/ml) and severe insufficiency (less than 10 ng/ml) [14, 21]. Patient characteristics and outcomes were analysed and compared according to the severity of Vit D deficiency. Patients after 6 months were re-evaluated for Vit D levels and all-cause mortality.

2.4. Statistical analysis

Where appropriate, data is presented as proportions, medians or mean ±SD. Wherever applicable the continuous variables were analysed using Student's tests or one-way ANOVA. Also a non-parametric Mann–Whitney test was used for skewed continuous data. Definite variables among groups were compared using the chi-square test; estimating the associations between Vit D deficiency and demographic and clinical index. Age and its correlation with HDL-C, HbA1c and Vitamin D levels was also studied using Pearson's correlation method.

A two adjusted $P < 0.05$ was considered significant. All data investigation was carried out using the Statistical Package for Social Sciences version 18 (SPSS Inc., USA).

3. Results

The total number of patients studied was 889; 66% were female of which 77% were Qataris and 34% male, with the mean age of between 75 ± 8.7 years. The upper range of age limit among the study group was 107 years old male (see **Tables 1–3** below for results). Sixty per cent of the patients were recruited from Home Healthcare Services (HHS) followed by the out-patient (31.8%) and in-patient (7.2%) departments. Findings identified in terms of percentages were included; 24.4% had stroke (cerebrovascular accident) and 23.65% had coronary artery disease, respectively, 26.25% had dementia, 76.5% had hypertension, 63.2 % had type 2 diabetes and 47.5% dyslipidemia (see **Table 1**).

The Vit D serum level mean baseline utilized was, 24.4 ±13.5 ng/ml; 72% of patients had Vit D deficiency with 31.4 and 29.6% being mildly and moderately deficient, respectively, while 10.8% were severely deficient (see **Table 1**).

As a result, 33.5% of patients were prescribed oral supplementation of Vit D. When tested at the follow-up 6 months later, Vit D levels available in 325 of these patients had increased to 28.5 ± 13.4 ($P < 0.001$).

Table 2 discusses the rate of recurrence and association of sociodemographic and clinical variables due to Vit D levels.

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Vit D deficiency was common in females than males, mildly affected patients female to male percentage was 70.3 vs. 29.7%, moderate 68.5 vs. 31.5% and severe 70 vs. 30%, $P < 0.91$; though, this was not significant. Patients admitted to HHS had notably more Vit D deficiency than other admitting services; mildly affected patients female to male percentage was 54.2 vs. 45.8%, moderate 68.0 vs. 32% and severe 87.5 vs. 15%, $P < 0.001$.

Age (years)*	74.9 ± 8.7	**Overall vitamin D levels***	24.4 ± 13.5
Female	589 (66.3%)	Optimal	175 (28.2%)
Unit		Mild deficiency	195 (31.4%)
Home care	421 (59.3%)	Moderate deficiency	184 (29.6%)
Out patient	283 (31.8%)	Severe deficiency	67 (10.8%)
In-patient	64 (7.2%)	**Medication**	
Nationality		Multi vitamin	147 (16.5%)
Qatari	655 (76.6%)	Proton pump inhibitors	304 (34.2%)
Non-Qatari	200 (23.4%)	Vitamin D 50000 IU (orally)	298 (33.5%)
Marital status		Calcium supplement	79 (8.9%)
Married	473 (60.1%)	Combined fosamax + Vit D	7 (0.8%)
Non-married	314 (39.9%)	Calcium + vitamin D	2 (0.2%)
Diagnosis		**Baseline**	
Hypertension	680 (76.5%)	Vitamin-D (ng/ml)*	24.4 ± 13.5
Diabetes mellitus (Type II)	562 (63.2%)	Calcium (mmol/L)*	2.3 ± 0.14
Dyslipidaemia	422 (47.5%)	Phosphorus (mmol/L)*	1.17 ± 0.29
Cerebrovascular accident	217 (24.4%)	Parathyroid hormone (pmol/L)**	65 (4-625)
Dementia	233 (26.2%)	**Follow-up**	
Coronary artery disease	210 (23.6%)	Vitamin-D (ng/ml)*	28.5 ± 13.4
Hypothyroidism	110 (12.4%)	Calcium (mmol/L)*	2.28 ± 0.2
Heart failure	37 (4.2%)	Phosphorus (mmol/L)*	1.19 ± 0.3
Renal dysfunction	99 (11.1%)	Parathyroid hormone (pmol/L)**	85 (4-848)
Fracture	32 (3.6%)	**Mortality**	11 (1.2%)
Traumatic injury	21 (2.4%)		
Aspiration pneumonia	24 (2.7%)		
Urinary tract infection	12 (1.3%)		

*Mean ± SD.
**Median (range).

Table 1. Demographics, clinical presentation and outcome in geriatric patients ($n = 889$).

	Vitamin D deficiency				*P*
	Optimal VDL (*n* = 175)	**Mild (*n* = 195)**	**Moderate (*n* = 184)**	**Severe (*n* = 67)**	
Gender					
Female	126 (72.0%)	137 (70.3%)	126 (68.5%)	47 (70%)	0.912
Male	49 (28%)	58 (29.7%)	58 (31.5%)	20 (30%)	
Unit					
HHS	66 (42.0%)	91 (54.2%)	102 (68.0%)	49 (87.5%)	0.001
Out patient	67 (42.7%)	55 (32.7%)	34 (22.7%)	5 (9.4%)	
In-patient	24 (15.3%)	22 (13.1%)	14 (9.3%)	2 (3.6%)	
Nationality					
Qatari	135 (79.4%)	146 (77.2%)	133 (75.6%)	49 (76.6%)	0.354
Non-qatari	35 (20.6%)	43 (22.8%)	43 (24.4%)	15 (23.4%)	
Marital status					
Married	104 (69.8%)	88 (52.0%)	89 (54.0%)	28 (43.8%)	0.008
Non-married	45 (30.2%)	81 (48.0%)	76 (46.0%)	36 (56.2%)	
Diagnosis (on-admission)					
Diabetes mellitus	107 (61.1%)	128 (65.6%)	124 (67.4%)	46 (68.7%)	0.566
Hypertension	135 (77.1%)	159 (81.5%)	148 (80.4%)	47 (70.1%)	0.217
Dementia	43 (24.6%)	60 (30.8%)	44 (23.9%)	16 (23.9%)	0.388
Coronary artery disease	37 (21.1%)	36 (18.6%)	49 (26.6%)	22 (32.8%)	0.055
Heart failure	7 (4.0%)	7 (3.6%)	11 (6.0%)	3 (4.5%)	0.703
Dyslipidaemia	85 (48.6%)	97 (49.7%)	95 (51.6%)	31 (46.3%)	0.879
Renal dysfunction	24 (13.7%)	15 (2.4%)	31 (5%)	10 (1.6%)	0.055
Cerebrovascular accident	46 (26.3%)	50 (25.6%)	47 (25.5%)	18 (26.9%)	0.996
Hypothyroidism	26 (14.9%)	33 (16.98%)	25 (13.6%)	11 (16.4%)	0.824
Fracture	6 (1%)	5 (7.7%)	9 (16.8%)	5 (14.9%)	0.302
Traumatic	2 (1.1%)	3 (1.5%)	9 (4.9%)	2 (3.0%)	0.100
Social admission	1 (0.6%)	2 (1.0%)	2 (1.1%)	3 (4.5%)	0.101
Aspiration pneumonia	5 (2.9%)	3 (1.5%)	4 (2.2%)	3 (4.5%)	0.565
Urinary tract infection	1 (0.6%)	4 (2.1%)	4 (2.2%)	0 (0.0%)	0.376

	Vitamin D deficiency				*P*
	Optimal VDL (*n* = 175)	**Mild (*n* = 195)**	**Moderate (*n* = 184)**	**Severe (*n* = 67)**	
Infected bedsore	1 (0.6%)	2 (1.0%)	2 (1.1%)	2 (3.0%)	0.461
Medication					
Multi vitamins	34 (19.4%)	41 (21.0%)	25 (13.6%)	15 (22.4%)	0.209
Proton pump inhibitors	52 (29.7%)	79 (40.5%)	76 (41.3%)	34 (50.7%)	0.038
Vitamin D 50,000 IU (Orally)	65 (37.1%)	81 (41.5%)	96 (52.2%)	39 (58.2%)	0.003
Calcium supplement	22 (12.6%)	21 (10.8%)	19 (10.3%)	8 (11.9%)	0.910
Fosamax + vitamin D	1 (0.6%)	1 (0.5%)	1 (0.5%)	0 (0%)	0.946
Calcium + vitamin D	0 (0%)	1 (0.5%)	0 (0%)	1 (1.5%)	0.237
Mortality	2 (1.1%)	1 (0.5%)	1 (0.5%)	0 (0%)	0.755

HHS: Home Healthcare Services.

Table 2. Comparison of qualitative variables according to vitamin D levels (VDL).

Further it was found that married patients had a considerably higher number of ideal Vit D levels. On admission, diagnoses were compared according to Vit D levels. Proton pump inhibitors $P < 0.038$ and oral Vit D supplementation $P < 0.003$ were prescribed and administered more in Vit D–deficient patients (see **Table 2**).

The mean blood glucose level was noticeably higher in the severe Vit D-deficient group compared to the ideal group 9.5 ± 5 vs. 7.2 ± 3.2 ng/ml, $P < 0.005$. The mean age was compared between the different Vit D–deficient groups, $P < 0.462$ (see **Table 3**).

In patients with T2DM and an estimated glomerular filtration rate (eGFR) _30 ml/min/1.73 m^2, the mean eGFR was 55.3 ± 8.5 ml/min/1.73 m^2. Note that 55 (19.9%) of those had kidney disease outcomes quality initiative CKD stage 1 disease (eGFR _90 ml/min/1.73 m^2), 142 (51.4%) had stage 2 disease (eGFR 60–89 ml/min/1.73 m^2) and 79 (28.6%) had stage 3 disease (eGFR 30–59 ml/min/1.73 m^2). There was no considerable link between eGFR in type 2 DM and Vit D levels ($P = 0.43$). No correlation analysis was conducted between Vit D levels and eGFR in nondiabetic patients as the study population was negligible.

Figures 1 and **2** refer to the connection between Vit D deficiency and HDL-C and HbA1c. There was a positive link noted however between HDL-C and Vit D levels ($r = 0.173$, $P = 0.001$), whereas HbA1c levels indicated a negative association with Vit D levels ($r = 0.152$, $P = 0.009$).

	Vitamin D deficiency				*P*
Baseline	**Optimal VDL (*n* = 175)**	**Mild (*n* = 195)**	**Moderate (*n* = 184)**	**Severe (*n* = 67)**	
Age (years)	74 ± 8.4	75.3 ± 8.3	74.8 ± 7.6	75.5 ± 9.8	0.462
Body mass index	24.7 ± 5.7	23.1 ± 5.2	26.7 ± 6.5	27.2 ± 7.4	0.263
Vitamin D (ng/ml)	41.2 ± 11.5	24.6 ± 2.9	14.9 ± 2.9	6.5 ± 1.9	0.001
Calcium (mmol/L)	2.3 ± 0.14	2.28 ± 0.12	2.29 ± 0.15	2.26 ± 0.13	0.307
Cholesterol (mmol/L)	4.3 ± 0.9	4.4 ± 0.96	4.5 ± 1.2	4.5 ± 1	0.464
Triglycerides (mmol/L)	1.28 ± 0.65	1.38 ± 1.1	1.45 ± 0.7	1.53 ± 0.9	0.304
TSH (mIU/L)	2.2 ± 1.6	3.9 ± 8.9	3.8 ± 10.2	7.1 ± 17.8	0.081
ALP (IU/L)	82.4 ± 45.1	89.6 ± 57.8	99.3 ± 63.4	105 ± 84	0.049
Glucose (mmol/L)	7.2 ± 3.2	7.7 ± 3.7	8.2 ± 5.1	9.5 ± 5	0.005
HbA_{1c} (%)	7.05 ± 1.5	7.3 ± 1.4	7.2 ± 1.8	8 ± 1.9	0.034
LDL (mmol/L)	2.5 ± 0.73	2.6 ± 0.8	2.7 ± 1	2.8 ± 0.8	0.133
eGFR (ml/minute)	55.3 ± 8.5	47.9 ± 18.1	56.1 ± 6.7	50 ± 17.3	0.432
T4 (ng/L)	18 ± 17.5	16 ± 6.8	13.6 ± 2	12.9 ± 2.8	0.381
Phosphorus (mmol/L)	1.17 ± 0.2	1.2 ± 0.4	1.15 ± 0.3	1.05 ± 0.27	0.118
Parathormone (pmol/L)	96.8 ± 124.3	108.5 ± 105.3	161 ± 164	130.2 ± 104.7	0.212
Haemoglobin (g/dl)	12.1 ± 1.6	12 ± 1.8	12.1 ± 1.9	12.07 ± 1.7	0.959
HDL-C (mmol/L)	1.4 ± 0.9	1.3 ± 0.3	1.2 ± 0.4	1.1 ± 0.4	0.040
Ejection fraction (%)	51.9 ± 11.4	54.4 ± 5.5	53.4 ± 9.6	52.8 ± 8.2	0.916
Albumin (mmol/L)	38.4 ± 6.1	38.5 ± 4.5	38.2 ± 9.9	36.7 ± 5.2	0.344
Follow-up					
Vitamin D (2) (ng/ml)	38.2 ± 15.9	26.9 ± 9.4	25.6 ± 11.5	22.3 ± 13.8	0.001
Parathyroid hormone (pmol/L)	104 ± 81.8	122.2 ± 111.4	154.2 ± 171.9	151.7 ± 185.9	0.807
Calcium (mmol/L)	2.28 ± 0.13	2.32 ± 0.37	2.25 ± 0.15	2.27 ± 0.12	0.516
Phosphorus (mmol/L)	1.1 ± 0.25	1.2 ± 0.29	1.2 ± 0.23	1.2 ± 0.26	0.693

TSH: Thyroid stimulating hormone; ALP: Alkaline Phosphatase; LDL-C: low density lipoprotein cholesterol; HDL-C: High Density lipoprotein cholesterol; all variable are expressed as mean ± standard deviation; T4: Thyroxin; eGFR: estimated glomerular filtration rate.

Table 3. Comparison of quantitative variables according to vitamin D levels (VDL).

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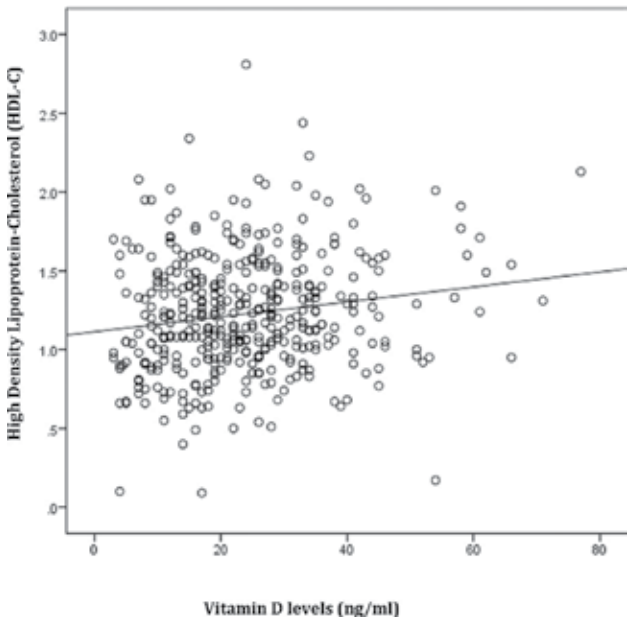

Figure 1. Correlation between HDL-C and vitamin D levels in geriatric patients.

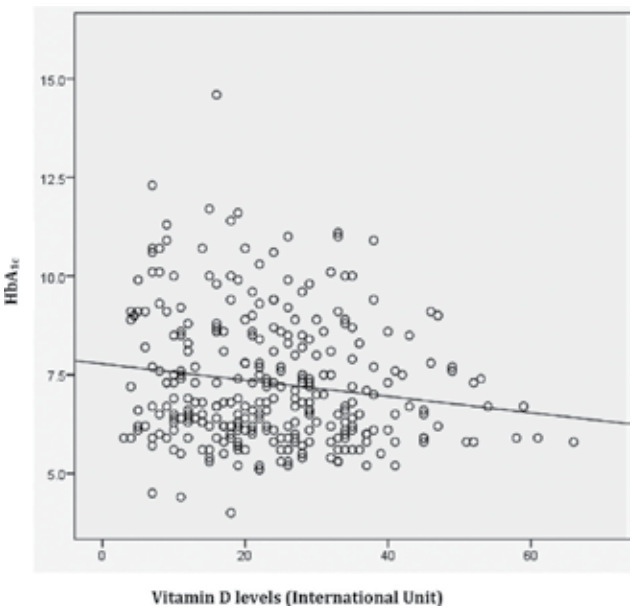

Figure 2. Correlation between type 2 DM patients HbA_{1c} and vitamin D levels in geriatric patients.

4. Discussion

It has been suggested that, lifestyle and socio-cultural practices may be related to the high Vit D deficiency reported among the young recently in Qatar [25]. However, the relationship between Vit D deficiency and its impact on the health of the elderly is still lacking.

Unfortunately, there are no accurate figures for the confident determination of vitamin D deficiency in Qatar due to the geographical and/or demographical nature of studies conducted [41].

However, centred on data gathered from a review of the system, about 90% of the Qatari population may be deficient in serum levels of the vitamin. This exclusive study from the region tries to address the impact of age, diabetic status and dyslipidaemia on Vit D deficiency among the geriatric population.

This study found the existence of an extraordinary large number of Vit D deficiency (71.8%) among the elderly in Qatar, which may be attributed to their limited exposure to sunlight as they age, generally due to the inactive lifestyle, clothing, extreme summers and minimal outdoor activity that characterises life in Qatar. A similar study in Indonesia found that 78.2% Vit D deficiency was present amongst its elderly population [21].

What is more, with increasing age the capacity of the skin to produce Vit D on sunlight exposure also decreases [23]. Likewise, advanced age reduces Vit D (1,25(OH)2D) production by the kidneys [24]. Accordingly, physiological changes and climate conditions together with advanced age influence Vit D metabolism among the elderly [25]. In the elderly, Vit D deficiency is associated with an increased risk of falls, osteoporosis and fractures [26]. At present, investigation of plasma 25(OH)D is considered as a reliable marker for Vit D level assessment [27, 28, 43].

In our study, considerably more patients from the home-based run services had a Vit D insufficiency as well as severe Vit D deficiency in comparison to the in- and out-patient departments of hospitals ($P = 0.001$). This corroborates reports from western researchers that found a higher occurrence of Vit D insufficiency among communal living elderly [25]. However, in the U.S., Vit D insufficiency was incidentally lower in the elderly except in those patients who sustained hip fracture [25]. Lund et al. [29] found Vit D deficiency in 25% of hip fracture cases, of which 5% had severe Vit D deficiency. However, the incidence of hip fractures in our study (14.9%) was relatively lower compared with earlier studies [29].

The present study observed severe Vit D deficiency in 70% of elderly females. Even though the Qatar demographics showed that the sex ratio for male:female is 1.7:1. The majority of population assigned for this study is female patients; this was decided up on the willingness of participation. Other possible explanation for this might be due to the higher life expectancy in females [21]. Also, Indonesia reported a higher incidence of Vit D deficiency among its elderly females in comparison to elderly males. This high number of women with Vit D deficiency in the Middle East and Asia may be attributed to socio-cultural practices such as the use of the veil outside in the Sun.

Aging has been associated with T2DM [30]. Moreover, several epidemiological studies have found a negative correlation between T2DM, obesity and Vit D deficiency [31–33]. Mathieu et al. advocated Vit D supplementation for improving glucose tolerance in patients with Vit D deficiency [34]. Further, Hidayat et al. [21] reported that in overweight elderly patients, their high BMI was pointedly associated with an increased Vit D deficiency.

Our findings previously published online [35] confirm these earlier reports on the link between type 2 diabetes (T2DM), body mass index and Vit D deficiency. In this study, markers of T2DM (raised HbA1c and high fasting blood glucose levels) had a negative association with levels of circulating vitamin D3. It was observed that significantly high levels of blood glucose ($P < 0.005$) and HbA1c ($P < 0.03$) were associated with acute Vit D deficiency. Pittas et al. [36] in a double-blind, randomized, controlled trial reported that in healthy adults with impaired fasting blood glucose, supplementation with vitamin D may attenuate increases in glycaemia and insulin resistance that occur over time.

In addition, well documented also has been the association between low Vit D levels and chronic kidney disease (CKD). Previous research has shown that in a large majority of patients with advanced CKD (stage 2), their levels of Vit D have a tendency to be lower than the normal limit, although in patients with advanced CKD (stage 3 and 4), Vit D levels fell significantly [37, 38]. Furthermore, elderly patients (<65 years) are likely to have an increased association of Vit D deficiency and renal dysfunction with lower glomerular filtration rate (GFR) [39].

The present study showed that eGFR was not associated with Vit D levels. Our findings are consistent with an earlier study showing no association between an impaired eGFR and Vit D deficiency ($P < 0.432$) [40]. Fraser et al. [41] explored the role of serum 25(OH)D, parathyroid hormone and calcium in the development of cardiovascular sicknesses. A positive association was identified between HDL-C and 25(OH) D levels; considerably lower HDL-C levels were detected in patients with acute Vit D deficiency, consistent with the findings of Fraser et al. [41]. As a result, HDL-C levels and Vit D deficiency had a substantial opposite relationship, as patients with lower levels of HDL-C had acute Vit D deficiency. LDL-C and triglyceride were less in patients with acute Vit D deficiency in comparison to those with ideal levels [42].

New research has found in recent times that the presence of vitamin D receptors and the vitamin D activating enzyme (1-hydroxylase) in the brain has advised a possible role of vitamin D in cognitive function. It is suggested that the vitamin D receptor and catalytic enzymes are confined to a small area of the brain involved in complex planning, processing and the creation of new memories. These findings in theory link the role of vitamin D to cognitive impairment, depression and also multiple sclerosis. Although, the current findings cannot be supported as dementia is associated with severely low Vit D levels.

Epidemiological research recently has underlined the significance of Vit D and calcium supplementation for communal living, hospitalized and care home elderly. The vitamin D requirements may vary only based on customary calcium intake. The therapeutic potential of vitamin D will not be affected by age and sex difference but may be affected by ethnicity [44, 45].

Taking into consideration the socio-cultural and hereditary factors predominant in the Middle East, the proper controlling of Vit D deficiency together with metabolic disorders should be made an essential part of treatment for the ageing people. Additionally, the eluding of Vit D deficiency among the elderly may possibly be useful for the optimum managing of high-risk metabolic disorders such as diabetes mellitus and dyslipidaemia which in time will improve the care provided for the population.

This study exposed certain limitations, such as the lack of cause-specific mortality data, as well as details of Vit D supplementation. Another limitation was that the influence of seasons and Vit D deficiency was not considered in the analysis; as the study was retrospective in nature. Even though these limitations exist, the large sample size is representative of the geriatric population in the Middle East. Therefore, this study gives an understanding into the occurrence of Vit D deficiency and its attendant issues among the elderly in Qatar.

In conclusion, vitamin D as a nutrient performs several functions, fundamental to the biological system of the human body including the endocrine and metabolic systems. A large occurrence of Vit D deficiency was detected in the elderly. Vit D serum levels were lower, the wrong way round with HbA1c and HDL-C levels. The follow-up indicated a small but major improvement in Vit D levels after Vit D supplements had been administered. For that reason, further research is required to assess whether or not administering Vit D supplements improves low HDL-C levels and/or glycemic control in T2DM.

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Chapter 5

Vitamin D Affects Neuronal Peptides in Neurodegenerative Disease: Differences of V-D2 and V-D3 for Affinity to Amyloid-β and Scrapie Prion Protein In Vitro

Yoichi Matsunaga, Midori Suenaga,
Hironobu Takahashi and Akiko Furuta

Additional information is available at the end of the chapter

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Abstract

The misfolding of neuronal peptides such as Aβ40/42 in Alzheimer's disease and cellular prion protein in scrapie induce abnormal aggregation of the peptides in the brain. The seeding of peptides' oligomers from monomers is the initial step to form molten-globule states before abnormal aggregation. Therefore, compounds targeting the step are useful to clarify the mechanisms underlying aggregation of the proteins and Vitamin D derivatives, which can interact with both Aβ40 and cellular prion protein; however they show different effects in the oligomerization step of the proteins. We discuss the different effects of Vitamin D_2 and Vitamin D_3 in the interaction with these peptides in brain.

Keywords: Alzheimer's disease, prion disease, amyloid-β, human PrP^c, Vitamin D derivatives, oligomerization

1. Introduction

Recently, involvement of Vitamin D (V-D) in cognitive impairment is reported.

V-D is a secosteroid and occurs in two distinctive major forms: Vitamin D_2 (V-D_2) and Vitamin D_3 (V-D_3). V-D_3 is a 27-carbon derivative of cholesterol, and V-D_2 is a 28-carbon derivative from plant ergosterol. The structure of V-D_2 differs from V-D_3 by containing an extra methyl group and a double bond between carbon 22 and 23 (**Figure 1**). Both V-D derivatives appear to have

similar biological effects in humans [1, 2]. V-D_3 is about four times as potent as V-D_2 [3]. Interestingly, V-D_2 is a naturally occurring V-D form derived from a fat extract of yeast by the exposure to UV light, and the metabolites were not detectable in the blood of vertebrates such as humans, unless administered from an external source [3, 4]. Thus, V-D_2 is not synthesized in vivo and is regarded as a supplement. The metabolites derived from V-D_2 are not equivalent to those for V-D_3 [5]. In contrast to V-D_2, V-D_3 is the naturally synthesized within the skin and oils of fur. Although both microsomal and mitochondrial 25-hydroxylases act on V-D_3, they do not act on V-D_2 [4, 6, 7], and furthermore the V-D binding protein shows lower affinity for V-D_2 than V-D_3 and its metabolites [8]. Currently, clinical applications of V-D for immunosuppression and reduction of pro-inflammatory immune pathways demonstrate that V-D is a prosteroid hormone rather than a vitamin [9, 10]. V-D cross blood-brain barrier by passive diffusion and enter the cerebrospinal fluid and brain. The beneficial effects in reducing the relapse risk in multiple sclerosis through its immune-regulatory effects were reported [11].

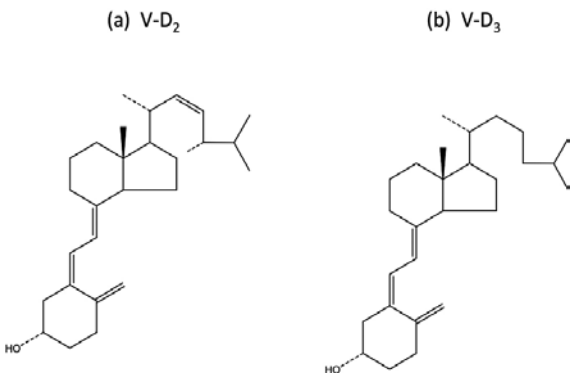

Figure 1. Structural differences between Vitamin D_2 and Vitamin D_3.

Recent epidemiologic studies report V-D_3 deficiency as a risk factor of cardiovascular disease including cardiac hypertrophy, myocardial remodeling developed to heart failure (HF) [12, 13] and some prospective studies report the relationship between hypovitaminosis-D and an increased risk of cognitive decline in elderly population [14] and suggested that supplementation of V-D could prevent the cognitive disorders [15–17], and its effects for the clearance of aggregated amyloid-β (Aβ) in AD brain [18].

In this chapter, we present the different binding affinity of V-D_2 and V-D_3 to amyloidogenic protein in brain: Aβ and prion protein.

2. Amyloid-β protein in Alzheimer's disease and scrapie prion protein in prion disease

AD and prion diseases are neurodegenerative diseases in brain and cause dementia. AD is the most common case of senile dementia and the number of AD patients is increasing and recent

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study shows that 46.8 million of AD patients live in the world and it is estimated to reach 131.5 million by 2050 [19]. It is featured by memory loss, deterioration of cognitive and behavioral process, and diminished social life. These symptoms do not improve and progress with life time.

The main pathological hallmark with AD patients is the senile plaque in the brain [20]. Extracellular accumulation of insoluble Aβ protein is the main component of the plaque that induces synaptic dysfunction and neuronal loss resulting in progressive dementia [21]. The Aβ is composed of 39–43 amino acids, naturally produced by proteolytic cleavage of integral membrane protein, 100–135 kDa amyloid precursor protein [22]. The majority of the secreted Aβ alloform includes the C-terminal Aβ40 and Aβ42. Quantitative analyses have shown that, on average, 60% of all plaques contain Aβ42 and 31% contain Aβ40 [23]. The misfolding and aggregation of Aβ and tau proteins are two principal aggregating proteins in AD brain [24, 25]. Growth of the fibrils occurs by assembly of the Aβ seeds into intermediate protofibrils, and self-associates to form mature fibers [26]. This multistep process may be influenced at various stages by factors that promote Aβ fiber formation and aggregation, and the seeding of Aβ40 oligomers is the initial step of the process [27, 28].

The emergence of a prion disease in cattle is known as bovine spongiform encephalopathy (BSE) and a possible transmission to humans by the exposure to BSE has been suggested [29, 30]. Gerstmann-Straussler-Scheinker disease and Creutzfeldt-Jacob are well-known naturally occurring prion diseases in human and they are transmissible and fatal. The main event contributing to the pathogenesis of prion disease is the conversion of the cellular prion protein (PrP^{c}) into scrapie prion protein (PrP^{sc}), which is a protease-resistant, insoluble protein [31, 32]. PrP^{c} is predominantly expressed in neurons, and attached to extracellular space of plasma membrane through a glycophosphatidylinositol. It is a sialoglycoprotein with a molecular weight of approximately 33–35 kDa [33, 34]. Studies have shown that PrP^{c}(90–231), which is N-terminal truncated fragments of PrP^{c} and corresponds to the core of the protease K (PK) resistant prion protein, preserve the pathogenic features of PrP^{sc} [35, 36]. Gerstmann-Straussler-Scheinker disease and Creutzfeldt-Jacob disease are caused by mutations in the PrP gene [37] and the mutations directly link to conformational conversion from PrP^{c} to PrP^{sc} and amplification of PrP^{sc} without exogenous PrP^{sc} [38, 39], and the infectivity can be explained by the direct PrP^{sc}-PrP^{c} interaction [40]. In vitro generation of infectious PrP^{sc} has demonstrated the protein-only hypothesis of prion propagation [41, 42]. Many reports have suggested that the multistep process of conversion from PrP^{c} into PrP^{sc} includes an oligomerization/polymerization step [43, 44]. The oligomerization or molten-globule state is a preliminary step required for the formation of insoluble protein in the brain like that of Aβ aggregates in AD brain, and soluble oligomers appear to be more cytotoxic than mature aggregates [45].

2.1. V-D-induced Aβ40 oligomerization

Quartz-crystal microbalance (QCM) measurement is a highly sensitive mass-measuring system [46, 47]. The instrument is equipped with a 27 MHz QCM plate at the bottom with a stirring bar. Changes of frequency are calculated by Sauerbrey's equation [48] as below.

We applied Sauerbrey's equation for the QCM in the air phase:

$$\Delta F = -2F_0^2 \Delta m / A\sqrt{\rho_q \cdot \mu_q}$$

where ΔF is measured frequency change (Hz), Δm is mass change, F_0 is fundamental frequency of the quartz-crystal, A is an electrode area, ρ_q is density of quartz-crystal, and μ_q is the shear modulus of quartz-crystal.

The equation indicates that a 0.61 ng/cm^2 increase in mass means a −1 Hz decrease in frequency. The change of frequency is proportional to that of mass.

The change of mass in Aβ40 with V-D_2 or V-D_3 was determined [49]. A significant decrease in frequency started after 15 min upon addition of Aβ40 to the V-D_2 solution, and found that the frequency decrease depends on both V-D_2 concentration and incubation time. Aβ40-V-D_2 complex formation occurs in solution and accelerated after 15–60 min later (**Figure 2a**). After 60 min, the calculated Aβ40 molecules aggregated by an Aβ40 was 4.21394 e^{19} at 0.1 M of V-D_2, 6.74725 e^{19} at 0.5 μM of V-D_2, and 8.85697 e^{19} at 1.0 μM of V-D_2 (**Table 1**). In case of V-D_3, however, no considerable decrease in frequency upon Aβ40 addition was observed (**Figure 2b**). These results show a different potential of V-D_2 and V-D_3 for Aβ40 oligomerization in vitro.

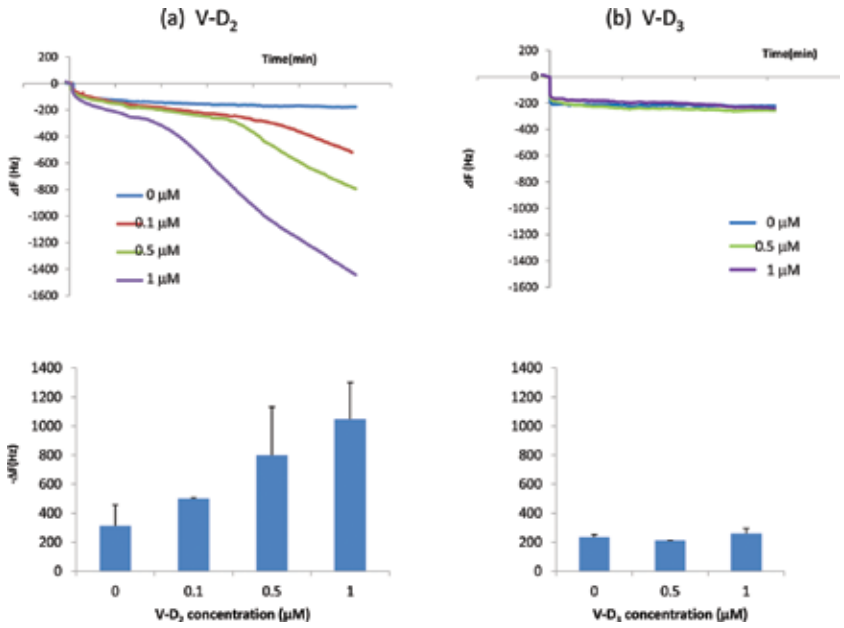

Figure 2. Quartz-crystal microbalance pattern for Aβ40 aggregation with V-D derivatives. Typical real-time monitoring of Aβ40 (12 μM) aggregation with V-D_2 at the concentrations of 0, 0.1, 0.5, and 1 μM (a) or with V-D_3 at the concentrations of 0, 0.5, and 1 μM (b) in quartz-crystal microbalance measurements. The changes of frequency of Aβ40 with V-D_2 or V-D_3 for 60 min are shown. The data are representative of three experiments. Total amount of Aβ40 aggregates with V-D_2 (a). V-D_2 induced potential dose-dependent Aβ40 aggregates. Total amount of Aβ40 aggregates with V-D_3 (b). V-D_3 did not induce Aβ40 aggregation after 60 min.

$V\text{-}D_2$ (μM)	$-\Delta F$ (Hz)	Δm (ng/cm^2)[a]	Δn (atoms/cm^2)[b]
Control*	3111 ± 44	190	2.63507×10^{19}
0.1	498 ± 6	304	4.21394×10^{19}
0.5	798 ± 332	487	6.74725×10^{19}
1	1048 ± 252	639	8.85697×10^{19}

* Direct binding of Aβ40 peptide to the Au electrode without $V\text{-}D_2$.
[a] A 1 Hz decrease in frequency results in a 0.61 ng/cm^2 increase in mass.
[b] Calculation using a molecular weight of 4331 for Aβ40.
Data are presented as mean ± SD values for three independent experiments.

Table 1. Measurement of frequency decrease in Aβ40 aggregation by $V\text{-}D_2$ on performing QCM 60 min later.

2.2. Electron microscopic observation exhibited $V\text{-}D_2$-induced Aβ40 oligomerization

Aβ40 in artificial cerebrospinal fluid without V-D as a control induced weak self-oligomerization and $V\text{-}D_3$ induced no enhancement to the control, however $V\text{-}D_2$ enhanced potent oligomerization for Aβ40 as **Figure 3**(**a–c**) [49].

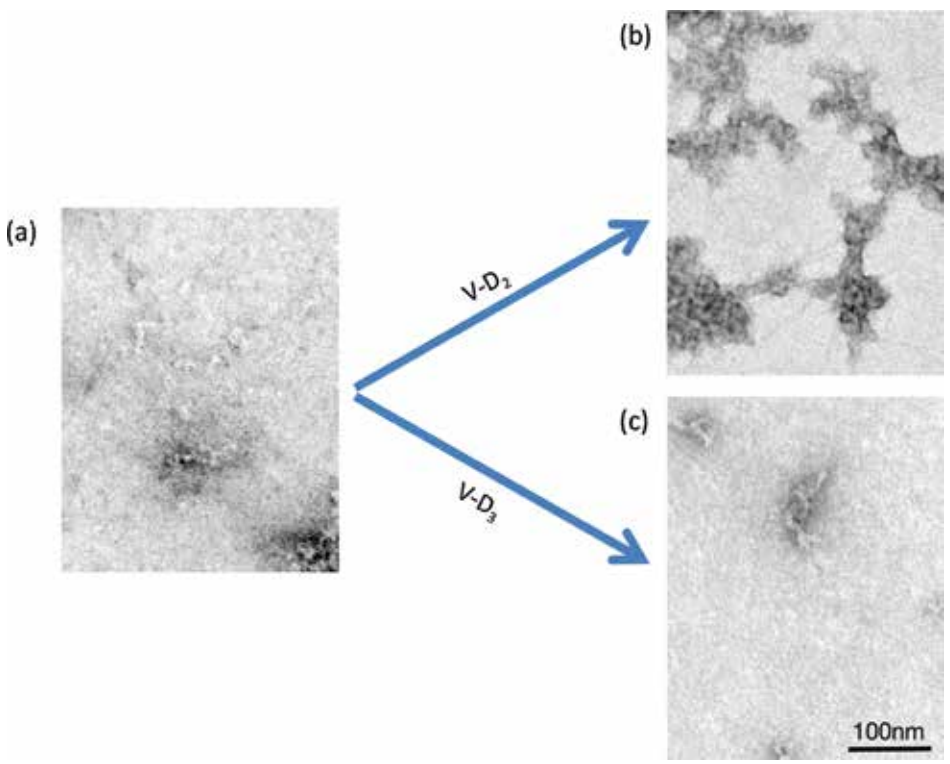

Figure 3. Electron microscopic observation for Aβ40 without or with $V\text{-}D_2$ or $V\text{-}D_3$. Aβ40 without V-D as a control (a), Aβ40 with $V\text{-}D_2$ (b), and Aβ40 with $V\text{-}D_3$ (c). Aβ40 in the photo indicates 100 nm for (a), (b), and (c).

2.3. Thioflavin-T assay revealed β-sheet formation of Aβ40 with V-D_2

Amyloid fibers are ordered β-sheet-rich proteins. Benzothiazole dye, Thioflavin-T (Th-T) is used to probe amyloid fibril formation due to specific noncovalent interactions that yield strong fluorescence upon binding [50]. Aβ40 showed a peak at 490 nm, indicating β-sheet formation [51]. V-D_2 increased peak intensity at 490 nm dose-dependently, indicating that V-D_2 facilitates β-sheet formation in Aβ40. The V-D_3 do not increase peak fluorescent intensity at 490 nm, indicating that V-D_3 facilitate no β-sheet formation in Aβ40 peptide (**Figure 4**) [49].

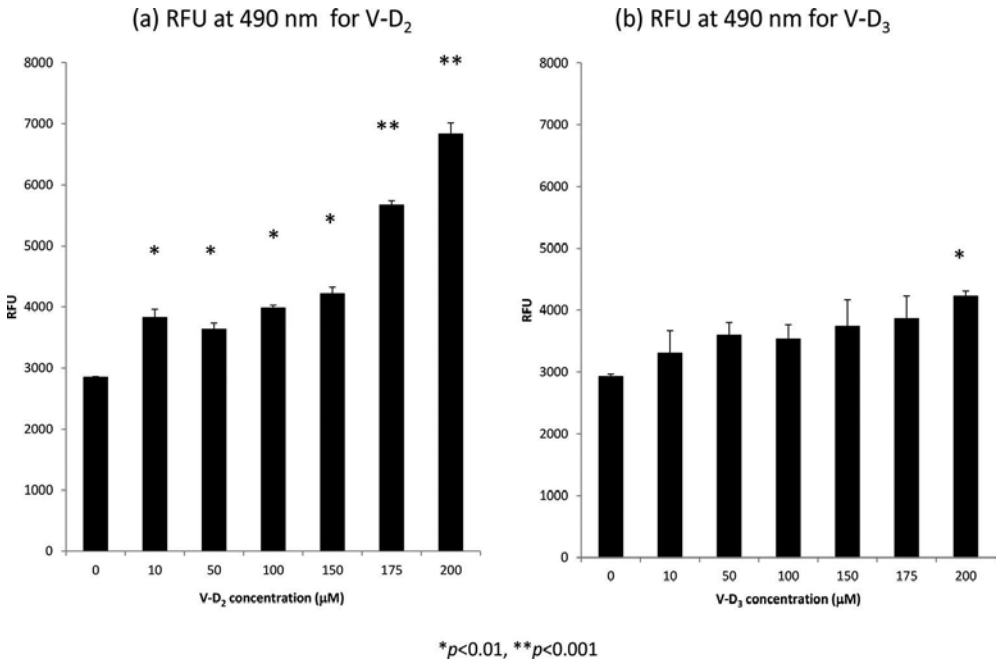

Figure 4. Th-T fluorescence monitored β-sheet formation of Aβ40 in the presence of V-D_2, V-D_3. (a) V-D_2 facilitates strong β-sheet formation in Aβ40 and (b) V-D_3 induced weak β-sheet formation in Aβ40. Y-axis indicates relative fluorescence units (RFUs). Data represent the mean ± SD values (bar) for three independent experiments. *$p < 0.01$, **$p < 0.001$ vs. without V-D derivatives.

2.4. Docking simulation between Aβ40 and V-D_2 or V-D_3

In silico docking analysis at the tertiary structure level by Molecular Operating Environment (MOE) software indicates the different interactions between V-D_2 or V-D_3 and Aβ40 peptide. The calculated minimum energy for V-D_2 was −40.36 kcal/mol and for V-D_3, −12.46 kcal/mol; for cholesterol, the calculated minimum energy was −25.89 kcal/mol. The result showed that both V-D_2 and V-D_3 bind common amino acid residues 7–8, 11–12, and 15–16 of Aβ40, and the C22–C23 double bond in V-D_2 stacks with the benzene ring of Phe19 in Aβ40, whereas V-D_3 has no double bonds and showed no stacking (**Figure 5**) [49].

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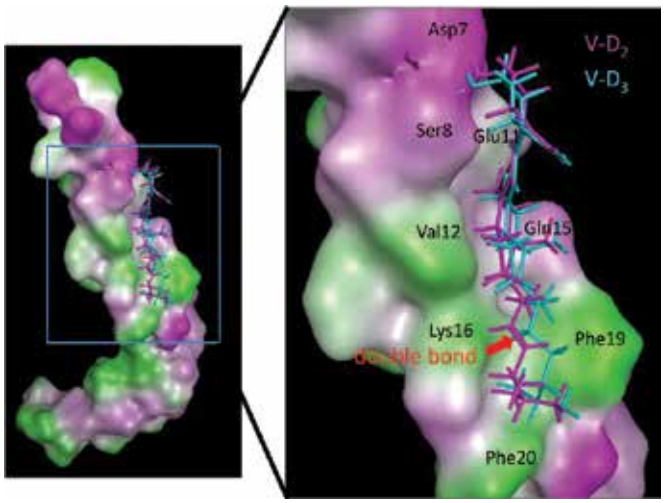

Figure 5. Docking simulation between Aβ40 and V-D_2 or V-D_3. Purple indicates hydrophilic residues and green indicates hydrophobic residues in Aβ40 (backbone). The double bonds (red arrow) of V-D_2 stack on the benzene ring of Phe19 in Aβ40. The minimum energy between Aβ40 and V-D_2 was −40.36 kcal/mol, that between Aβ40 and V-D_3 was −12.46 kcal/mol.

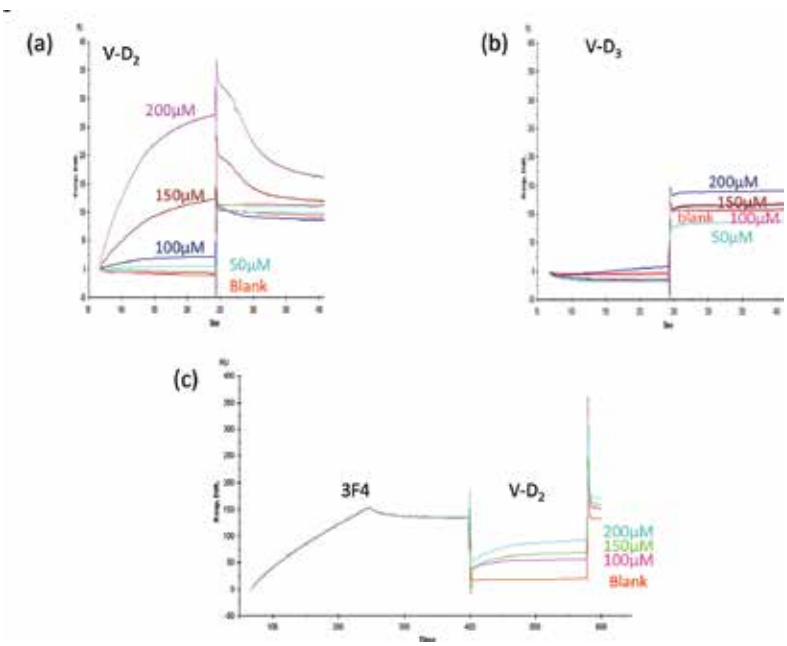

Figure 6. Affinity of V-D to PrP, as measured using the Biacore system. (a) The interaction between PrP^c(90–231) and V-D_2 showed high binding affinity, with a K_a of 6.17 e^8 and a K_d of 1.62 e^{-9}. (b) The interaction between PrP^c(90–231) and V-D_3 showed no binding affinity. (c) The interaction between PrP^c(90–231) and V-D_2, after saturating with the anti-3F4 mAb.

2.5. Affinity of V-D_2 to PrP^c(90–231), as a Biacore assay

A Biacore assay indicates a high affinity of V-D_2 for human recombinant cellular prion protein (Hu-rPrP^c)(90–231), and after saturating PrP^c(90–231) with the anti-3F4 antibody, specific for amino acid fragment 109–112 of PrP^c, V-D_2 binding to PrP^c(90–231) was decreased (**Figure 6a and c**), indicating that within the 3F4 epitope, PrP^c(90–231) was responsible for the interaction with V-D_2. However, V-D_3 showed no affinity for PrP^c(90–231) (**Figure 6b**), The binding kinetics of V-D_2 to Hu-rPrP^c(90–231) was shown in **Table 2** [52].

Ligand	Analyte	K_a (1/M)	K_d (M)
	V-D_2	6.17 e^8	1.62 e^{-9}
PrP^c(90–231)	V-D_3	ND*	ND*
	3F4 + V-D_2	1.12 e^8	8.95 e^{-9}

* ND, not detected.

Table 2. Binding kinetics of V-D_2 and V-D_3 to Hu-rPrP^c(90–231).

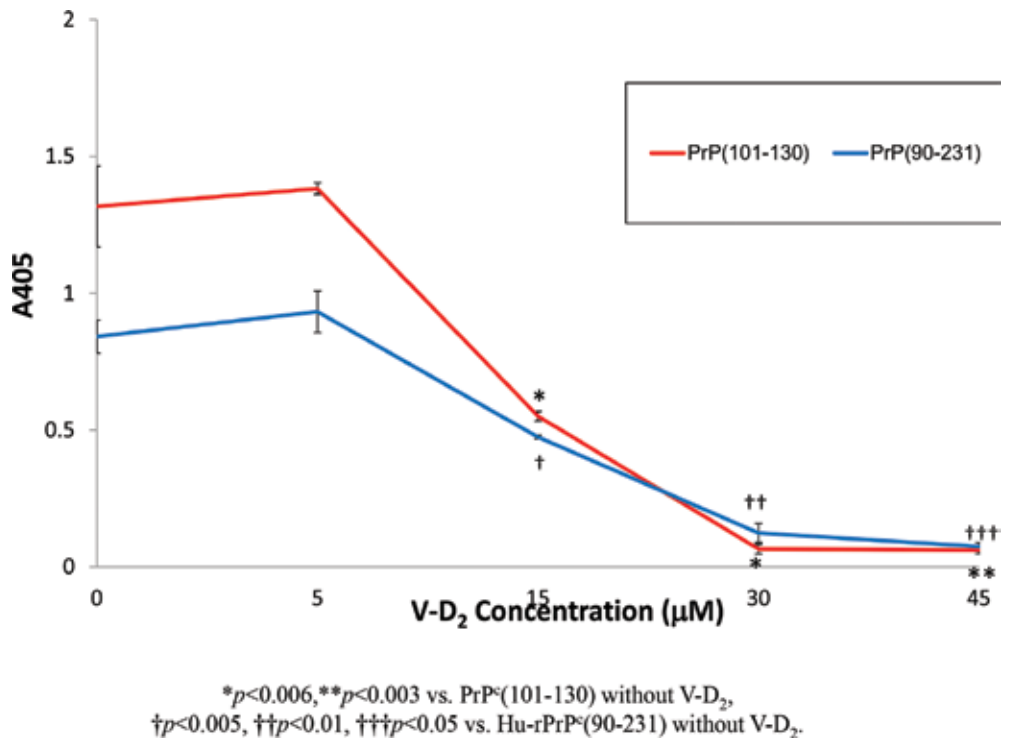

Figure 7. Reactivity of mAbs against PrP^c with V-D_2 by ELISA. The 3F4 epitope on PrP^c was affected by V-D_2 in a dose-dependent manner. The blue line indicates signals for PrP^c(90–231) and the red line f PrP^c(101–130).

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2.6. Reactivity of 3F4 antibody with Hu-rPrP^c(90–231) bound to V-D$_2$, as monitored by ELISA

The responsible fragment within Hu-rPrP^c(90–231) that was affected by V-D$_2$ was determined by ELISA. The reactivity of the 3F4 antibody to PrP^c(90–231) that was incubated with V-D$_2$ showed decreasing signals toward PrP^c(90–231) bound with V-D$_2$ in a dose-dependent manner (**Figure 7**) [52]. These results confirm the observation by Biacore assay (**Figure 6**).

2.7. Specific sequences of Aβ40 and PrP^c(90–231) responsible to conformational transition

The Aβ hydrophobic core 16–20 (KLVFF) was the minimum sequence required in a binding screen of full-length and trimeric to decameric peptides spanning the entire Aβ40 sequence. Alanine substitution demonstrated that Lys16, Leu17, and Phe20 are critical for this interaction [53]. The stereospecific binding of KLVFF to the homologous Aβ sequence was later confirmed as the product of specific hydrophobic and electrostatic interactions. Controlling amyloid β-peptide fibril formation with protease-stable ligands was reported [53]. The sequences of Aβ (9–14): GYEVHH and Aβ (17–21): LVFFA, are responsible to pH-shifts [54] and thermal-induced structural transformation from α-helix/random coil to β-sheet in Aβs were Aβ (16–23) and Aβ (17–24) [55]. The conformational transition from Aβ40 monomer to oligomers may occur in response to small chemical compounds and may be dependent on specific Aβ40 sequences. The key amino acid of Aβ40 for interaction with V-D$_2$ is the Phe at a position 19, and it is around the sequences responsible to pH shifts and thermal stress [54, 55].

In case of PrP^c(90–231), the sequence of PrP^c(107–112): TNMKHM is pH dependent [56], and it contains PrP^c(109–112), the responsible sequence for the interaction with V-D$_2$.

2.8. Structural difference of V-D$_2$ and V-D$_3$ could explain different potential for the affinity to Aβ40 and PrP^c(90–231)

The C22–C23 double bond contained in V-D$_2$ structure may influence the conformational flexibility of the molecule through allylic strain and rigidity of the double bond against rotation [57, 58]. Therefore, we hypothesize that conformational restriction by the double bond in the V-D$_2$ side chain facilitated binding of V-D$_2$ to the recognition site of Aβ40 and PrP^c(90–231).

3. Conclusion

We detected V-D$_2$-induced Aβ40 oligomerization by QCM, and electron microscopic observation demonstrated the potential of V-D$_2$ for Aβ40 oligomerization through β-sheet formation as revealed by Th-T study. V-D$_2$-mediated Aβ40 oligomerization occurs through interaction between the Phe19 benzene ring of Aβ40 and the C22–C23 double bond of V-D$_2$. In case of prion, the fragment of V-D$_2$ binding to PrP^c(90–231) is around 3F4 epitope, 109–112 amino acid in PrP^c(90–231). These fragments are involved in the sensitive fragments to pH shifts and thermal stress. The binding of V-D$_2$ to amyloidogenic peptides in brain might give some insights to oligomerization of these peptides in the brain.

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Vitamin D and Cancer

Chapter 6

Vitamin D and Colorectal Carcinogenesis

Argjira Juniku-Shkololli

Additional information is available at the end of the chapter

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Abstract

Colorectal cancer is the second leading cause of cancer-related death in the Western industrialized world. Many epidemiological studies have shown a negative association between colorectal cancer incidence and vitamin D levels. It has been suggested that the antitumoral action of $1,25(OH)_2D_3$ in colorectal cancer relies on several mechanisms at the cellular level. This prompted us to evaluate expression of certain immunohistochemical markers during tumor progression in colorectal human tissue and to study for the first time the relationship between histological type and grade of colorectal tumors with the expression of these markers. The investigated markers were the ones responsible for apoptosis (PAK1 and p53), cell adhesion (beta-catenin), differentiation (p53), and proliferation (Ki67). We also analyzed the correlation of their expression with vitamin D blood levels in these patients. Our results showed that the expression of these biomarkers increased with progression from colorectal adenomas to carcinomas. Expression of PAK1, beta-catenin, and p53 in the nucleus correlated with advanced stages of carcinoma. Low vitamin D blood levels correlated with nuclear accumulation of p53, nuclear beta-catenin expression, and expression of Ki67.

Keywords: vitamin D, colorectal cancer, beta-catenin, PAK1, p53, Ki67

1. Introduction

Colorectal cancer (CRC) is one of the commonest malignancies affecting both males and females. It is the second leading cause of cancer-related death in the Western industrialized world. The incidence of colorectal cancer increases with age, with nearly two-thirds of patients diagnosed aged over 65 years. Colorectal cancer (CRC) is the third most frequent tumor, which affects the inhabitants of developed and developing countries. Among males, CRC comes after lung and prostate tumors; among females it follows breast cancer,

occupying the second place in terms of incidence [1]. As a result of early detection of colonic polyps by screening and removal before they can develop into outright cancer, death rates have been dropping. In addition, screening and treatment for colorectal cancer at early stages have improved over the last several decades, resulting in increasing number of survivors of colorectal cancer.

Vitamin D is a secosteroid. Though it is named a vitamin, it is rather recognized as a prohormone given its synthesis in the skin and the multiple systemic actions of its metabolites [2]. In 1980, Garland brothers had suggested that vitamin D could be a protective factor against colorectal cancer, based on their observation of geographic distribution for colorectal cancer mortality in regions where population was less exposed to sunshine [3]. Few years later, the same authors confirmed this association, by reporting an inverse correlation between vitamin D status and CRC [4]. Different studies in the upcoming years [5–7] have also confirmed the relationship between plasma 25-hydroxyvitamin D concentrations and risk of colorectal cancer.

Many epidemiological studies have shown a negative association between colorectal cancer incidence and vitamin D levels [4, 7], as well as colorectal cancer risk and calcium intake [8, 9]. 1,25-Dihydroxyvitamin D3 directly affects growth factor and cytokine synthesis and signaling in colonic epithelium and modulates the cell cycle, apoptosis, and differentiation [10]. $1,25(OH)_2D_3$ exerts its biological effects by binding to the vitamin D receptor (VDR), thereby regulating gene expression. The active metabolite has prominent antiproliferative, anti-angiogenic, and pro-differentiating action in a wide range of tumor cells due to the VDR being expressed in almost all tissues. Several important cellular signaling pathways can thus be acted upon. However, for clinical trials, the problem remains of how to administer side effect-free doses of $1,25(OH)_2D_3$ [11].

A frequently measured range for serum levels of $25OHD_3$ in adults is 10–50 ng/ml. Intestinal calcium absorption is optimized at levels above 30–32 ng/ml. Parathyroid hormone levels start to rise at 25OHD levels below 30 ng/ml, marking vitamin D insufficiency [12]. Numerous investigations reported an increased risk for colorectal cancer in individuals with $25OHD_3$ blood levels below 12 ng/ml [13, 14].

In kidney cells, 25OHD is converted by the hydroxylase into the active metabolite $1,25(OH)_2D_3$. However, also other cell types, such as colonocytes, express vitamin D hydroxylases [15], indicating an autocrine/paracrine function of the active metabolite. Low serum $25OHD_3$ precursor levels could result in colonic $1,25(OH)_2D_3$ production that is insufficient for maintenance of autocrine/paracrine regulation of cellular growth and function [16].

It has been suggested that the antitumoral action of $1,25(OH)_2D_3$ in colorectal cancer relies on several mechanisms at the cellular level, such as inhibition of cell proliferation, sensitiveness to apoptosis, induction of epithelial differentiation, cell detoxification metabolism, inhibition of angiogenesis, and cell-cell adhesion [2]. This prompted us to evaluate immunohistochemical expression of beta-catenin and PAK1 (which are involved in Wnt-beta-catenin pathway) as well as expression of p53 and Ki67 (markers of apoptosis and cell proliferation, respectively) in colorectal polyps and cancer. We therefore studied for the first time the

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relationship between histological type and grade of colorectal tumors with the expression of these markers and attempted to correlate these results with vitamin D blood levels.

2. The role of vitamin D in CRC

2.1. Relevant data about CRC

Colorectal cancer develops through a multistage process of histopathologic and molecular changes, as a result of complex interactions between genetic predisposition and environmental factors. This type of cancer presents an ideal research model of carcinogenesis, since it progresses through multistep histopathologic changes and lesions of each stage are available for studying. Studies regarding colorectal carcinogenesis are focused on the genetic changes in three fundamental categories of genes: (1) tumor suppressor genes, (2) proto-oncogenes, and (3) DNA repair genes [17].

Comparative studies in human and animal samples have proved their similarity as organisms, regarding many pathological processes, including cancer, the same cell structure, and the same function of organ systems. Furthermore, in favor of this similarity of human and animal organisms, an additional argument would be using the same medicines both in humans and animals. Moreover, a range of surgical techniques and treatments such as transplantations and surgical techniques were established and sophisticated being applied firstly in animals. Many disease developments can be investigated in animal models in physiologically relevant conditions with humans. Colon carcinogenesis, as widely known, is a multifactorial process influenced by many interactive variables, making it difficult to determine an exactly specified mechanism. Using animal models as an investigation approach will make possible in vivo molecular, pathological, physiological, and anatomical possibilities to researchers of animals, giving us the favor to understand many disease features.

Mouse models have served as a basis for investigations in the field of colorectal cancer etiology as well as for mechanisms underlying the oncogenic process. On the other side, xenograft models are used to examine the response of the human tumor to a specific therapeutic regime. However, it is not clear if drug efficacy data obtained from xenograft models translate into clinically relevant treatment modalities.

2.1.1. Genetic changes during colorectal carcinogenesis

In colorectal cancer, it is the progressive accumulation of multiple genetic mutations, which results in the transition from normal mucosa to benign adenoma, to severe dysplasia, and to frank carcinoma [18]. More than two decades ago, Fearon and Vogelstein presented the model for the genetic basis of colorectal neoplasia, the adenoma-carcinoma sequence [19]. According to this genetic model, in most colorectal cancers, the primary event is an aberrant activation of APC/beta-catenin pathway, followed by RAS mutations and loss of function of p53 in later stages, while the total accumulation of changes, rather than their order, is responsible for

determining the tumor's biologic properties. Ten years later, it was concluded that mutations in all three genes happen in only 6.6% of colorectal cancers, indicating that these mutations lie on alternate pathways of colorectal tumor development; the heterogeneous pattern of tumor mutations suggests that multiple alternative genetic pathways to colorectal cancer exists [20].

2.1.2. Epigenetic changes in colorectal neoplasia

In the later years, colorectal cancer development is seen from a different point of view: genetic alterations represent only one piece of a complicated process [21–25]. Epigenetic changes in cancer-related genes and noncoding RNAs also have a function which contributes to the malignancy status [26, 27]. Factors that may induce epigenetic changes in colorectal neoplasia involve environmental, as well as inherited factors:

2.1.2.1. Environmental factors

The influence of environmental factors on epimutagens most of all includes dietary factors, and among them folate is the most investigated in connection to colorectal neoplasia. Folate is a donor of one-carbon units and therefore is important in methylation reactions and in DNA synthesis and repair [28]. According to epidemiological and experimental studies, dietary folate correlates inversely with the risk of colorectal neoplasia [29–31]. However, the effect of folate intake on tumorigenesis is very complex and depends on the stage of tumor development [10, 32].

Advancing age also correlates closely with epigenetic changes in normal colorectal mucosa, where methylation of certain genes, including the ESR1 [33, 34], MLH1 [35], HIC1, and IGF2 [36], has been shown to increase progressively with age. This process seems to be accelerated in patients with colorectal cancer, for at least some of these genes [37, 38].

2.1.2.2. Inherited factors

Given that epigenetic changes are stable and potentially heritable through meiosis, some of the ways in which inheritance may influence epigenetic changes associated with colorectal neoplasia should be considered [28]. Groups of authors have reported germline epimutations in MLH1, which creates predisposition to young-onset MSI tumors in colon and at extracolonic sites [39, 40]. Germline epimutation provides a mechanism for phenocopying of genetic disease [39] and causes transcriptional silencing of the affected allele [40]. According to these observations, germline epigenetic changes can mimic hereditary cancer syndromes and may be inherited [41, 42]. The timing when these changes occur, as well as the combination of genetic and epigenetic events, rather than their merely accumulation, gives selective priority to the cancer cells, resulting in activation of certain pathways [43].

2.1.3. Clinical relevance of epigenetic changes in colorectal cancer

Increasing recognition of epigenetic changes in the histologically normal colorectal mucosa and in precursor lesions can make these changes serve as a marker for patients at risk for colorectal cancer [33]. Epigenetic markers are progressively finding their place in screening

tests for colorectal neoplasia [44]. Regarding the impact that epigenetic changes may have in colorectal cancer treatment decisions, there is a growing evidence that MSI tumors respond differently to traditional chemotherapeutic agents [45, 46]. Moreover, the outcomes for some patients with these cancers may be worse with standard treatments [47].

It was observed that low folate status predisposes to development of several malignancies including colorectal cancer [48]. Experimental studies confirmed that older age and inadequate folate intake are strongly implicated as important risk factors for colon cancer and each is associated with altered DNA methylation [49]. Keyes and colleagues concluded that folate supplementation, which can enhance methyl availability, increases both genomic DNA methylation and *p16* promoter methylation in old mice; this epigenetic change by aging and dietary folate affects the expression of *p16*, a critical gene for both aging and carcinogenesis [49]. Methylation/demethylation processes in promoter sequences of vitamin D hydroxylases may lead to reduced or enhanced expression of these enzymes, respectively [50] (see Section 2.3.3.).

Numerous studies have provided evidence that women may be better protected against colorectal cancer than men. Several investigations have reported a lowered colorectal cancer risk associated with enhanced phytoestrogen intake (see, e.g., [51]). Phytoestrogens resemble structurally and act functionally as estrogen agonists; their affinity for ER-β is higher than that of estradiol itself and is lower for ER-α [52]. Therefore, phytoestrogens can act as estrogen agonists or antagonists depending on the type of estrogen receptors available, as well as on the level of endogenous circulating hormones [53, 54]. In Asian countries, populations with high consumption of soy foods (which are very rich in phytoestrogens) have a clearly reduced risk of colorectal cancer incidence. The major phytoestrogen in soy—genistein—beside other mechanisms (see Section 2.3.4.) is also involved in regulation of gene activity by modulating epigenetic events such as DNA methylation and/or histone acetylation.

2.2. Vitamin D: general concepts

Bound to the vitamin D receptor (VDR), $1,25(OH)_2D_3$ not only regulates calcium and phosphate metabolism but also exerts a wide range of non-calcemic biological effects, the most important of which are suppressing hyperproliferative growth and supporting cell differentiation [16]. When Zehnder and coworkers demonstrated that many types of cells were positive for CYP27B1, it was recognized that there is a widespread potential for extrarenal synthesis of $1,25(OH)_2D_3$ [55]. Further, Cross and coworkers demonstrated the synthesis and degradation of $1,25(OH)_2D_3$ by high-pressure liquid chromatography [56], and it was accepted that vitamin D could have alternative roles in extrarenal tissues, for instance, in the colon.

2.2.1. VDR in normal and in malignant colon cells

Vitamin D actions as a steroid hormone are mediated through the vitamin D receptor (VDR) [57], which is a high affinity ligand-activated transcription factor [58]. Activated VDR heterodimerizes with the retinoid X receptor (RXR); this complex binds to the vitamin D response elements (VDREs) in the promoter of target genes and recruits coactivators and corepressors to induce or inhibit gene transcription [59]. The expression and functionality of the VDR are

mandatory for the anticancer effects of vitamin D; therefore, the loss of this transcription factor, as seen in some cells after malignant transformation, results in calcitriol resistance [58].

An important step toward understanding of the complexity of vitamin D interactions was recognition of the fact that VDR is also expressed in tissues other than those responsible for calcium and phosphorus metabolism. The presence of VDR in malignant cells suggested that regulation of cancerous cell functions could be another target of 1,25-$(OH)_2D_3$ and provided the biological basis for many epidemiological studies. Shabahang et al. reported that the more differentiated is the cell line, the expression of VDR is higher [60]. Cross and coworkers found that expression of the VDR increases during transition from normal mucosa to polyps, as well as in the course of progression from adenomas to well-differentiated and moderately differentiated tumors, and then declines during further progression [61]. This suggests that cancerous colon cells express VDR until they reach a certain level of differentiation. Such model of regulation suggests existence of a physiologic protective mechanism of tumor progression, which is reduced in the later stages. Investigations by Evans and coworkers found that in colorectal cancer, a high level of VDR expression was associated with a favorable prognosis [62].

VDR can function as a receptor for secondary bile acid, lithocholic acid, which is a hepatotoxic compound and a potential enteric carcinogenic [63]. Binding of both lithocholic acid and vitamin D to the VDR results in induction of CYP3A, the enzyme that detoxifies lithocholic acid in the liver and intestine [7, 64].

2.2.2. Expression of VDR, CYP27B1, CYP27A1, and CYP24 as a function of malignant transformation in the colon

Cross et al. [56] were the first to demonstrate the conversion of the precursor 25 OH D_3 into $1,25(OH)_2D_3$ in Caco-2 cells by finding constitutive expression of the CYP27B1 in almost every growth phase of this cell line and the sequential metabolism of the secosteroid along the C-24 and C-23 oxidative pathways. The authors concluded that human colon cells can control their growth through $1,25(OH)_2D_3$ in an autocrine/paracrine manner dependent upon the presence of the vitamin D receptor [56]. Cross and coworkers were also the first to demonstrate that expression of CYP27B1 and of the VDR rises (approximately fourfold) in the course of progression from adenomas to well-differentiated and moderately differentiated (G1 and G2) tumors and then substantially declines during further progression [65]. Other authors also reported that in hyperproliferative, premalignant colon cells, expression of CYP27B1 (synthesizing hydroxylase) is increased, as well as VDR expression, while in high-grade colon tumors, CYP27B1 expression is again repressed (in contrast with CYP24A1—degrading hydroxylase expression) [15]. According to this, the extrarenal synthesis of $1,25(OH)_2D_3$ provides a physiological mechanism for prevention of malignant growth.

Holt and coworkers [66] demonstrated for the first time that rectal crypt proliferation correlated inversely with serum $1,25(OH)_2D_3$ levels. These data support the abovementioned hypothesis that colon cancer cells posses an intrinsic physiological defense which can prevent hyperproliferation and progression into malignancy [56]. In colon tissue derived from a large patient cohort, VDR and CYP27B1 mRNA expression was low in normal tissue but rose

early during colon tumor progression [65, 67]. This system fails in the late stage, high-grade colon cancer, while there is increased expression of CYP24, which could cause rapid catabolism of 1,25$(OH)_2D_3$ at the tumor site, counteracting its local inhibition of tumor growth [68]. Physiologic regulation of vitamin D hydroxylases in normal and malignant human colonic tissue suggests a role for the locally accumulated hormone in prevention of tumor progression; during low-grade early-stage malignancy, colonic synthesis of 1,25$(OH)_2D_3$ could potentially provide a block to progression, if its catabolism could have inhibited [69].

2.2.3. Epigenetic regulation of vitamin D hydroxylases

Expression of CYP27B1 and CYP24A during colorectal cancer progression is under epigenetic control [16]. Differences in expression of vitamin D hydroxylases during tumor progression, as seen in colorectal cancer patients [65, 68], could be caused by epigenetic regulation of gene activity through methylation/demethylation processes, as well as histone acetylation/deacetylation [16]. DNA methylation at CpG islands in the promoter region of genes is associated with transcriptional silencing of gene expression in mammalians, whereas reduction of methylation in CpG islands results in increase of gene activity.

In low-grade cancer, CYP27B expression is very high, comparing with its expression in colorectal mucosa of non-tumor patients [15, 65]. Increased synthesis of 1,25$(OH)_2D_3$ in colon mucosa could be responsible for higher transcriptional activity of CYP24A1, as well as for autocrine/paracrine inhibition pf tumor cell growth. If transcriptional repression of CYP24A1 expression could be affected by methylating agents, advanced colorectal cancer patients could also benefit from treatment with vitamin D substances [10].

2.2.4. The antitumoral action of 1,25$(OH)_2D_3$ in different tissue types

Numerous observations have indicated a much broader range of action for 1.25$(OH)_2D_3$, including the regulation of cell differentiation, proliferation, apoptosis, invasion, and angiogenesis in several types of tumor cells and animal models of cancer [70–72]. 1-Alpha,25$(OH)_2D_3$, also known as calcitriol, as well as vitamin D analogs, might have potential as anticancer agents because their administration has antiproliferative effects, can activate apoptotic pathways, and inhibit angiogenesis [70]. A general deregulation of the vitamin D system was observed in most malignancies. Evidence for an inverse correlation between serum vitamin D_3 status and cancer incidence, e.g., the colon, breast, and prostate, has increased over the last years [73]. Anticancer property of vitamin D has been studied in a wide variety of commonly occurring cancers (both in vitro and in vivo) of which the actions on colorectal, breast, and prostate cancers have been found to be most promising [74]. In 16 types of cancer, there is an evidence of a beneficial role of vitamin D (gastrointestinal: colon, esophageal, gallbladder, gastric, pancreatic, and rectal; urogenital: bladder, kidney, and prostate; female: breast, ovarian, and vulvar cancer; and blood cancers: Hodgkin's lymphoma, non-Hodgkin's lymphoma, and multiple myeloma) [75]. A strong association between a low vitamin D status and cancer incidence or mortality has been reported for colon, rectal, breast, prostate, and ovarian cancer [76]. However, data regarding the role of vitamin D in cancer risk, incidence, and mortality are still controversial [58].

2.3. Mechanisms of colorectal cancer: vitamin D interaction

The molecular basis of the idea that vitamin D has the potential to prevent cancer lies in its role as a nuclear transcription factor, which regulates cell growth, differentiation, apoptosis, and many cellular mechanisms, with a key role for cancer development.

2.3.1. Effects of vitamin D on cell proliferation, differentiation, apoptosis, and angiogenesis

Since the 1980, 1,25-OHD_3 has been recognized as a potent cellular antiproliferative and pro-differentiating agent in the colon [69]. During the last two decades, vitamin D was proven as a potential inhibitor of cell growth and angiogenesis, as well as a stimulator of cell maturation and apoptosis [70]. The classic signaling pathway is through the vitamin D nuclear receptor (VDR), which is a transcription factor.

Over recent years there has been an expanding consideration for non-calcemic functions of $1,25(OH)_2D_3$, including its antitumoral effects [73]. There is an increasing evidence about inverse correlation of vitamin D status and colorectal, breast, and prostate cancer [6, 75, 77]. Moreover, many observational studies have reported various actions of $1,25(OH)_2D_3$, as regulation of cell differentiation, proliferation, apoptosis, cell invasion, and angiogenesis; vitamin D exerts its actions mainly via its high affinity receptor VDR through a complex network of genomic (transcriptional and posttranscriptional) and also nongenomic mechanisms, which are partially coincident in the different cells and tissues studied [70–72].

Angiogenesis is an essential process for growth of solid tumors; therefore, anti-angiogenic agents contribute to the anticancer treatment. Anti-angiogenic drugs can cause inhibition of tumor progression, stabilization of tumor growth, and regression of tumor mass and prevent metastases. Experimental studies have reported anti-angiogenic action of $1,25(OH)_2D_3$, as well as his analog, 22-oxacalcitriol [78]. Today, there is a growing evidence that high vitamin D intake and a plasma level of 25(OH) D_3 reduce the incidence of colorectal cancer by modifying cancer angiogenesis, cell apoptosis, differentiation, and proliferation. Results from Kang et al. [79] suggest that vitamin D supplementation alone, or in combination with anticancer agents, might reduce the incidence of colorectal cancer.

Vitamin D also increases the level of cystatin D—an endogenous protein, which shows antitumor and antimetastatic property, by facilitating the expression of the gene coding for it [74].

Induction of apoptosis is a mechanism by which $1,25(OH)_2D_3$ inhibits tumor cell growth and may contribute to tumor suppression and explain the reduction in tumor volume found in various in vivo animal studies [63]. Besides its physiological function in maintaining constancy of cell numbers in different tissues, apoptosis also prevents the possibility of mutational changes leading to malignancy after DNA damage by removal of such damaged cells [74]. $1,25(OH)_2D_3$ is able to modulate apoptosis mediators by diverse mechanisms that favor the elimination of malignant cells [58]. Apoptosis sensitization by vitamin D in colorectal adenoma and carcinoma cells involves the upregulation of the proapoptotic proteins and the downregulation of anti-apoptotic proteins. The proapoptotic BAX component of the Bcl-2 competes with the antiapoptotic Bcl-2 on mitochondrial cell surface for release of cytochrome c from it; the proapoptotic

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branch tends to stimulate the release, while the antiapoptotic branch inhibits it. In normal cells, survival factors continuously oppose apoptosis by several mechanisms, out of which activation of antiapoptotic Bcl-2 is important. Proapoptotic compounds may have a favorable role in the prevention of cancer development, growth, and metastasis while aiding to its chemotherapy [74]. Vitamin D may also induce cell death by alternative pathways, such as increasing the calcium concentration, releasing cytochrome c and reducing intracellular glutathione [58].

Our study summarizes changes in the expression of certain immunohistochemical markers during tumor progression in colorectal human tissue. We have investigated markers responsible for apoptosis (PAK1 and p53), cell adhesion (beta-catenin), differentiation (p53), and proliferation (Ki67), as well as correlation of their expression with vitamin D blood levels [80].

In our study, average vitamin D blood level in patients with colorectal cancer was 5.99 ng/ml (range: 3–23.04 ng/ml), whereas average vitamin D blood level in patients with colorectal adenoma was much higher, i.e., 21.4 ng/ml (range: 11.3–30.66 ng/ml). The difference was statistically highly significant ($p = 0.0001$). Interestingly, among our patients with adenocarcinoma, only one had a vitamin D blood level in a normal range (23.04 ng/ml), while only one adenoma patient approached vitamin D deficiency (11.3 ng/ml).

PAKs are a family of serine/threonine protein kinases with six isoforms (PAK1-6), which play important roles in cytoskeletal dynamics, cell survival, and proliferation [81]. Although PAKs are not mutated in cancerogenesis, they are overexpressed, hyperactivated, or amplified in several human tumors. PAK1 has been reported to be overexpressed in colorectal cancer, but its role in CRC remains unclear [82]. Some recent studies have implicated PAK's role in activation of Wnt-beta-catenin signaling through direct interaction and phosphorylation of beta-catenin (see, e.g., [83]). In colorectal cancer, nuclear PAK1 is associated with advanced tumor stage. In adenocarcinomas, overall PAK1 (nuclear and cytoplasmic staining) was expressed in 80% of cases. Nuclear PAK1 expression was negative in all adenomas, as well as in grade I adenocarcinomas. In grade II adenocarcinomas, nuclear PAK1 was expressed in 20% of patients, while in grade III adenocarcinomas, it was expressed in 50% of cases (**Figure 1**). Our results of PAK1 expression were similar to those from Ye [81] and Zhu et al. [84], who found PAK1 expression in 70% of cells. Correlations with tumor grade and stage, as well as with the nodal status [85], indicate PAK1 as a very important marker during colorectal tumor progression.

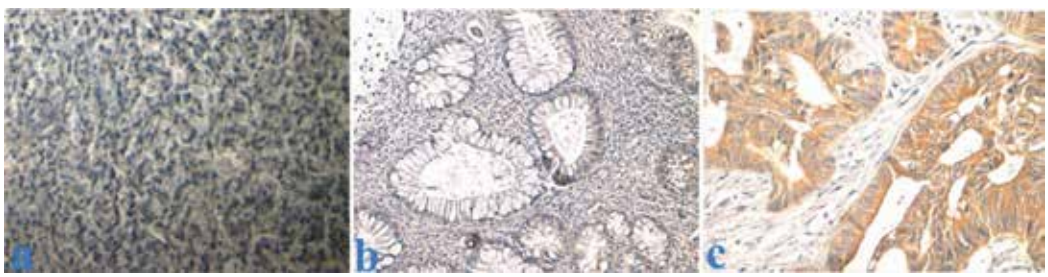

Figure 1. PAK1 expression in (a) colorectal mucinous adenocarcinoma (nuclear expression), (b) colorectal polyp (no expression), and (c) colorectal non-mucinous adenocarcinoma (cytoplasmic expression).

Numerous studies have focused on the clinical relevance of nuclear beta-catenin accumulation during colorectal pathogenesis, demonstrating its diagnostic, as well as prognostic significance [86]. A high density of beta-catenin nuclear accumulation was associated with higher mortality in selected groups of patients with colorectal cancer [87]. Under normal circumstances, beta-catenin is part of a complex of proteins that constitute adherens junctions necessary for creation and maintenance of epithelial cell layers, but the gene that codes for beta-catenin can function as an oncogene as well. When beta-catenin binds to the product of the mutated APC gene, free cytoplasmic beta-catenin is destabilized. This leads to the accumulation of nuclear beta-catenin, where it acts as a transcriptional activator of genes, specific for tumor formation [88]. Aberrant activation of the Wnt/beta-catenin signaling pathway due to mutation of adenomatous polyposis coli (APC), of beta-catenin or AXIN genes, is the most common and initial alteration in sporadic colorectal tumors [89]. It is significant that reduction of beta-catenin transcriptional activity is known to be mediated by $1,25(OH)_2D_3$ and is accompanied by the export of nuclear beta-catenin and its relocalization to the plasma membrane [90]. Our study showed a significant increase in beta-catenin nuclear expression during progression from adenomas to non-mucinous adenocarcinomas, as well as from non-mucinous adenocarcinomas to mucinous adenocarcinomas (**Figure 2**).

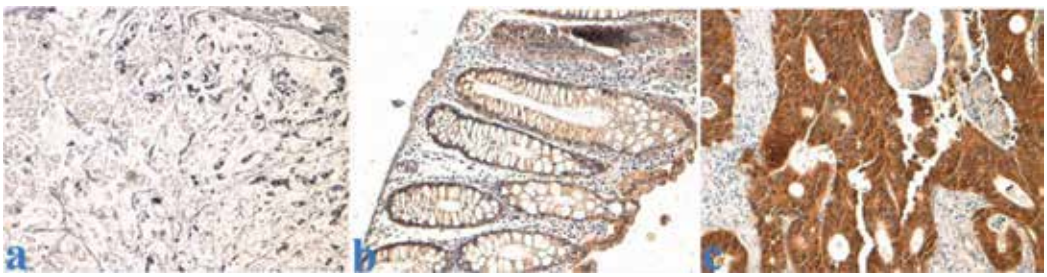

Figure 2. Beta-catenin expression in (a) mucinous adenocarcinoma (nuclear expression), (b) adenomatous polyp (membranous expression), and (c) non-mucinous adenocarcinoma (cytoplasmic expression).

Normal colorectal mucosa (tumor margins) expressed membranous beta-catenin staining and was used as internal positive control (**Figure 3**). In contrast to results of some other authors [91], we found beta-catenin overexpression to be most pronounced in mucinous adenocarcinomas. Nuclear beta-catenin expression correlated with both tumor grade and stage. Cytoplasmic beta-catenin expression was present in most of polyps and in all non-mucinous adenocarcinomas, whereas staining was positive in only 25% of mucinous adenocarcinomas. Cytoplasmic beta-catenin expression showed an association with better differentiation. This observation correlates with results from other authors. The presence of beta-catenin expression in the membrane and cytoplasm at an early tumor stage, and nuclear expression at advancing stages, illustrates the sequence of genetic mutations in normal epithelium developing into colorectal tumors.

p53 is a nuclear protein that induces cell cycle arrest or apoptosis in response to DNA damage. Its mutations are frequently associated with colorectal oncogenesis. The wild type of the p53 gene product has a short half-life and is not detectable by IHC. In contrast, mutant

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p53 protein has a much longer half-life, accumulates in the nucleus, and creates a stable target for IHC detection [92]. Frequency of p53 expression in our study correlates well with the frequency of p53 mutations found in studies using sequencing techniques for identification of p53 mutations in sporadic colorectal cancer [93, 94]. We detected nuclear p53 expression in 53% of adenocarcinoma patients, while cytoplasmic expression was present in 33% (**Figure 4**). In other studies the frequency of p53 staining ranges from 45 to 60% [95]. Nuclear p53 expression was increasing in parallel to tumor grade: in grade III adenocarcinomas, p53 was expressed in all cases, in grade II adenocarcinomas, p53 was expressed in 50% of cases, and in grade I adenocarcinomas, nuclear p53 was expressed in 33% of cases. In the group with colorectal adenomas, only one patient had nuclear p53 expression.

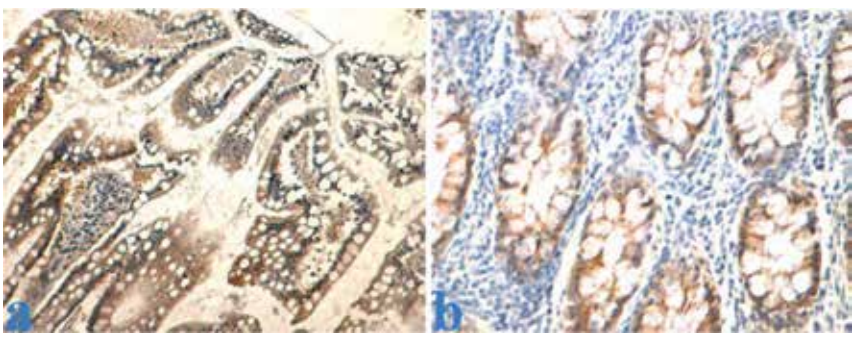

Figure 3. Membranous expression of beta-catenin in the margins of colorectal carcinoma: (a) mucinous adenocarcinoma and (b) non-mucinous adenocarcinoma.

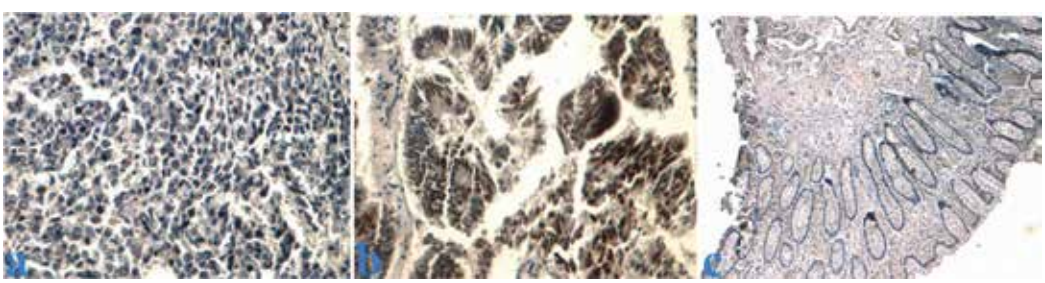

Figure 4. p53 expression in (a) mucinous adenocarcinoma (nuclear expression), (b) non-mucinous adenocarcinoma (nuclear and cytoplasmic expression), and (c) adenomatous polyp (no expression).

Ki67 is a nuclear nonhistone protein that is present at low levels in quiescent cells but is increased in proliferating cells, especially during G2, M, and the latter half of the S phase. Thus, Ki67 reactivity, defined as percentage of tumor cells staining positive in IHC staining, is a specific nuclear marker for cell proliferation. While the growth of malignant tumors is highly variable (although it might reflect their clinical course), proliferation still is a key feature of tumor progression. In our study, the mean Ki67 expression in all colorectal adenocarcinomas

was 47%. This is similar to those of Georgescu et al. (48%) [96] and Oshima et al. (44%) [97]. The proliferative activity as measured by Ki67 antibody was related to histological type and grade: Ki67 expression was higher in non-mucinous adenocarcinomas, compared with mucinous adenocarcinomas ($p = 0.0164$) (**Figure 5a** and **b**).

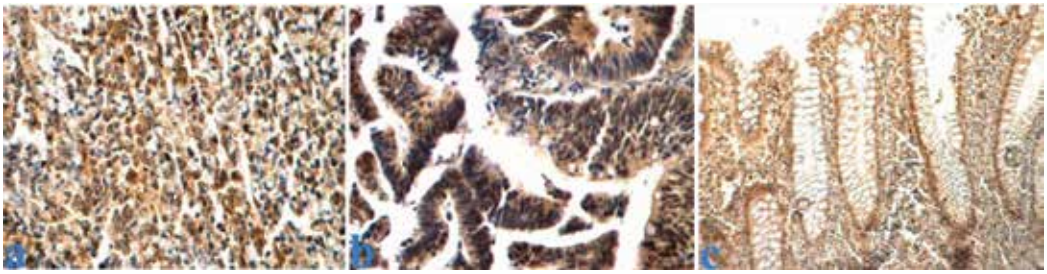

Figure 5. Ki67 expression in (a) mucinous adenocarcinoma (25%), (b) non-mucinous adenocarcinoma (70%), and (c) adenomatous polyp (10%).

Ki67 expression was high in well-differentiated (G1) and moderately differentiated (G2) adenocarcinomas (52%), compared with poorly differentiated (G3) adenocarcinomas (17%) ($p = 0.0314$). In colorectal adenomas, Ki67 expression was very low (16%) (**Figure 5c**). This indicates a low level of proliferative activity in these lesions. While our results are similar to those published by Nabi et al. [98], Georgescu et al. observed that Ki67 expression was higher in poorly differentiated (57%) than in moderately differentiated (34%) and well differentiated (20%) adenocarcinomas [96]. Correlation of Ki67 expression with the histological type of adenocarcinoma resulted in 26% in mucinous versus 55% in non-mucinous colorectal adenocarcinomas and was similar to that reported by Nabi et al. [98]. This suggests that proliferative activity in mucinous adenocarcinomas is lower than in non-mucinous adenocarcinomas.

Taken together, our data show that expression of these biomarkers increased with progression through the adenoma to carcinoma sequence. Accumulation of PAK1, beta-catenin, and p53 in the nucleus revealed correlation with advanced stages. Ki67 expression, however, was higher in well-differentiated than in poorly differentiated carcinomas.

Serum vitamin D levels in patients with positive nuclear PAK1 and beta-catenin expression had a negative trend, while patients with positive nuclear p53 expression had significantly lower vitamin D blood levels. Vitamin D levels were the lowest in mucinous adenocarcinoma and correlated with nuclear accumulation of p53, nuclear beta-catenin expression, and higher expression of Ki67. Considering the importance of an adequate $25OHD_3$ supply for synthesis of the active metabolite $1,25(OH)_2D_3$ in colon mucosal cells and the relevance of the latter for regulation of the Wnt/beta-catenin pathway, $25OHD_3$ blood levels could be considered as an indicator of Wnt/beta-catenin activity and increased cell proliferation [80]. This further emphasizes the chemopreventive role of vitamin D in CRC.

Garland et al. [99] in the early 1999 had suggested that ingestion of at least 800 IU (20 μg) of vitamin D, together with 1800 mg of calcium, is needed to significantly lower the incidence

and mortality of colorectal cancer. Matusiak and Benya concluded that nontoxic vitamin D precursors should be sufficient for CRC chemoprevention, but that neither vitamin D nor its precursors may be sufficient for CRC chemotherapy [100].

2.3.2. The expression of VDR and CYP27B1 in carcinogenesis and at the time of Vitamin D therapy

Sufficient blood levels of vitamin D provide a substrate for increasing the amount of vitamin D in the colonocytes, due to the presence of VDR and CYP27B1. Cross et al. [65] had shown (by RT-PCR, as well as by Western blotting and immunohistochemical methods) that in human large intestinal carcinomas expression of the genes encoding the 25-(OH)D_3-1α-hydroxylase (CYP27B1), as well as the VDR, increases in parallel with ongoing dedifferentiation in the early phase of carcinogenesis, whereas in poorly differentiated late-stage carcinomas, only low levels of the respective mRNAs can be detected [65]. According to this finding, colorectal cancer cells are able to increase their autocrine counter-regulatory response to neoplastic cell growth, through upregulation of the vitamin D/VDR system which mediates the antimitotic effects of the steroid hormone.

Expression of VDR, CYP27B1, and CYP24 determines the efficacy of the antimitotic action of 1,25-D3 and is distinctly related to the degree of differentiation of cancerous lesions. Bareis et al. [67] addressed the question of whether the effects of 1,25-D3 on VDR, CYP27B1, and CYP24 gene expression in human colon carcinoma cell lines also depend on the degree of cellular differentiation [67]. They showed that slowly dividing, highly differentiated Caco-2/15 cells responded in a dose-dependent manner to 1,25-D3 by upregulation of VDR and CYP27B1 expression, whereas in highly proliferative, less differentiated cell lines, such as Caco-2/AQ and COGA-1A and COGA-1E, negative regulation was observed. From the observed clonal differences in the regulatory effects of 1,25-D3 on VDR and CYP27B1 gene expression, the authors suggest that VDR-mediated growth inhibition by 1,25-D3 would be efficient only in highly differentiated carcinomas.

Bises et al. double stained colon tumors for CYP27B1 and VDR [15]; they found CYP27B1-enhanced expression in high- to medium-differentiated human colon tumors compared with tumor-adjacent normal mucosa or with colon mucosa from non-cancer patients. In high-grade undifferentiated tumor areas, expression was lost. Most colonocytes expressed both CYP27B1 and VDR, while some of them were positive only for VDR; this suggests that 1,25-D3 synthesized in colonocytes and bound to its receptor could exert its antimitotic function in both an autocrine and a paracrine fashion [15].

Because (as Matusiak and Benya [100] had shown) CYP27B1 is present in colonic epithelia, D3 alone may be sufficient for CRC chemoprevention and/or chemotherapy, providing that CYP27A1 and CYP24 are present in appropriate quantities and cellular compartments. To determine whether cholecalciferol may be useful for CRC chemoprevention and/or chemotherapy, Matusiak et al. reported on cellular CYP27A1 and CYP24 protein expression in human ACFs, polyps, and CRCs of defined differentiation along with associated lymph node metastases. Their findings suggest that cholecalciferol has potential for use in CRC chemoprevention but may be less efficacious for CRC chemotherapy.

2.3.3. Interactive role of calcium and vitamin D in colorectal prevention

Combined supplementation is required for optimal chemoprevention of cancer by calcium and vitamin D [76]. An interaction between nutritional calcium and vitamin D in protection against colorectal cancer may be due to the ability of luminal calcium to suppress degradation of $1,25(OH)_2D_3$ synthesized in colonocytes [101]. Calcium may also directly reduce hyperproliferation of the colonic mucosa [102] by binding to the calcium-sensing receptor (CaR) and by activating antimitotic, proapoptotic signal transduction mechanisms [103–105]. The effects of vitamin D and calcium on growth and differentiation of many epithelial cancers may be explained, in part, by their ability to control expression of E-cadherin and to regulate Wnt pathway [106]. Calcium regulates Wnt signaling through the calcium-sensing receptor as well: activation of the CaR enhances E-cadherin expression and suppresses TCF4 expression [107, 108]. It also stimulates secretion of Wnt5a, which inhibits beta-catenin signaling by increasing expression of an ubiquitin ligase that is involved in degradation of beta-catenin [109].

Several studies implied that the beneficial effect of calcium supplementation was due to the ability of calcium to form insoluble salts with potentially irritating and ultimately tumorigenic bile acids [110]. However, bile acids also interact with the vitamin D system: LCA is able to bind the VDR, thereby stimulating expression of CYP24A1 [111] and reducing availability of locally synthesized colonic $1,25(OH)_2D_3$. Bile acids are able to induce aberrant crypt foci, the precursors of neoplastic transformation in the colon; LCA activates the pregnane X receptor (PXR) and induces CYP3A expression [10]. In addition, bile acids interact with the vitamin D system: lithocholic acid can bind to the vitamin D receptor (VDR) and induce CYP24A1 expression [111].

Ingestion of considerable amounts of calcium and soy (phytoestrogens—see Section 2.3.4.) could provide accumulation of $1,25\text{-}OH_2D_3$ in colon, by enhancing the expression of synthesizing hydroxylase (CYP27B1) and reducing of the catabolizing hydroxylase (CYP24A). This is not the case with the renal vitamin D hydroxylases.

2.3.4. Role of folates in modulating the risk of colorectal cancer

As a vitamin of the B family, folate is essential for synthesis, repair, and methylation of DNA, while as a methyl donor, folate could play an important role in epigenetic regulation of gene expression [112].

The evidence from epidemiologic, animal, and human studies strongly suggests that folate status modulates the risk of developing cancers in selected tissues, the most notable of which is the colorectum [48]. Dietary folate influences DNA methylation, synthesis, and repair, and aberrations in these DNA processes may enhance carcinogenesis, particularly in rapidly proliferative tissues such as the colorectal mucosa [113]. Folates serve as physiological methyl donors during nucleotide precursor biosynthesis as well as during DNA, RNA, and protein methylation. Therefore, changes in folate metabolism have impact on two important determinants of carcinogenesis: on genetic expression and on maintenance of DNA integrity and stability. Folate deficiency affects the expression of key genes that are related to cell cycle control, DNA repair, apoptosis, and angiogenesis in a cell-specific manner [114]. Folate status affects

several cancer-related pathways including the p53 pathway, the Rb pathway, and the APC/*Wnt* pathway, as well as pathways involved in cell adhesion and cell migration and invasion [115].

Experimental results from Cross and coworkers suggest that, at least in mice, a "Western diet" resembling the high fat, low vitamin D, and calcium diet causes degradation of colonic $1,25(OH)D_3$, which can be stopped only by folate optimization [101]. The authors also concluded that folate optimization overrides the negative effects of low vitamin D and calcium intake [101]. (Regarding epigenetic regulation of vitamin D hydroxylases, see Section 2.2.3.)

According to results from Kim [116], dose and timing of folate intervention are critical in providing safe and effective chemoprevention; exceptionally high supplemental folate levels and folate intervention after microscopic neoplastic foci are established in the colorectal mucosa and promote, rather than suppress, colorectal carcinogenesis. Therefore, a "dual-modulator" role was suggested for folate in colorectal cancer, with a protective influence when ingesting only moderate amounts before development of aberrant crypt foci [117].

Extrarenal actions of $1,25(OH)_2D_3$, such as regulation of cell differentiation, proliferation, apoptosis, invasion, and angiogenesis in several types of tumor cells [70–72] suggest its potential therapeutic role against cancer. Nevertheless, the use of $1,25(OH)_2D_3$ is restricted by its hypercalcemic effect at therapeutic doses; this can be putatively overcome by the use of analogs that retain the antitumoral action but have less calcemic effect [2]. Numerous studies have shown that $1,25(OH)_2D_3$ and several analogs clearly reduce the growth of colorectal xenografts [70, 71].

In order to prevent premalignancies as well as their progression to tumors, we should focus on increasing the efficiency of the vitamin D system [16]. Especially in the colon, this can be accomplished by consuming calcium, soy, and folate [10]. Dietary modulation (using calcium, folate, and phytoestrogens) of extrarenal $1,25(OH)_2D_3$ synthesis in organs that are potentially prone to tumor incidence could lead to improved apoptotic and antimitotic activity by locally enhancing the concentration of $1,25(OH)_2D_3$ in these tissues, thereby preventing tumor cell growth [16]. A low folate status predisposes to development of several common malignancies including colorectal cancer [48]. Giovannucci et al. [30] demonstrated that prolonged intake of folate above currently recommended levels significantly reduced the risk of colorectal cancer [30].

Differences observed in the expression of vitamin D hydroxylases in patients with colon cancer during the course of tumor progression could be caused by the epigenetic regulation of gene activity via methylation/demethylation processes as well as by histone acetylation/deacetylation [16]. Methylation/demethylation processes (i.e., epigenetic regulation) in promoter sequences of vitamin D hydroxylases may lead to reduced, respectively, enhanced expression of these enzymes [50]. CYP27B1 expression is exceedingly high in low-grade cancerous lesions, compared with its expression in normal colonic mucosa of non-cancer patients [15]. Enhanced synthesis and accumulation of $1,25(OH)_2D_3$ in colonic mucosa could be responsible for the upregulation of transcriptional activity of CYP24A1 and also for the autocrine/paracrine inhibition of tumor cell growth [65]. Cross et al. [16] suggested that this enhanced expression of CYP27B1 could be due, at least in part, to epigenetic regulation (i.e., demethylation), while raised CYP24A1 expression may result from the normal regulatory

loop following the accumulation of $1,25(OH)_2D_3$ in colonic mucosa [16]. However, in highly malignant tumors, an efficient antimitogenic effect by $1,25(OH)_2D_3$ is unlikely, since the expression of the catabolic vitamin D hydroxylase by far exceeds that of CYP27B1 [68].

2.3.5. Estrogen pathway in CRC: interaction with vitamin D

Worldwide, colorectal cancer has a higher incidence rate in men than in women, suggesting a protective role of sex hormones in the development of disease [118]. In addition, studies from relationship between gender and mortality of colorectal cancer show lower mortality for women, especially premenopausal women; epidemiologic studies over several decades suggest a decline in mortality of women attributed to the use of hormone therapy [119, 120]. Expression of estrogen receptor (ER) subtypes α and β has been detected in cancer cell lines. The ER-α:ER-β ratio has been identified as a possible determinant of the susceptibility of a tissue to estrogen-induced carcinogenesis [10]. In both normal and cancerous colonocytes, ER-α expression levels remain low, while ER-β is the predominant ER in the normal colon [121, 122]. The expression level of ER-β in tumor tissue compared with normal colon mucosa is decreased and correlates with stage of the disease [123, 124].

In the colon adenocarcinoma-derived cell line Caco-2, which is ER-β positive but negative for ER-α, Lechner et al. demonstrated an increase of CYP27B1 after treatment with 17β-estradiol [125]. They proved that supraphysiological concentrations of 17β-estradiol not only elevated CYP27B1 mRNA expression and enzymatic activity but also reduced that of CYP24A1 [125]. By enhancing vitamin D accumulation in colon cancer cells, this could inhibit tumor progression. Transfection of SW480 cell lines with ER-β resulted in inhibition of proliferation and cell cycle arrest; SW480 xenografts with ER-β expression had 70% reduction in the tumor weight [126]. Transfection of colon cancer cell lines with ER-β also affects the MAPK signaling pathway [127].

In animal models the transcriptional activity of ERs changes over time and is influenced by estrogen level [128]. Therefore, it is likely that hormone replacement therapy (HRT) in women protects against colon cancer through an increased ratio of ER-β [118].

Regarding potential mechanisms for the interaction of estrogen with vitamin D on CRC risk, experimental evidence indicates that estrogen and vitamin D complexes undergo competitive binding for their common cellular uptake membrane receptor, megalin [129]. Megalin serves as a key endocytosis cell surface receptor for several vitamins and hormonal ligands (including vitamin D [130] and its recently identified ligands—estrogen and testosterone bound to sex hormone-binding globulin (SHBG) [131]. This is clinically relevant to the Women's Health Initiative (WHI) trial findings, as megalin gene knockout has been demonstrated to strongly induce both estrogen deficiency and vitamin D deficiency [131, 132].

Both estrogens and phytoestrogens exert their effects on target cells by genomic and nongenomic mechanisms [133]. Phytoestrogens reduce cell injury and DNA damage mediated by 5 μM oleic acid hydroperoxides in Caco-2 cells [134] and decrease levels of oxidative DNA damage in humans [135]. Genistein, a major phytoestrogen in soy, is antimitotic and proapoptotic in colon cells via the TGF-β/Smad pathway [136]. This phytoestrogen is also involved in regulation of gene activity by modulating epigenetic events such as DNA methylation and/or

histone acetylation (see, e.g., [137]). According to experimental studies, genistein enhances VDR expression in colon cancer cells; upstream and downstream events in the signaling cascade are all interrelated and all participate in the control of VDR expression by 17β-estradiol as well as by phytoestrogens [138]. Genistein also induces CYP27B1 and reduces CYP24A1 expression and activity in a mouse model and in human colon adenocarcinoma-derived cell lines [139].

Reduced incidence of cancer (including colon tumors) has been related to the consumption of a typical Asian diet containing soy; soy and red clover are important sources of phytoestrogens that bind preferentially to estrogen receptor-beta (ER-β) [16]. While the colon cannot be considered an estrogen-dependent tissue, it must be defined as an estrogen-responsive organ [112]. Expression of estrogen receptor (ER) subtypes α and β has been detected in cancer cell lines. In normal colonic mucosa, it is ER-β that is mainly expressed. When mice were fed a diet containing soy, the expression of the synthesizing vitamin D hydroxylase, CYP27B1, was enhanced, and that of the catabolic hydroxylase, CYP24A1, was decreased [50]. In a clinical pilot trial [140], postmenopausal women with a past history of rectal adenomas were given a daily dose of 17 β-estradiol for 1 month to reach premenopausal hormone levels. Rectal biopsies were obtained at the beginning and end of the trial. A predominant result was that VDR mRNA was increased. Such data suggest a protective role of female sex hormones, particularly of estrogens, against CRC. This could provide a rationale for the observation that the age-adjusted risk for CRC is lower for women than for men, even though men and women suffer from similar rate of CRC deaths in their lifetime [16].

Regarding the mechanism behind the gender differences of CRC, Hartman et al. [126] investigated the molecular function of ER-β in colon cancer cells, focusing on cell cycle regulation. They concluded that ER-β inhibits proliferation and tumor growth of colon cancer cells by regulating G1-phase cell cycle genes. Furthermore, this ER-β-mediated cell cycle repression is dependent on functional estrogen response element (ERE) binding. To dissect the processes that ER-β mediates and to investigate cell-specific mechanisms, Edvardsson et al. [127] reexpressed ER-β in three colorectal cancer cell lines (SW480, HT29, and HCT-116) and performed genome-wide expression studies in combination with gene-pathway analyses and cross correlation to ER-β-chromatin-binding sites [127]. Overrepresentation analysis of functional classes indicated that the same biological themes, including apoptosis, cell differentiation, and regulation of the cell cycle, were affected in all three cell lines [127]. ER-β-mediated downregulation of IL-6 has an important impact on inflammation process involved in colon carcinogenesis. The influence of ER-β on apoptosis was further explored using functional studies, which suggested an increased DNA repair capacity. Edvardsson et al. propose that enhancing ER-β action has potential as a novel therapeutic approach for prevention and/or treatment of colon cancer [127].

It has been established that postmenopausal hormone replacement therapy (HRT) is associated with decreased incidence and death rate of colon cancer in both epidemiologic [141, 142] and intervention [142, 143] trials. Additional data suggest that this action may, at least in part, be mediated through VDR signaling. RT-PCR results confirmed that estrogen administration increased mRNA expression of VDR as well as of a downstream target of vitamin D action, E-cadherin [140]. Besides HRT, high estrogen content of soy is implicated to be

protective against colorectal cancer; however, evidence suggests that higher soy consumption is protective against colon cancer only in women [144]. The use of estrogens for prevention of colon cancer is an attractive concept in women; however, the increased rates of cardiovascular events with HRT limit the use of these agents in clinical practice [118].

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Chapter 7

Vitamin D, Its Receptor Gene Polymorphism and Breast Cancer

Mehir un Nisa Iqbal and Taseer Ahmed Khan

Additional information is available at the end of the chapter

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Abstract

Vitamin D is synthesized within skin followed by the peripheral maturation in liver and kidneys. Vitamin D is most essential secosteroid produced its systemic functions via complex with steroid/thyroid nuclear receptor called vitamin D receptor (VDR). The binding of the vitamin D3 to VDR causes conformational changes that permit VDR-RXR heterodimer formation and VDR/ SRC-1 (transcriptional co-activator proteins) interactions. Functional expression and nuclear activation of VDR is necessary to produce its effects upon binding with vitamin D response element (VDRE) on target gene where it causes transcriptional activation resulting in the prevention of breast cancer by inhibiting proliferation, impeding differentiation and stimulating pro-apoptosis. Season, latitude, pigmentation of skin, aging, sunscreen use, obesity, and smoking all affect the production of vitamin D. In case of vitamin D deficiency or VDR gene polymorphisms, vitamin D responses are altered and probably are involved in the risk of breast cancer. Since many epidemiological, observational and interventional studies have been done to illustrate the role of vitamin D and its receptor gene polymorphism in breast cancer development but controversial findings have been observed. Therefore, the role of vitamin D and its receptor gene polymorphisms in development of breast cancer are still a matter of discussion.

Keywords: breast cancer, vitamin D, VDR, vitamin D receptor gene polymorphisms, VDR gene polymorphisms

1. Introduction

1.1. Breast cancer

Breast carcinoma is one of the most frequently diagnosed cancers among women worldwide with a high frequency reported in the West [1, 2]. This highest incidence of breast cancer in American whites and in most European countries reveal the long-standing high prevalence of reproductive factors associated with increased risk of breast cancer, including early menarche, late child bearing age, few pregnancies, hormone replacement therapy and increased mammography [3, 4]. In Israel, the increased incidence of breast cancer may reflect the disproportionately high prevalence of BRCA1 and BRCA2 mutations [5, 6].

Western lifestyle is another most important factor for Britain's high number of breast cancer cases fuelled by the women overeating, too much drinking and too little exercise doing in routine life. In addition, breastfeeding is also an important factor, which reduces the chance of developing breast cancer. Eastern women do not drink alcohol than women in the United Kingdom, and obesity ratio is much lower in Asian women than in western women, whereas breastfeeding rates are much higher in Asians (http://www.dailymail.co.uk/news/article-1301445/Western-lifestyle-blame-soaring-breast-cancer-rates.html). Affected women with breast cancer are usually young and often present with advanced disease [7]. According to a World Health Organization (WHO) estimate, around 25.2% people are diagnosed with breast cancer annually. The exact reason why a woman develops breast cancer is still unrevealed; though certain risk factors enhance a person's probability of getting breast cancer.

The factors that play a significant role in the aetiology of breast cancer include genetic [8, 9], hormonal [10, 11], environmental [12], lifestyle [13] and reproductive factors [14]. In addition, ovarian hormones (endogenous estrogen) are the key risk factors for the development of breast cancer and their progression among post-menopausal women [15, 16]. However, it is unclear that to what degree the effects of other risk factors may be mediated by their links with circulating free estradiol. Intake of vegetables and fruits are related with a substantial decrease of breast cancer risk [17, 18]. Vegetables are rich in antioxidants and certain phytochemicals may contribute to the reduced risk of breast cancer [19–21]. Plant-based diets are also high in fibres, which can decrease serum estrogen and could, in this way, contribute to reduced risk of breast cancer [22, 23]. In addition, increased consumption of fruit and vegetables are associated with lower rates of obesity, which is a crucial risk factor for post-menopausal breast cancer [24]. High energy intake, physical sluggishness, high body mass index (BMI) and weight put on are coupled to an increased breast [25] cancer risk. Low levels of HDL-C in breast cancer patients than in control subjects have also been documented [26]. But still, data from prospective studies are very limited (Moorman, 1998). Furthermore, consanguineous marriages are common in certain racial groups, which will increases the risk of breast cancer [27].

Among these contributing factors, vitamin D and its receptor gene polymorphisms may play a pivotal role in the development of mammary gland tumourigenesis [28].

1.2. Vitamin D and vitamin D receptor (VDR)

Vitamin D and VDR are the two most important participants playing a key role in vitamin D endocrine system in the prevention of breast cancer. Vitamin D is a sunshine vitamin, which is involved in a variety of actions and also reduces the risk of many cancers [29, 30].

VDR is a member of nuclear receptor (NR) superfamily and transcription regulating factor also called NR1I1 or nuclear receptor subfamily 1, group I and member 1. VDR is a high-affnity, low-capacity receptor having a molecular weight of about 48–55 kD. VDR is expressed in majority of human tissues. But some cells have decrease or no VDR expression including RBCs, mature cardiac and skeletal muscles and cerebellar Purkinje cells [31]. Its actions are preceded by the formation of heterodimer with retinoid X receptor (RXR), which causes the conformational changes in VDR and allow the binding of vitamin D3 at ligand binding domain (LBD). In addition, the heterodimer complex then binds with a specific sequence present in the DNA called vitamin D response element (VDRE). Genomic pathway involves the expression of genes in a tissue-specific manner [28].

1.2.1. VDR domains

VDR contain five functional domains (**Figure 1**) including:

1. A and B domains both are shortest domains contain 20 amino acids.
2. C domain (DNA binding domain or DBD) having two Zn fingers [32] motifs. Two alpha helices are found at the carboxy terminus of each Zn finger (namely helix A and B which constitutes DNA recognition and phosphate binding sites respectively).
3. Flexible hinge D domain is present in between C and E domains having the ability to change structural conformation after VDR ligand binding.
4. E domain (ligand binding domain or LBD) consists of 12 alpha helices along with 3 short beta strands, organized in a manner that it forms three dimensional hormone binding pockets of which vitamin D3 is attached.

Both N-ter and C-ter has activation function (called AF-2) in translation [33].

1.2.2. Vitamin D/VDR actions

1. **Genomic actions:** Vitamin D3 produces its pleiotropic effects by genomic and non-genomic actions. It mediates its genomic actions upon binding to intracellular nuclear transcription factor called VDR.
2. **Non-genomic actions:** Vitamin D also plays various non-genomic actions. Non-genomic actions are also called rapid actions, which are caused by the interaction of vitamin D with the membrane VDR to perform its biological effects through intracellular signalling pathways [35]. However, the contribution of non-genomic pathway in the development of anti-neoplastic effects on breast remains unclear.

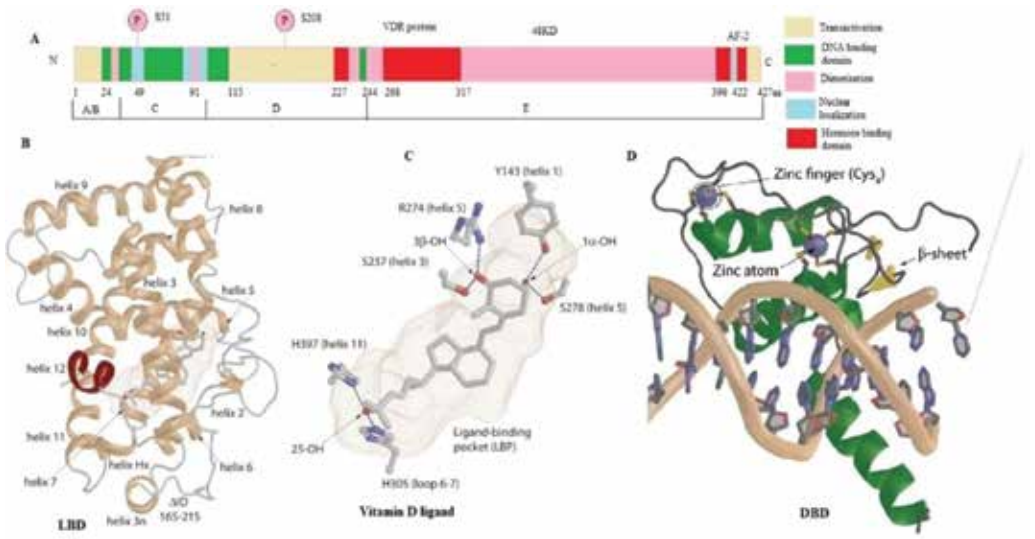

Figure 1. The crystal structure of VDR showing its functional domains [34]. (A) Schematic representation of VDR domains. (B) LBD of the VDR which contains 12 alpha helices. (C) The binding mode of Vitamin D in the hormone-binding pocket. (D) The DBD of the Vitamin D. The two zinc atoms are represented in blue in colour, whereas beta sheets are represented in yellow colour.

2. Bio-activation and metabolism of Vitamin D in normal breast

It is already known that vitamin D affects the breast cancer cell growth but limited information is available about its delivery, uptake and metabolism in mammary cells. Vitamin D is either derived from the gastrointestinal (GIT) absorption or synthesized within the skin under the exposure of UVB radiations, which then undergoes the 25-hydroxylation in liver in presence of 25-hydroxylase resulting in the production of 25(OH)D3. 25(OH)D3 is the precursor molecule for the synthesis of active Vitamin D3 (1,25(OH)2D3). It is a major circulating form of vitamin D, which is stored in adipose tissues. It is also an accurate biomarker of vitamin D, which determines the overall status of vitamin D in the body. However, the precursor does not readily binds to the VDR and must be converted into its active form, 1,25(OH)2D3, which has high binding affinity to VDR. The conversion of precursor vitamin D into its active metabolite occurs in the presence of 1-α-hydroxylases. Immunohistochemistry and *in situ* hybridization studies indicated strong expression of 1α-hydroxylase protein and mRNA in the distal convoluted tubule, the cortical and medullary part of the collecting ducts and the papillary epithelia. Lower expression was observed along the thick ascending limb of the loop of Henle and Bowman's capsule. Weaker and more variable expression of 1α-hydroxylase protein and mRNA was seen in proximal convoluted tubules, and no expression was observed in glomeruli or vascular structures [36]. Whereas lesser expression of 1α–hydroxylase was also observed in non–renal cells including keratinocytes, macrophages, prostatic epithelium, colonocytes [37, 38] and breast epithelium [39] to lesser extent. Kidneys and non-renal 1-α-hydroxylases are

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encoded by the same gene mapped on the chromosome 12 [40]. However, the presence of this enzyme on non-renal tissues indicated that the non-renal tissues have the ability of vitamin D bio-activation, responsible to convert 25(OH)D3 into 1,25(OH)2D3. 1,25(OH)2D3 is virtually not detected in human serum under anephric conditions, which means that kidneys are the major source of 1,25(OH)2D3 in circulation. These observations emphasize that 1,25(OH)2D3 produced by the non-renal tissues is not released in the bloodstream. However, they act locally upon binding to VDR on the same tissues from where it is synthesized. Such local actions of vitamin D are likely included in proliferation, differentiation and apoptosis, which are discussed below in later sections.

2.1. Bio-activation pathways in breast cells

The above information supports the hypothesis that two distinct pathways may be involved in the bio-synthesis and bio-activation of vitamin D in breast such as 1,25(OH)2D3 and 25(OH)D3 (vitamin D precursor) pathways [41, 42].

2.1.1. Endocrine pathway

The endocrine pathway is involved with the circulation of 1,25(OH)2D3, which reaches the mammary tissues and produces anti-neoplastic effects through genomic pathway.

2.1.2. Autocrine/paracrine pathway

The other pathway is the autocrine/paracrine pathway involved with the 25(OH)D3, which reaches the mammary gland and converts into 1,25(OH)2D3 [43] in the presence of 1-α-hydroxylase to prevent breast cancer [41]. Most of the extra-renal tissues of the body have its own 1-α-hydroxylase enzyme needed for the production of active metabolite of vitamin D [37]. The circulating level of 25(OH)D3 seems to be the key regulator of tissue-specific synthesis of active vitamin D [37, 44]. The locally produced active vitamin D binds with VDRs of mammary epithelium in order to regulate the expression of more than 200 genes, which are involved in controlling cell proliferation, inhibit cell growth, stimulate cell differentiation, induce apoptosis and inhibit angiogenesis [45] and contribute in the prevention of breast tumourogenesis [46]. Moreover, mammary epithelial cells also contain 24-hydroxylase enzyme (CYP24), which converts active vitamin D into less active metabolites including 24,25-dihydrohydroxyvitamin D3 and 1,24,25-trihydroxyvitamin D3 [43]. For this reason, we can say that breast tissues contain all the elements of vitamin D signalling axis, which involve in the local synthesis as well as metabolism of vitamin D and its signal transduction through VDRs.

3. Vitamin D signalling in the prevention of breast cancer

3.1. VDR expression in breast

Several extra-renal epithelial cells of body express VDR, for example, epithelial cells of rat, mouse and human mammary glands. VDR expression is highest in breast tissues during

puberty, pregnancy and lactation in women [47]. In mice, the expression is highest in ductal epithelium when compared to terminal end-buds epithelium of mammary gland. In human, VDR-positive cells are found in basal and luminal layer of breast epithelium [39]. Cap cells and stromal compartments of breast are also rich in VDR [48–50]. The presence of VDR in different cells of breast highlights the complexity of vitamin D signalling in breast tissues.

3.2. Mechanism of vitamin D signalling in breast cancer prevention

Despite these consistent data, the exact mechanism of breast cancer prevention by vitamin D has yet to be discerned. Both 25(OH)D3 and 1,25(OH)2D3 exert its profound effects on normal VDR-positive breast epithelium such as hormone-stimulated growth inhibition, ductal elongation, ductal branching and induction of biomarkers involved in breast differentiation. The expression of VDR and 1-α-hydroxylase in mammary adipocytes also takes part in the prevention of cancer in whole tissue since adipocytes secrete diffusible signals in response to 25(OH)D3, which constrain morphogenesis of the nearby ductal tissues [48].

Furthermore, alteration in cellular energy metabolism, immune responses and other processes of vitamin D signalling in the prevention of breast cancer on non-tumourigenic breast epithelium is described below.

3.2.1. Anti-proliferation

Vitamin D causes cell-cycle arrest by direct or indirect involvement of growth factors and does not allow the cell to enter in the S phase from G1 phase [51]. It increases the expression of cyclin-dependent kinases (CDKs) inhibitors, including p21 and p27, and reduces the expression of CDK2, CDK4, cyclin D1, cyclin A1 and cyclin E1, which results in the arrest of cell-cycle progression [52, 53]. It is also involved in the downregulation of c-myc oncoprotein and inhibits the cell proliferation [54]. However, all these consequences describe that vitamin D hampers the cell proliferation by affecting the crucial controllers of cell-cycle progression. Furthermore, vitamin D also enhances the transcription factor CCAT enhancer-binding protein alpha (C/EBPα), which mediates the anti-proliferative effects of vitamin D observed in *in vitro* study on MCF-7 cells [55]. Tumour suppressor TCF-4 also hinder cell-cycle progression [56]. Beside these, vitamin D also causes the induction of BRCA 1 (breast cancer 1) gene, which is inversely associated with the cell proliferation, promotes tumour suppression and inhibits cell-cycle progression [57].

3.2.2. Growth arrest and pro-apoptosis

Vitamin D plays an important role in the induction of apoptosis in mammary tissues, since *in vitro* conditions, such as shrinkage of cell, condensation of chromatin network and fragmentation of DNA, have been observed in MCF-7 cells upon treatment with vitamin D [58]. The mechanism by which vitamin D induced apoptosis has not been fully understood. However, the most probable mechanism is the downregulation of anti-apoptotic protein, called Bcl2 (51). Vitamin D increases the tumour necrotic factor alpha (TNFα) with or without caspase 3 activation. In the caspase 3-independent mechanism, vitamin D-mediated induction of

apoptosis in MCF-7 cells is thought to be correlated with mitochondrial disruption, which causes the release of cytochrome C and formation of reactive oxygen species (ROS) resulting in the apoptosis [59]. Other mechanism of caspase-independent apoptosis induced by vitamin D-dependent Ca+ absorption is most likely associated with the increased activation of lysosomal proteases [60]. Finally, vitamin D also acts a pro-oxidant for breast cancer cells, which generally increase the redox potential [61] of carcinogenic cell, may be one of the most important underlying pro-aptototic mechanisms of vitamin D. The pro-oxidant action of vitamin D in MCF-7 cells could result from increased intra-cellular reactive oxygen species production during aerobic metabolism. Vitamin D inhibits the expression of one of the major constituents of the cellular defence system against ROS, like superoxide dismutase (SOD) [62]. This decrease could be one of the mechanisms underlying the pro-oxidant action of vitamin D. Indeed, it was previously reported that overexpression of SOD protects MCF-7 cells from being injured [63, 64] . Decrease in SOD levels would cause a shift in the balance between superoxides and hydrogen peroxide (H_2O_2). Increased levels of superoxides can, in turn, cause increased oxidative damage attributable to interaction with NO to form the highly toxic peroxynitrite [65] and to increased availability of free iron that supports hydroxyl radical formation through the Fenton reaction [66].

Changes in the redox state could translate into reversible oxidation of cysteines in major proteins that determine cell fate, such as protein kinases, protein tyrosine phosphatases and transcription factors (e.g. Sp1, activator protein-1, nuclear factor-κB and p53) [67–73]. The key components of the apoptotic process, such as mitochondrial permeability transition pores and increase caspases, are also subjected to redox regulation [74]. Oxidation of the cysteine in the active site of GAPDH may be considered a sensitive, easily accessible marker for these processes. It is noteworthy that the increase in the cellular redox potential was shown to abolish the DNA-binding ability of the transcription factors activator protein-1 and nuclear factor-κB [75] can cause apoptosis and prevent breast cancer. Notably, a recent study describes the relationship between p53 and VDR. Mutant P53 (mutp53) converts the Vitamin D pro-apoptotic activity into anti-apoptotic activity and attain oncogenic activity which demonstrate gain of function (GOF) [76].

3.2.3. Anti-angiogenesis

Vitamin D inhibits angiogenesis, which is another important feature for tumour growth and progression. It also has the ability to impede angiogenesis at very minute concentration [77] mediated through the downregulation of vascular endothelial growth factor (VEGF), tenascin-C, tumour growth factor α (TGF-α) and epidermal growth factor (EGF) [78, 79].

3.2.4. Anti-invasion or anti-metastasis

Vitamin D inhibits the invasion of tumour in nearby tissues but its deficiency promotes the growth of breast cancer cells in the bones of nude mice and alters the bone micro-environment [80]. This ability of vitamin D is supposed to be caused by the decrease expression of metal-loproteinases (MMP-9) and serine proteinases (such as urokinase-type plasminogen activator and tissue-type plasminogen activator) along with the increased expression of their inhibitors

[81]. In addition, vitamin D also downregulates P-cadherin [82] and upregulates E-cadherin [83].

3.2.5. Anti-inflammation

Vitamin D reduces the expression of cyclooxygenase-2 (COX-2), which plays a crucial role in the synthesis of prostaglandin in many breast cancer cell lines in human. It increases the upregulation of 15-hydroxyprostaglandin dehydrogenase, an enzyme which is involved in catalysing the conversion of active prostaglandins into biologically inactive ketoderivatives [84]. Prostaglandins have been supposed to play a role in the breast cancer development and its progression [85]. Prostaglandins are secreted by the breast cancer cells or surrounding tissues promote tumour progression caused by cell proliferation, resistant to apoptosis, tumour invasion and angiogenesis [85]. An increased expression of COX-2 in breast cancer has been assumed to correlate with high-grade, large tumour size and poor prognosis [86].

3.2.6. Anti-estrogen

Vitamin D inhibits estrogen biosynthesis (steroidogenesis) and its biological actions [84]. Vitamin D suppresses the estrogen pathway by inhibiting the expression of gene which encodes aromatase (the enzyme which converts androgens to estrogen) [84]. Vitamin D also reduces the expression of estrogen receptor alpha (ERα-) [87]. The combined actions of vitamin D can decrease the estrogen and the receptor, which mediates their signalling in the prevention of breast cancer.

3.3. Vitamin D deficiency and breast cancer

The half-life of circulating vitamin D is approximately about 2–3 weeks which is a better indicator of blood vitamin D. Active vitamin D3 (1,25(OH)2D3) is locally synthesized from its precursor (25-(OH)D3) in almost all body cells because of the universal presence of 1α-hydroxylases in all cell type including breast [88]. So, the deficiency of 1-α hydroxylase may augment the deficiency of vitamin D and thereby associated with increased breast cancer risk and mortality [89].

Serum vitamin D concentrations and vitamin D supplementations are the independent predictors of breast cancer risk. Serum level of vitamin D of more than 50 ng/ml is associated with the 50% lower risk of breast cancer in women than serum values less than 30 ng/ml [90, 91]. In addition, breast cancer risk reduces in the pre-menopausal women who consume calcium and vitamin D orally [92]. Locally advanced breast cancer patients have more severe vitamin D deficiency than those with early-stage disease [93].

Deficiency of vitamin D is related with secondary hyperparathyroidism, which causes increased bone resorption, release of calcium from bones osteoclasts into the blood and may exacerbate osteoporosis with subsequent harsh effects on bone mineral density (BMD). In breast cancer patients, osteopenia and osteoporosis mostly occur because of early menopause and vitamin D deficiency, which is then augmented by chemotherapy and hormone replacement therapy [94]. Therefore, breast cancer patients are necessary to suffer a baseline metabolic

bone evaluation along with circulating vitamin D levels and bone mineral densitometry [94, 95].

Vitamin D deficiency is also associated with the recurrence, tumour size and death from breast cancer. It means that having enough amount of vitamin D may be able to keep a cancer from getting worse. In fact a recent meta-analysis concluded that high circulating level of vitamin D is weakly related with breast cancer incident; however, strong association was found with better breast cancer survival [89]. So, the maintenance of an optimal vitamin D status at the time of diagnosis and during 1-year follow-up period is necessary for improving survival of breast cancer patient.

There are four types of studies which illustrated whether exposure of ultraviolet B (UVB) radiations and low levels of vitamin D decrease the risk of breast cancer.

3.3.1. Geographical studies

In these studies, the geographical variation in the incidence or mortality of breast cancer is compared statistically with solar UVB radiations. The lower breast cancer incidence rate was found in the regions of high solar UVB radiations such as in Australia, China, France, Nordic countries, Spain and the United States [96].

3.3.2. Observational studies

Observational studies do comparison of vitamin D levels with the incidence of breast cancer among cases and controls. There are two categories of observational studies:

1. The studies in which vitamin D levels is measured near the time of breast cancer diagnosis are called case-control studies.
2. The studies in which vitamin D is measured at the time of women enrolment in studies prior to the breast cancer diagnosis are called nested case-control studies.

Only the case-control studies have reported that low levels of vitamin D are associated with breast cancer risk [97]. The reason why nested case-control studies have not reported the same results may be due to

1. breast cancer develops very rapidly, and
2. without supplementation, vitamin D levels tend to change little over time.

Observational studies have also documented that those females have higher vitamin D levels at the time of diagnosis live longer as compared to those with low vitamin D levels [46, 96]. In addition, the chances of mortality are higher in black women after diagnosis of breast cancer than in white women.

3.3.3. Laboratory studies

Laboratory studies have focused on the mechanisms of vitamin D in the contribution of reduced risk of breast and other cancer types. According to these studies, vitamin D allows

the cells to stay alive if they are the right type and present at the right place, or it helps the cells to commit suicide (apoptosis) if cells are not the right type or not present at the right place. Vitamin D also reduces the formation of blood vessels around tumours and decreases the ability of tumours to invade [98]. According to the randomized controlled trials, vitamin D reduced the risk of cancer, including breast cancer [99, 100].

4. Vitamin D receptor gene

The human VDR (hVDR) gene is located at long arm of chromosome 12 bands 13-14 (12q13-14) [101, 102]. The gene is 75 kb long and contains 11 exons [103]. This gene is divided into three regions: one coding region and two non-coding regions.

4.1. Non-coding regions

The 5′ promoter region of VDR lacks initiator (TATA and CAAT boxes) and is rich in GC content. It provides the putative site for binding of many transcription factors [103]. The promoter region is present at exon 1(1a, 1b, 1c, 1d, 1e, 1f). The promoter region facilitates the transcription activity of VDR target gene. The 3′ UTR contains poly (A) repeats, which is reported to be associated with the mRNA stability.

4.2. Coding region

Coding region comprises of exon 2–9. Exon 2, which have translation start codons, contains DNA-binding site, whereas exon 7, 8 and 9 have ligand (vitamin D) binding site [104].

5. Single nucleotide polymorphism (SNP)

Polymorphism is defined as the presence of two or more clearly different phenotypic variants of a particular DNA sequence in the same population of a species. The most common form of polymorphism is the single nucleotide polymorphism in which variation occurs at a single base pair usually present in approximately 1% of the population. These types of changes can be present in non-coding region of genes and in introns, which would not affect the translation of proteins, but these changes can affect the degree of gene expression and levels of proteins. The changes can also be present in coding regions of DNA or exons resulting in the formation of an altered protein sequence. Sometimes variation in exons do not cause the change in the structure of protein called synonymous polymorphisms.

These changes often produce or eliminate restriction sites for endonuclease to digest the DNA. As a result, fragments of DNA with a different length will be obtained which can be identified by gel electrophoresis. This process is called restriction fragment length polymorphisms (RFLPs). The produced fragments will be the undigested fragments, which is homozygous dominant, whereas the digested fragments are heterozygous and homozygous recessive.

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Sometimes polymorphic alleles are linked with each other and within a population in non-random proportion is known as linkage disequilibrium (LD), [105] and the combination of alleles (blocks) or set of SNPs present on the same chromosome which tends to be inherited together is termed as haplotype. The size of these blocks is different ranging between 10 and 20 kb and could be important in determining the reason of genetic disorder.

6. SNPs in the VDR gene

The variation in the 5′-promoter region of VDR gene can change the sequence of mRNA as well as protein levels, whereas alteration in 3′ UTR sequence can disturb the stability of mRNA thereby affecting the efficacy of translated protein. Some SNPs have been existed in the VDR gene, including *Cdx*2 [106], *Fok*1 [107], *Bsm*1, *Taq*1, *EcoR*V [108], *Apa*1 [101] and *poly* (A) [109] microsatellite repeats.

6.1. *Cdx*2 SNP

The VDR *Cdx*2 (G-1739A) is the single nucleotide polymorphism, which was recognized by the sequence analysis of promoter region. It is an adenine to guanine (A to G) SNP situated at the promoter region of VDR gene at exon 1e. It was initially reported to be located at the 3731 bp upstream exon 1a of promoter region of VDR gene among Japanese women [106], but later identified to be located at 1739 kbp upstream of 1e exon just 2 kb away from the exon 1a among many ethnic population [110]. It is the binding region of *Cdx*2 protein, a most important intestine-specific caudal-related homeodomain protein, which increases the transcription of VDR. When A allele is present in *Cdx*2 promoter, the Cdx2 protein is bound more strongly as compared to when a G allele is present. The A allele stimulates the initiation of transcription, whereas G allele inhibits [106].

6.2. GATA SNP

GATA (A-1012G) is located at exon 1a in the core sequence of DNA called AGATAT [111]. It provides the binding of GATA protein and the binding site is present in A allele and absent in G allele. The mechanism of this polymorphism is not identified yet; however, this polymorphism alters the immune responses to cancer cells. A allele is responsible to reduce cytotoxic response to cancer cells. In addition, it is also an important element that if the transcription is begun in exon 1a or 1d. In presence of G allele, exon 1d comprises an alternate start codon which results in a formation of N-ter extended protein called VDRB1. G allele is most likely associated with the VDRB1 (long) protein, whereas A allele is related with the VDRA (short) protein.

6.3. *Fok*1 SNP

*Fok*1 polymorphism is also called start codon polymorphism (SCP). It is a thymine to cytosine (ATG to ACG) polymorphism located at the 10 bp upstream 5′ end of exon 2 on the DNA-

binding domain, which results in a formation of more active transcription factor that is three amino acids shorter [103, 112]. Those individuals who have ACG sequence in the start codon, the initiation of translation occurs at the second ATG site which results in a formation of three less amino acids at NH2 terminus containing 424 amino acids. If the initiation occurs at first ATG sequence, it produces full-length VDR protein containing 427 amino acids. In the presence of restriction site, alleles are designated as 'f', whereas its absence is designated as 'F' (active form) [113]. The restriction recognition site of *Fok*1 is 5′-GGATG*-3′; 3′-CCTAC*-5′ and enzyme cleaves 9/13 nucleotide downstream of the recognition site.

6.4. *Bsm*1-*Apa*1-*Taq*1 SNP

Most of the functional sequence variants identified near the 3′ region of VDR gene were *Bsm*1, *Apa*1 and *Taq*1 SNP. These SNPs are in linkage disequilibrium with each other and are located in the same haplotype block. Therefore, these SNPs may have the potential to influence the mRNA stability. The *Apa*1 and *Bsm*1 are located at intron 8, whereas *Taq*1 is located at exon 9 [114].

The presence of restriction enzyme site in these SNPs is designated as lower case letter such as b, a and t, whereas absence is designated as upper case letter including B, A, T. The restriction site for Bsm1 is 5′-GAATGCN*-3′, Apa1 is 5′-GGGCC*C-3′ and Taq1 is 5′-T*CGA-3′.

6.5. *Poly* (A) repeats

Poly (A) tail is a variable number of tandem repeats (VNTR) or short tandem repeats (STR) containing variable numbers of adenine nucleotide present at the 3′ UTR of VDR. *Poly* (A) is also linked with Bsm1, Apa1 and Taq1 polymorphisms and also involved in the mRNA stability of VDR. It varies in length and can be divided into two types:

1. The long (L) *Poly* (A) sequence in which 18–24 adenine nucleotide is present, and
2. The short (S) *Poly* (A) sequence in which 13–17 adenine nucleotide is present.

Because all four polymorphisms (*Bsm*1, *Apa*1, *Taq*1 and *Poly* (A)) are present in close proximity on the VDR gene, strong linkage disequilibrium exists among them. The two most common haplotypes are:

1. baTL haplotype in which *Bsm*1 and *Apa*1 restriction sites are present, whereas *Taq*1 site is absent along with the presence of long *Poly* (A) repeats.
2. BAtS haplotype *Bsm*1 and *Apa*1 restriction sites are absent, whereas *Taq*1 site is absent along with the presence of short *Poly* (A) repeats [115].

The baTL haplotype is reported to be associated with the increase incidence of breast cancer [116].

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7. VDR gene polymorphisms and breast cancer

VDR gene polymorphism is associated with the breast cancer risk [117–125] but insufficient data are available to find the relationship with breast cancer risk [126]. The studies have pointed out allelic variations in VDR gene, such as *Cdx*2, *Fok*1, *Bsm*1, *Taq*1, *Apa*1 and *Poly* A in different ethnic groups with breast cancer incidence with contradictory results [117, 118, 121, 126].

7.1. *Cdx*2 polymorphism and breast cancer

The contradictory observations were reported on the association of *Cdx*2 polymorphism and breast cancer susceptibility [125]. Recently, a meta-analysis has documented that *Cdx*2 polymorphism is linked with breast cancer susceptibility only in Africans [127]. However, no profound relations was observed between *Cdx*2 polymorphism and breast cancer risk among Pakistani population [126].

7.2. *Fok*1 polymorphism and breast cancer

*Fok*1 polymorphism contain large consensus sequence has no relationship with breast cancer incidence [116, 117]. But the association between *Fok*1 polymorphism and breast cancer was reported in several ethnic groups [113, 120], mainly in Caucasians [128, 129]. Nemenqani et al. [121] found that *Fok*1 polymorphism is associated with the ER and PR status of breast cancer and described that *Fok*1 polymorphism has a significant interaction with the ER status but not with PR status of breast cancer.

7.3. *Bsm*1 polymorphism and breast cancer

*Bsm*1 polymorphism is the most important functional VDR gene polymorphism, which is found to be associated with the risk of developing breast cancer [124]. However, it has also been documented that there is no relation between *Bsm*1 and breast cancer [119]. Rollison et al. [123] describe that *Bsm*1 is involved to alter the vitamin D intake and overall breast cancer risk. McCullough et al. [130] found that B allele of *Bsm*1 decreases breast cancer incidence by 20%.

7.4. *Taq*1 polymorphism and breast cancer

Many case-control studies suggested that *Taq*1 polymorphism is not associated with breast cancer risk [119–121]. But it has been reported that *Taq*1 is one of the functional polymorphisms which is linked with increased breast cancer incidence [131, 132]. A meta-analysis on large ethnic groups revealed that the *Taq*1 polymorphism increases the risk of breast cancer development in Caucasians; however, no profound association was observed among Asians [133].

7.5. Other polymorphisms and breast cancer

Positive association of poly A [118] or Apa1 [119] was found to be reported with breast cancer risk, showing a connection between polymorphism and likelihood of having a tumour.

8. Conclusion

This chapter concluded that women with breast cancer are more likely to have low vitamin D levels. Those women who do not get adequate vitamin D may be more likely to develop breast cancer later in life as compared to those who have higher vitamin D levels, who are less likely to develop breast cancer and less likely to die from breast cancer.

Because of the broad spectrum of vitamin D effects on mammary tissue, it is suggested to be a most important physiological growth regulator of mammary gland and could be a potential therapeutic agent. Additionally, due to the expression of VDR to a higher extent on breast epithelial cells, vitamin D signalling should also be monitored during breast cancer treatment. Since breast cancer is a complex disease which may or may not be associated with the decreased vitamin D levels or VDR polymorphisms. However, the functions and role of vitamin D and VDR cannot be neglected during breast cancer treatment.

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Chapter 8

Vitamin D3 and Neurofibromatosis Type 1

Juichiro Nakayama

Additional information is available at the end of the chapter

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Abstract

Vitamin D3 (VD3) and its analogs have been shown to inhibit growth of various cell types found in neurofibromas and pigmented lesions of patients with neurofibromatosis type 1 (NF1). Excimer light irradiation at 150–300 mJ/cm^2 in combination with VD3 efficiently inhibited growth of cultured fibroblasts, mast cells, Schwann cells, and melanocytes. Long-term whole body irradiation with narrowband ultraviolet B (UVB) in patients with NF1 significantly increased serum levels of VD3, which was accompanied by a brightening of generalized skin hyperpigmentation. Irradiation with either laser or intense pulsed-radio frequency in combination with topical application of VD3 analogs yielded moderate to fair improvement of café-au-lait macules, small pigmented spots, and skin-fold freckling in NF1 patients. Thus, topical or systemic application of VD3 or one of its analogs may provide beneficial effects to treat skin lesions for patients with NF1.

Keywords: neurofibromatosis type 1, café-au-lait macule, neurofibroma, vitamin D3, narrowband UVB

1. Introduction

Neurofibromatosis type 1 (NF1), also known as von Recklinghausen's disease, is an autosomal dominant disorder affecting approximately 1 in 3500 people. Common to all ethnic origins, NF1 appears to occur in similar proportions for all individuals without sex or ethnic specificity. Riccardi [1] classified heterogenous neurofibromatosis disorders into eight categories. Hallmarks of NF1, the classic type of neurofibromatosis, include café-au-lait macules (CALMs), and multiple benign cutaneous neurofibromas (NFs), comprising dermal, subcutaneous, and plexiform types. Clinical diagnosis is based on the presence of two or more of the following findings: six or more CALMs (largest diameter >0.5 cm in prepubertal individuals, or >1.5 cm

in postpubertal individuals); two or more NFs of any type, or one plexiform NF; axillary freckling; optic glioma; two or more Lisch nodules within the iris; a distinctive osseous lesion; or first-degree relative diagnosed with NF1 according to the preceding criteria [2]. While significant advances in understanding both the pathoetiology and genetics of NF1 have been made in the last decade, no therapeutic modalities are currently available for NF1 patients; although several studies are currently examining various agents specifically directed at plexiform NF, such as clinical trials for sirolimus and imatinib mesylate [3].

We have previously investigated the effects of vitamin D3 (VD3) and its analogs on skin lesions of patients with NF1 and observed inhibited growth of fibroblasts primarily isolated from NFs (previously called fibroblastic cells) [4]. We next performed in vivo experiments in which we found topical application of a VD3 analog onto a CALM grafted to nude mouse skin inhibited uptake of bromodeoxyuridine into cells on the basal layer of grafted epidermis [5]. In addition, cell density of NF tissues subcutaneously grafted onto the skin of nude mice decreased significantly with direct local injection of a VD3 analog [6]. Consequently, as an initial human study, we evaluated the effect of 1 month of VD3 analog application twice a day to an NF1 patient's large pigmented plaque. We observed moderate improvement of the applied macule, including a remarkable decrease in intensity of Fontana Masson staining in the epidermal basal layer [6]. We extended our study to examine whether VD3 or its analogs inhibit growth of Schwann cells and mast cells isolated from primary NFs. We also investigated the capacity of these agents to inhibit growth of human epidermal melanocytes. Our results indicated that with the exception of Schwann cells, all cell types examined were inhibited by VD3 and its analogs in vitro.

From a molecular aspect, the NF1 gene encodes a Ras GTPase-activating protein called neurofibromin; mutations in this gene affect Ras-mitogen-activated protein kinase (MAPK) signaling [7]. Inhibition of this signaling, also known as the Ras/Raf/MEK/ERK or MEK pathway, has also been shown to be efficacious in treating human NF [8]. Therefore, we examined inhibitory effects of a mechanistic target of rapamycin (mTOR) inhibitor (rapamycin) and a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor (lovastatin) [9] on growth of Schwann cells isolated from primary NFs. Our results demonstrated that these agents significantly inhibited Schwann cell growth in vitro.

We examined the potential of other therapeutic modalities to be combined with VD3 for even greater improvement of skin lesions in patients with NF1. Previous reports indicate narrow-band ultraviolet B (NB-UVB) irradiation directly induces conversion of vitamin D to VD3 in various cell types including human keratinocytes [10, 11]; thus, we analyzed whether long-term whole body NB-UVB irradiation increased serum levels of VD3 in NF1 patients. We also observed the effects of irradiation on patient skin lesions by photographing treatment areas before and after irradiation procedures. Our results showed that 6 months of irradiation or more increased patient VD3 serum levels significantly. This increase was accompanied by changes in the patients' skin color including lightening of generalized hyperpigmentation.

It has been established that laser treatments have little effect on pigmented skin lesions, such as CALMs or small freckling, of patients with NF1. Instead, we investigated the effects of laser or intense pulsed-radio frequency (IPL-RF) irradiation in combination with topical application

of VD3 analogs. In one young patient, treatment with Q-switched Ruby laser irradiation in combination with a topical VD3 analog treatment resulted in almost complete disappearance of a CALM located on the nose. A similar therapy combining IPL-RF irradiation with topical VD3 application was very effective at diminishing multiple small pigmented freckles on the face and body of another patient. Herein, our recent investigative results concerning the effects of VD3 and its analogs on NF1 are described in greater detail.

2. Methods

2.1. Cell culture

Primary fibroblasts isolated from NFs of patients with NF1 were cultured as previously described [4]. Human epidermal melanocytes (Melanocel 1; Kurabo, Japan) were cultured according to the manufacturer's instructions. Isolation of Schwann cells and mast cells was performed as previously described [12]. Briefly, NF pieces were dissociated in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific K.K., Yokohama, Japan) containing collagenase (Life Technologies, Carlsbad, CA) and dispase (Roche Diagnostics, Basel, Switzerland), and then cells were resuspended in DMEM containing fetal calf serum, antibiotics, 3-iso-butyl-L-methlxanthine (Sigma, St Louis, MO), β-heregulin (Wako, Japan), forskolin (Sigma) and insulin (Sigma). Isolated Schwann cells were seeded onto culture flasks coated with poly-L-Lysine (Sigma) and laminin (Life Technologies). To isolate mast cells, NF pieces were incubated in a digestion buffer containing Hank's Balanced Salt Solution (Life Technologies), collagenase (Worthington Biochemical, Lakewood, NJ), hyaluronidase (Worthington Biochemical), and DNase (Sigma). Cells were layered over 75% Percoll® (Sigma) and centrifuged, and then nucleated cells were collected from the buffer-Percoll interface. Percoll-enriched gradient cells were suspended in AIM-V® Medium (Life Technologies) containing human stem cell factor (PeproTech, Rocky Hill, NJ). The purity of Schwann or mast cell populations was assessed with S-100 or toluidine blue staining, respectively, and a purity grade of more than 90% was confirmed for both cell-type isolations. Cells were used after 2–3 passages.

2.2. Treatment of cells with VD3 and its analogs with or without excimer light

Fibroblasts from NFs and human epidermal melanocytes were seeded at a density of 2×10^4 cells/ml onto 35-mm cell culture dishes for 2 days. Next, cells were treated for 3 days with 10^{-9}–10^{-7} M calcitriol (VD3, 1α,25-dihydroxyvitamin D3; Enzo Life Sciences, Farmingdale, NY), tacalcitol [1,24-dyhydroxyvitamin D3; Teijin, Japan], or 22-oxacalcitriol (OCT; 1α,25-dihydroxy-22-oxavitamin D3; Chugai Pharmaceuticals, Japan). Cells were trypsinized and then dissociated cells were collected by centrifugation and counted by Coulter counter. Melanocytes treated with agents were also labeled with 1 μCi of ^{3}[H]-thymidine (^{3}[H]-TdR); incorporation into melanocytes was counted by liquid scintillation counter, as previously described for counting fibroblasts isolated from NFs [4]. Both cell types were exposed to excimer light (308 nm) at doses of 150–300 mJ/cm^2 in the presence of calcitriol and tacalcitol and were then

cultured for 3 days. Excimer light (308-nm UVB) was used to irradiate culture dishes instead of 308 nm of UVB because the tip of the excimer light was small and handy and it allowed for precise irradiation of an expected dose. In the following experiments, isolated primary fibroblasts (three patients), mast cells (five patients), and Schwann cells (six patients) were cultured as described above and then treated with 0.1 μM calcitriol or tacalcitol for 3 days. Cells irradiated with excimer light in the presence or absence of agents were cultured for 3 days after irradiation. Floating mast cells were centrifuged before counting. Mean counts of triplicate measurements were determined.

2.3. Treatment of cells with rapamycin and/or lovastatin

Isolated primary Schwann cells and fibroblasts from NFs were seeded at a density of 1.0–2.0 × 10^4 cells/ml onto 35-mm culture dishes. After 2 days, cells were treated with rapamycin (0.1–100 nM), lovastatin (0.1–10 μM), or a combination of both for 3 days. Number of cells per dish was evaluated by Coulter counter and mean counts of triplicate measurements were determined for each dish, as previously described [13].

2.4. NB-UVB irradiation

In the dermatology outpatient clinic at Fukuoka University Hospital, NF1 patients who had complained of itching or painful sensation were administered whole body NB-UVB irradiation (312 ± 2 nm) at doses of 0.2–0.5 J/cm^2 once weekly or biweekly (every 2 weeks). Approximately, half of these patients presented with complicating atopic dermatitis. During irradiation, serum VD3 levels of patients who had received NB-UVB irradiation for more than 18 months were measured and compared with those of patients without any treatment. Next, nine patients who had not received any previous treatment were irradiated with NB-UVB for 6 months and serum VD3 levels were measured before and after irradiation for a kinetic study. Patients not showing an increase in serum VD3 levels, even after 6 months of irradiation, were further irradiated for more than a year to study whether additional irradiation increased serum VD3 levels. Special Laboratory References (Tokyo, Japan) measured serum VD3 concentration of patient serum samples using a VD3 Radioimmunoassay Kit (Immunodiagnostic Systems, UK) in accordance with the manufacturer's instructions. As a preliminary clinical investigation, we also examined whether increases in serum (and possibly skin) VD3 levels brought about by long-term NB-UVB irradiation bestowed any benefit to NF1 patient skin lesions [10, 11]. We photographed the examined areas before and after irradiation, especially focusing on generalized pigmentation of the skin.

2.5. IPL-RF or laser irradiation with or without topical VD3 analogs

We investigated combinations of topical VD3 analogs with various forms of irradiation including NB-UVB, IPL-RF (Aurora; Syneron Medical, Israel), Q-switched Ruby (JMEC, Japan), and neodymium-doped yttrium-aluminum-garnet (Nd:YAG) laser (MedLight C6; Cynosure, Westford, MA) treatments. IPL-RF irradiation was performed according to previously described methods [14, 15]. Laser toning procedures using a Q-switched Nd:YAG laser were carried out during 5–10 sessions over 1- to 2-week interval with 2.5 J/cm^2 at 5 Hz. Clinical

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improvement of skin lesions was determined by photographs or using a colorimeter (Crystaleye; Olympus, Japan) before and after treatments.

All clinical studies were performed with prior approval from an ethics committee at Fukuoka University Hospital, and all enrolled patients agreed to the studies and provided informed consent.

3. Results

We previously reported that VD3 and its analogs inhibited growth of primary fibroblasts isolated from NFs, whereby around 30–50% of fibroblast growth inhibition was observed with 0.1 μM calcitriol, tacalcitol, or OCT [4]. This inhibition rate almost corresponded to that of isolated primary normal human fibroblasts. A decreased number of fibroblasts was accompanied by 50–70% inhibition of ^{3}[H]-TdR uptake, observed as early as 2–4 days after addition of agents to culture medium [4]. We also tested whether growth of human epidermal melanocytes was inhibited by VD3 and its analogs and found similar levels of cell growth inhibition and ^{3}[H]-TdR uptake (**Figure 1**).

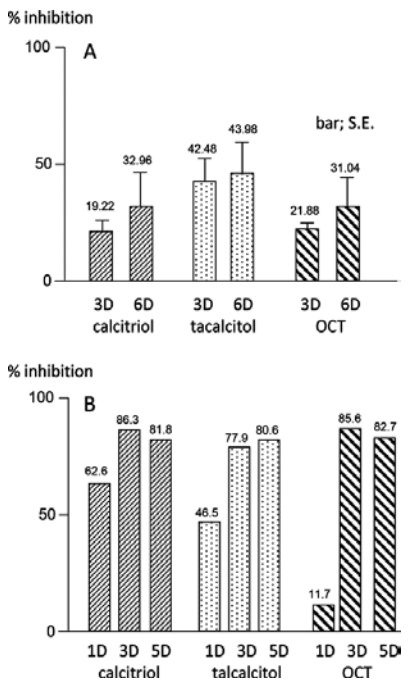

Figure 1. Growth inhibition of human melanocytes with addition of calcitriol, tacalcitol, or OCT. After treatment, number of cells was counted with a Coulter counter and ^{3}[H]-TdR uptake was measured by liquid scintillation. Percent inhibition of cell growth (A) and ^{3}[H]-TdR uptake (B) are shown.

We observed growth inhibition in fibroblasts from NFs and human melanocytes was increased by 10^{-9} to 10^{-7} M calcitriol or tacalcitol in combination with excimer light irradiation. Indeed, calcitriol and tacalcitol inhibited growth of both cell types in a dose-dependent manner, and this inhibition was further augmented by around 20–30% with 150–300 mJ/cm^2 of excimer light irradiation (**Figure 2**). Recent progress in cell culture techniques allowed us to isolate and culture Schwann and mast cells from NFs with a purity of more than 90%, as described in the Methods section. Primary fibroblasts, mast cells, and Schwann cells were isolated from NFs and examined for how they responded to 0.1 μM calcitriol or tacalcitol and/or excimer light irradiation. Growth of mast cells was inhibited by 16–20% with 0.1 μM calcitriol or tacalcitol; whereas with excimer light irradiation at a dose of 300 mJ/cm^2, this value rose with statistical significance to 26% (almost the same as that of fibroblasts). Also, a small additive inhibition was observed with 0.1 μM calcitriol or tacalcitol and excimer light irradiation at a dose of 300 mJ/cm^2 in mast cells. With regard to Schwann cells, neither VD3 nor tacalcitol had an effect on their growth, an unexpected result. Excimer light irradiation inhibited growth of Schwann cells by approximately 38% in a statistically significant manner (**Figure 3**).

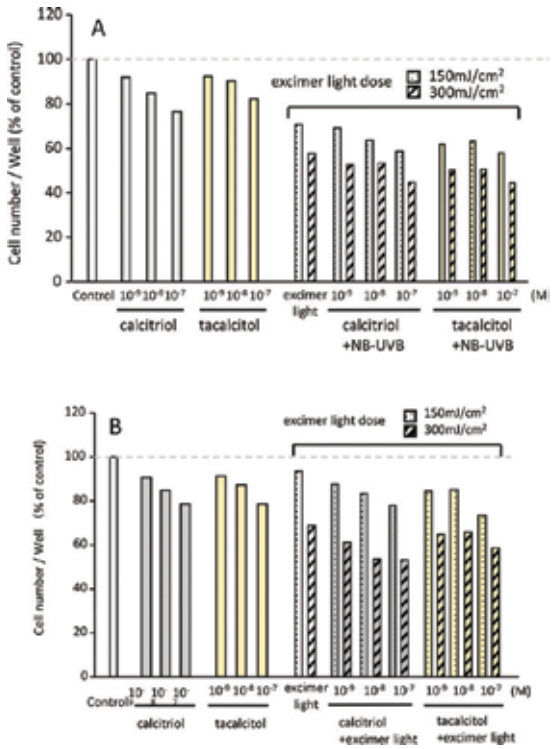

Figure 2. Inhibition of cell growth by calcitriol or tacalcitol in combination with excimer light irradiation. Fibroblasts isolated from NFs and human melanocytes were irradiated with excimer light in the presence or absence of calcitriol or tacalcitol, and number of cells per dish was counted. Data for fibroblasts (A) and melanocytes (B) are shown.

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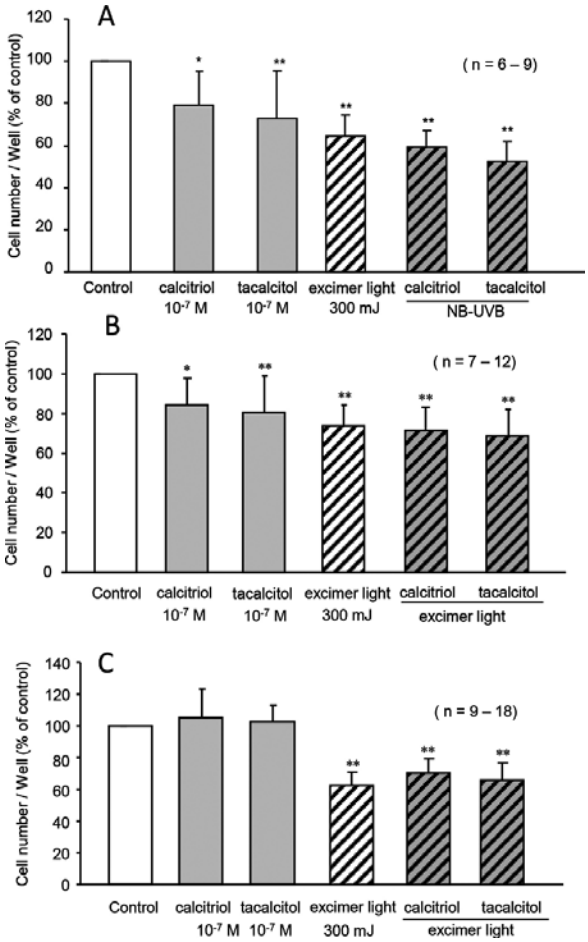

Figure 3. Inhibitory effects of excimer light irradiation with or without calcitriol or tacalcitol treatment on the growth of fibroblasts (A), mast cells (B), and Schwann cells (C). *$P < 0.05$ and **$P < 0.01$ vs. control cells. Values represent mean ± standard deviation.

As we did not see any effect of VD3 or its analogs on Schwann cells, we investigated whether rapamycin or lovastatin inhibited growth of these cells using isolated primary fibroblasts as a control. Growth of primary Schwann cells isolated from NFs was inhibited by 0.1–10 nM rapamycin or 0.1–10 μM lovastatin in a dose-dependent manner. Furthermore, combination of 0.3 nM rapamycin with 1 μM lovastatin resulted in a considerable additive inhibitory effect on the growth of Schwann cells. Growth inhibition rates for fibroblasts with either rapamycin or lovastatin were consistently less than those of Schwann cells (**Figure 4**). We also examined whether a combination of rapamycin and/or lovastatin with VD3 augmented growth inhibitory effects on Schwann cells and fibroblasts. Our experimental results showed that addition of 0.1 μM calcitriol slightly diminished the growth inhibitory effect of 0.3 nM of rapamycin and 1 μM of lovastatin. In contrast, the combination of these agents showed additive effects on fibroblast growth inhibition (data not shown).

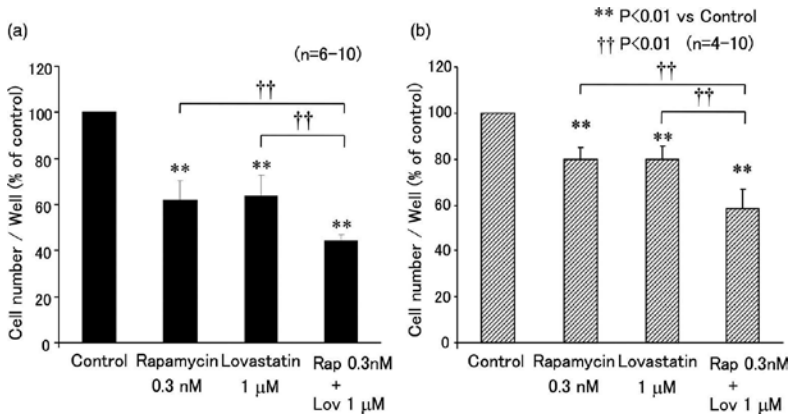

Figure 4. Significant growth inhibition of Schwann cells(A) and fibroblasts(B) isolated from NFs by 0.3 nM rapamycin and/or 1 μM lovastatin.

We investigated whether long-term whole body NB-UVB irradiation increased serum VD3 levels in patients with NF1 and found that patients irradiated once weekly or biweekly (0.2–0.5 J/cm^2 dose per irradiation) for at least 18 months had significantly increased serum VD3 levels compared with patients receiving no treatment (**Figure 5**). A kinetic analysis of this increase subsequently carried out in nine patients revealed that 6 months of irradiation was enough to significantly increase serum VD3 levels (**Figure 6**). Finally, we also observed that significant increases in serum VD3 levels occurred when irradiation with NB-UVB was continued for more than 1 year in patients previously demonstrating low serum VD3 levels after an initial 6 months of irradiation (**Figure 7**).

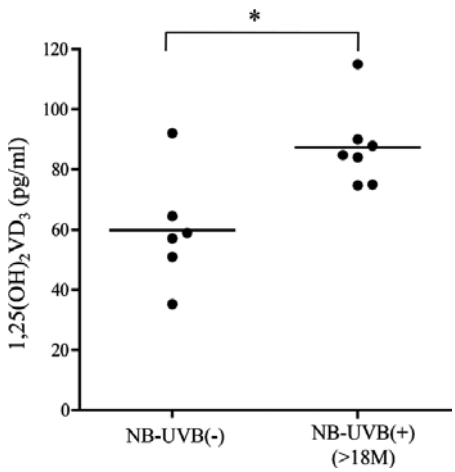

Figure 5. Increase in serum VD3 levels in NF1 patients irradiated with NB-UVB at doses of 0.2–0.5 J/cm^2 once weekly or biweekly for more than 18 months; levels compared with untreated patients (*$P < 0.05$).

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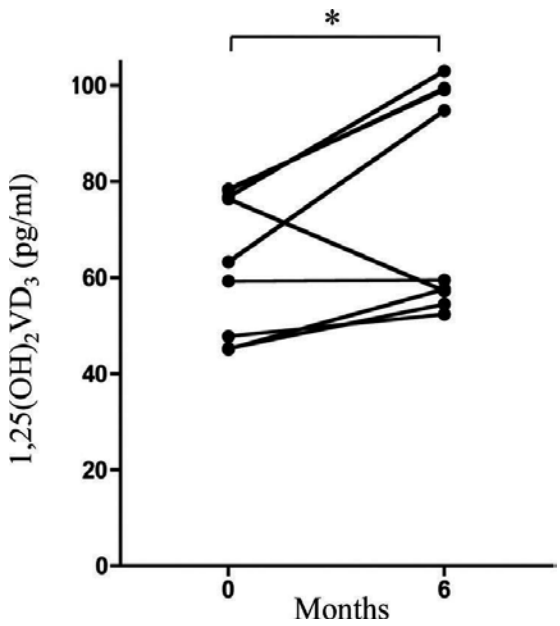

Figure 6. Time course of serum VD3 levels in NF1 patients irradiated with NB-UVB. Serum VD3 levels of patients before and after 6 months of NB-UVB irradiation were measured (*$P < 0.05$).

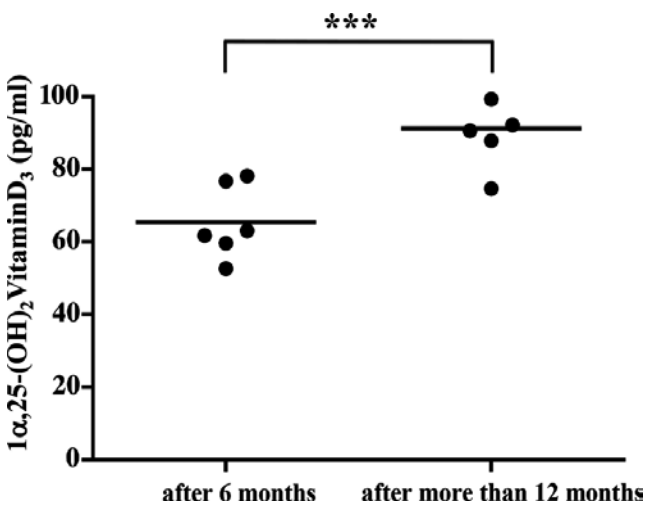

Figure 7. Increase in serum VD3 levels in NF1 patients who had not shown an increase in VD3 levels, even after an initial 6 months of NB-UVB irradiation, after continuing irradiation treatment for more than 1 year thereafter. Statistical analysis was performed using a paired *t*-test (***$P < 0.001$).

We examined whether CALMs, small pigmented freckles, or generalized hyperpigmentation improved in 10 patients after more than 6 months of NB-UVB irradiation (once weekly or

biweekly). Using photography, we observed general hyperpigmentation in most patients receiving NB-UVB irradiation became brighter; although effects on CALMs and small pigmented spots were not apparent within this period (**Figure 8**). A questionnaire completed by the patients indicated that 80% (8/10) believed they had either excellent or fair improvement of pigmented skin lesions, and all of the patients were satisfied with the results of NB-UVB irradiation.

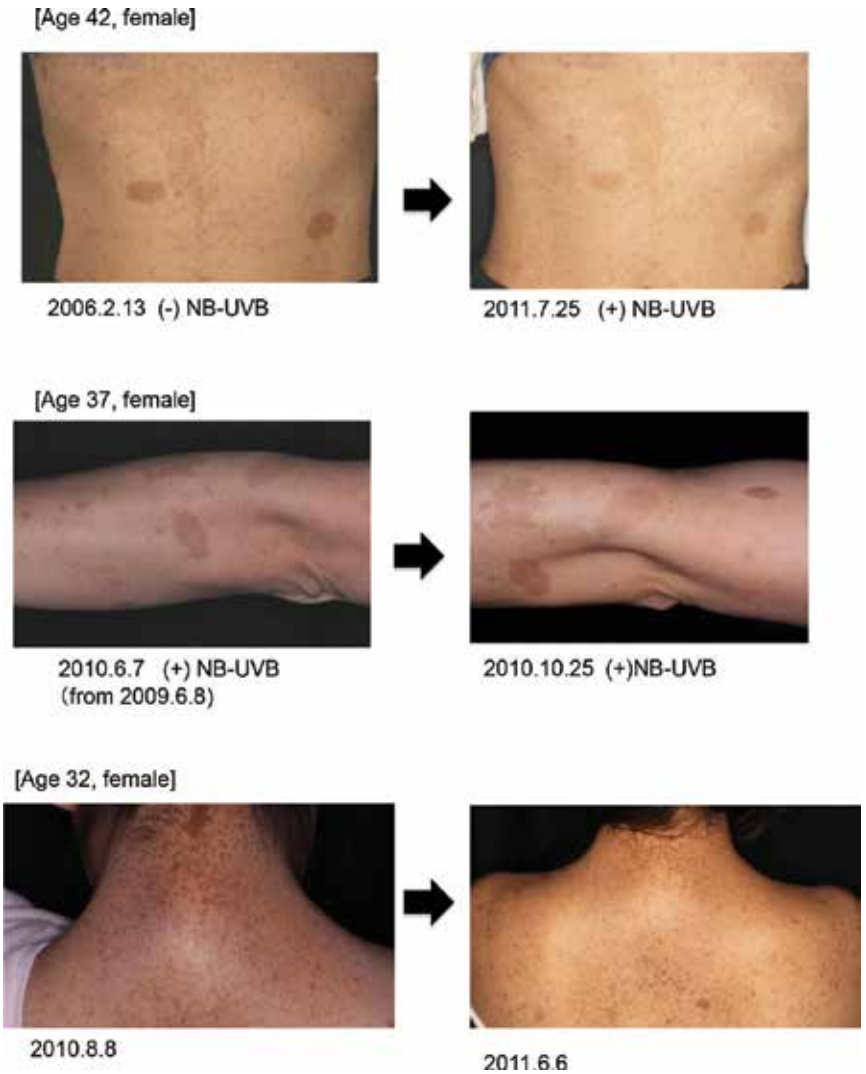

Figure 8. Photographs of three typical cases, showing lightened skin color of generalized hyperpigmentation after NB-UVB irradiation at doses of 0.3–0.5 J/cm^2 weekly or biweekly for more than 6 months.

Next, the effects of combining IPL-RF irradiation with topical OCT application on CALMs and multiple small pigmented spots were investigated. We IPL-RF irradiated multiple pigmented

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spots on the body of a 27-year-old female patient. Multiple pigmented spots received either OCT only or IPL-RF irradiation only for comparison to the area treated with both IPL-RF and OCT. Remarkable improvement of multiple pigmented spots was observed after six IPL-RF irradiation procedures and continuous topical OCT application over a 4-month period (**Figure 9**). In addition, the area treated only with IPL-RF irradiation showed similar improvements to the combination treatment area; however, areas treated with OCT only or OCT plus IPL-RF exhibited increased lightness of skin appearance, as measured by colorimeter [14]. We then treated an additional eight NF1 patients with this combination therapy and observed a moderate to good response with regard to lightening of pigmented spots in six patients [15].

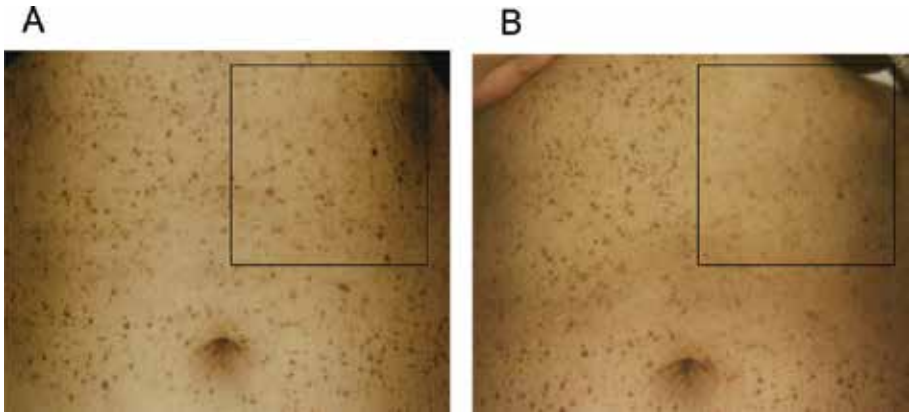

Figure 9. Effects of combining IPL-RF irradiation with topical OCT treatment on multiple pigmented spots on the torso of a 27-year-old female NF1 patient. Six courses of irradiation with a light fluence of 15–25 J/cm^2 and RF energy 15–25 J/cm^2 were carried out over a 4-month period. Irradiation of 70–100 shots/100 cm^2 was performed once every 2 or 4 weeks. Photographs shown were taken before (A) and 6 months after (B) treatment.

With regard to the effects of topical VD3 analogs in combination with laser irradiation on CALMs, we experienced one case of a young male patient who had a conspicuous CALM on his nose. The patient had previously been treated with Q-switched Ruby laser irradiation in a cosmetic clinic for a long time without success before he visited our dermatology clinic at Fukuoka University Hospital. The pigmented macule was first treated only with topical OCT application twice a day for 10 months. After that, Q-switched Ruby laser irradiation (8 J/cm^2) was performed twice every 3 months along with continuing topical OCT application. The CALM showed fair improvement after the initial 10 months of topical OCT application and then virtually disappeared over the course of the next 6 months with combination treatment (**Figure 10**).

Recently, an irradiation procedure known as laser toning has been applied to alleviate facial melasma of women for cosmetic purposes [16, 17]. Given that our previous experience using laser toning with an Nd:YAG laser for melasma brought about fairly good results, we applied this procedure to CALMs and small pigmented freckles on seven NF1 patients in combination with topical tacalcitol application. Side-by-side therapy with either laser toning alone or in

combination with tacalcitol treatment was adopted. A typical case, a 17-year-old female patient with NF1, is shown in **Figure 11**.

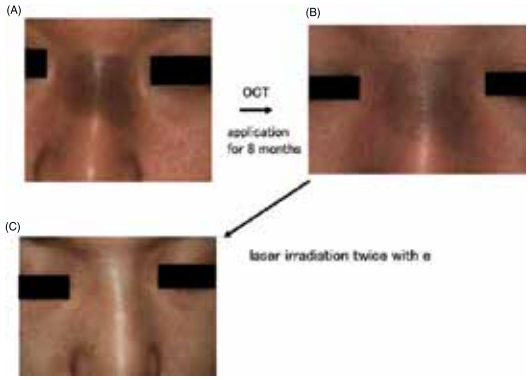

Figure 10. CALM on the nose of a 20-year-old male NF1 patient treated with OCT in combination with Q-switched Ruby laser irradiation. A) Before treatment, (B) after 10 months of topical OCT application twice a day, and (C) after 6 months of Q-switched Ruby laser irradiation twice every 3 months in combination with topical OCT treatment.

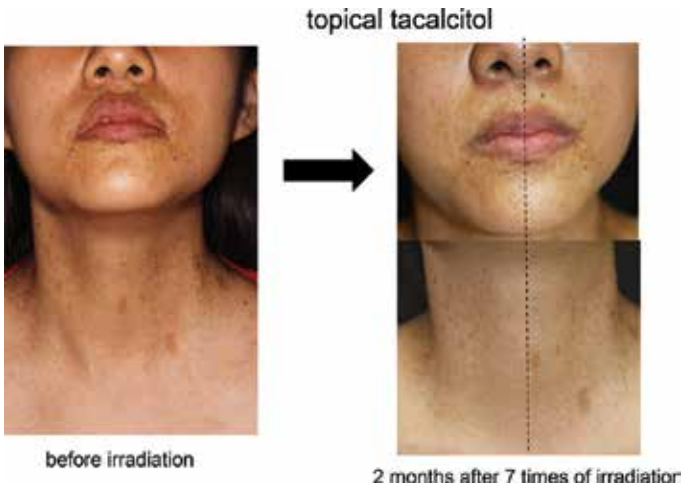

Figure 11. Side-by-side treatment of hyperpigmentation and small pigmented spots using Nd:YAG laser toning with or without topical tacalcitol ointment. Pigmented lesions of a 17-year-old female patient were treated seven times with laser toning only (right side) or seven times with laser toning in combination with topical tacalcitol ointment (left side). Pigmentation of her left side was more improved compared with the right side.

Her facial pigmented spots were treated with either laser toning alone (right side) or laser toning plus tacalcitol treatment (left side, **Figure 11**). Hyperpigmentation around her mouth and small pigmented spots around her neck became lighter on the left side compared with those on the right. Since laser toning with an Nd:YAG laser exerts effects mainly on epidermal melanocytes, this treatment was thought to have little beneficial effect on CALMs. However,

side-by-side therapy revealed that a combination of laser toning with topical tacalcitol application caused more lightening in the examined area compared with laser toning treatments alone (**Figure 12**).

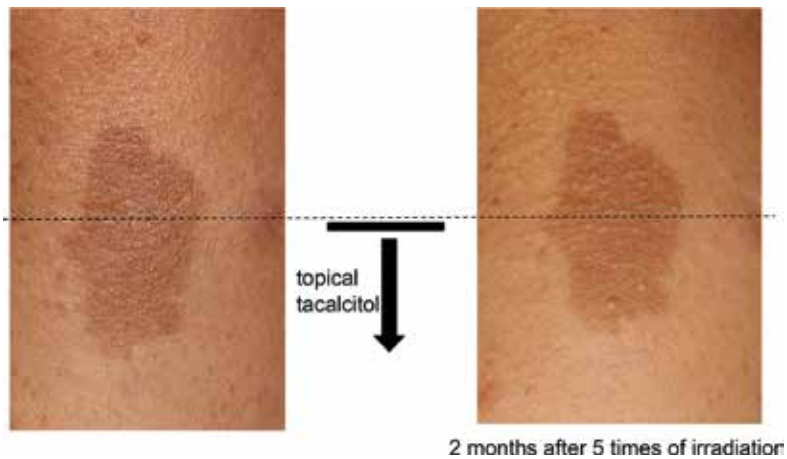

Figure 12. Side-by-side treatment of a CALM using Nd:YAG laser toning with or without topical tacalcitol ointment. A CALM of a 28-year-old male patient was treated five times with laser toning only (upper half) or five times with laser toning and topical tacalcitol ointment (lower half).

4. Discussion

NF1 is one of the major neurocutaneous syndromes. Patients with this disease experience decreased quality of life caused by cutaneous morbidities such as CALMs, skin-fold freckling, and NFs. Moreover, if malignant peripheral nerve sheath tumors should arise from plexiform NFs, it is potentially lethal to patients with NF1. Our understanding of the etiopathogenesis of NF formation in NF1 has progressed with use of mouse models and various cells isolated from NFs. Tumors are comprised of Schwann cells, mast cells, fibroblasts, and perineurial cells; however, Schwann cells are thought to be the primary tumor cell-type as they possess both germline and second-hit mutations in the NF1 gene ($NF1^{-/-}$) [18]. Other components, such as mast cells and fibroblasts with haploinsufficient NF1 gene mutations ($NF1^{+/-}$) are essential to sustain the formation of NFs. In a mouse model, $Nf1^{-/-}$ diploinsufficient Schwann cells rapidly proliferated and secreted KIT ligand at approximately sixfold higher levels compared with wild-type controls [19]. Mast cells haploinsufficient for the NF1 gene infiltrated into NFs in response to KIT ligand and exhibited potency to proliferate. Precise etiopathogenesis of CALMs remains obscure, but it has been suggested that melanocyte density is increased within CALMs [20]. Also, somatic mutation analysis yielded two NF1 hits in melanocytes isolated from CALMs [21]. A one-hit mutation in melanocytes has been thought to cause skin-fold freckling and global hyperpigmentation.

VD3 and its analogs have been found to inhibit in vitro growth of primarily isolated fibroblasts and mast cells, but not Schwann cells. We also have found that growth of commercially available human epidermal melanocytes is efficiently inhibited by VD3 and its analogs. We used these human epidermal melanocytes in our study because it was difficult to obtain informed consent from patients to excise a large enough sample of a CALM to get the required number of melanocytes. Thus, whether the growth inhibition rate of primarily isolated melanocytes ($NF1^{+/-}$) from NF1 patient CALMs is the same as that of the human epidermal melanocytes ($NF1^{+/+}$) remains to be examined.

Given that we observed that the growth of Schwann cells was not affected by VD3 and its analogs, we extended our study further to whether an mTOR inhibitor (rapamycin) or Ras-MEK pathway inhibitor (lovastatin) could inhibit the growth of primary Schwann cells and fibroblasts isolated from NFs. It was found that these agents could inhibit growth of both Schwann cells and fibroblasts isolated from NFs. A combination of VD3 with rapamycin and/or lovastatin did not increase the suppressive effects of either of these drugs on the growth of Schwann cells, but it did cause additive suppression of fibroblast growth. With regard to clinical use of VD3 or its analogs for NF1 patient skin lesions, our in vitro experimental results indicate a combination of rapamycin and/or lovastatin with VD3 should be more effective at suppressing NF growth in vivo. Although the tumorigenic cells in NFs are considered to be $NF1^{-/-}$ Schwann cells, supporting $NF1^{+/-}$ mast cells and/or fibroblasts are considered to be essential for NF formation.

We found definite inhibitory effects of 308-nm UVB irradiation (excimer light) on growth of all cells comprising the NF1 phenotype in vitro. We then studied whether NB-UVB irradiation brought about beneficial effects on skin lesions of patients with NF1. In addition, we measured changes in serum VD3 levels of these patients after long-term whole body NB-UVB irradiation. We observed that at least 6 months of irradiation significantly increased serum VD3 levels, which were accompanied by a lightening of generalized hyperpigmentation of the skin of most patients examined. Hyperpigmentation commonly resulting from UV or sun exposure, caused by induction of endothelin-1, was not detected with NB-UVB irradiation at doses of 0.2–0.5 J/cm^2 once weekly or biweekly, even if NB-UVB irradiation was continued for more than 3 years.

Increases in NF1 patient serum VD3 levels by NB-UVB irradiation are in accord with a previous report suggesting NB-UVB irradiation for patients with either atopic dermatitis or psoriasis causes upregulation of serum 25-hydroxyvitamin D (calcidiol) [22]. Other reports suggest serum calcidiol levels in patients with NF1 are significantly lower than those of control subjects [23], and low levels of calcidiol have a negative correlation with severity of NF formation [24]. Also, reduced bone density and increased incidence of calcidiol deficiency in adults with NF1 have been reported [25]. Therefore, oral supply of VD3 and long-term NB-UVB irradiation could bring about benefits for both skin and internal lesions of NF1 patients; although, care should be taken to identify and minimize any adverse events caused by long-term NB-UVB irradiation.

To date, no evidence supports use of laser therapy for removal of CALMs. However, we experienced the virtual disappearance of a CALM on the nose of a young male patient with

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NF1 after two treatments with Q-switched Ruby laser irradiation in combination with continuous topical OCT application during a 16-month period. We also observed multiple small pigmented spots on the torso of a female patient with NF1 virtually disappeared after treatment with IPL-RF in combination with topical OCT application. Our recent studies using laser toning with an Nd:YAG laser in combination with topical tacalcitol application showed fairly good results with regard to multiple facial small pigmented spots. So, we recommend adopting this irradiation with topical VD3 ointment for pigmented spots associated with severe cosmetic problems, especially those on the face, in patients with NF1.

Our clinical investigative results with VD3 have been obtained from a small number of patients with NF1, and no double-blind studies have been performed. Therefore, further studies of a larger scale are needed to clarify to what extent pigmented lesions such as CALMs, small pigmented spots, and skin-fold freckling can be improved by irradiation with laser or IPL-RF in combination with VD3. Additional studies examining suppression of new NF formation with long-term whole body NB-UVB irradiation, which significantly enhances serum VD3 levels in patients with NF1, would also be of great value. It is generally considered that either topical application or internal ingestion of VD3 should exert the same biological mechanism of action. On the one hand, direct topical application of VD3 or its analogs onto skin lesions may be more effective than internal ingestion because of locally higher concentrations of VD3. On the other hand, skin lesions related with NF1, such as CALMs or NFs, are generally scattered across the whole body, ingestion, or injection of VD3 may be more useful for practical therapeutic applications. In conclusion, use of VD3 or its analogs is encouraging for either improving pigmented skin lesions or suppressing new NF formation in patients with NF1.

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Section 4

Therapeutic Measurements of Vitamin D

Chapter 9

Pathogenic and Therapeutic Role of Vitamin D in Antiphospholipid Syndrome Patients

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Additional information is available at the end of the chapter

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Abstract

In this chapter, the novel findings on interrelationship between vitamin D status and two well-known prothrombotic states, antiphospholipid syndrome, particularly its thrombotic phenotype, and metabolic syndrome will be reviewed. We shall present the results obtained from patients included in Serbian National Antiphospholipid Syndrome Registry, 68 patients with primary antiphospholipid syndrome (PAPS) and 69 patients with antiphospholipid syndrome associated with certain autoimmune rheumatic disease (sAPS), as well as 50 patients with pure metabolic syndrome (MetS). These results will be analysed and compared with the novel literature data. Prevalence of MetS in APS is high, with the atherogenic dyslipidaemia as its most prevalent characteristic. Prevalence of thrombotic events was significantly higher in APS patients with coexisting MetS, compared with those without MetS. Among APS patients, prevalence of VitD deficiency was significantly higher than in patients with pure MetS. VitD level was also significantly lower in APS patients with coexisting MetS or previous thrombotic events than in those without them. Elucidating interrelationships between VitD deficiency, MetS and thrombotic events in APS patients open up the possibility of distinguishing those subjects with the particularly high cardiovascular risk and ensuing need for the strict control of modifiable risk factors and VitD supplementation.

Keywords: vitamin D, antiphospholipid syndrome, metabolic syndrome, classification criteria, thrombosis

1. Introduction

The antiphospholipid syndrome (APS), primary or associated with certain autoimmune rheumatic diseases, especially systemic lupus erythematosus, represents prothrombotic state. Coexistence of metabolic syndrome (MetS) and autoimmune rheumatic diseases is already recognized [1, 2], while clinical significance of MetS in patients with APS has not been systematically studied [3]. Recent recognition of certain pleiotropic functions of vitamin D (VitD) has enabled us to hypothesize on its role in the pathogenesis of obesity, MetS, APS, autoimmunity and thrombosis. Therefore, the aim of this review will be: (1) to clarify the possible linking role of VitD between APS and MetS, (2) to critically assess the need for estimation of VitD status in APS patients, depending on the coexistence of MetS and (3) to explore the potential therapeutic role which VitD, as an immunomodulator and anti-thrombotic agent, could have in these patients.

2. Basic definitions

Metabolic syndrome (MetS) and antiphospholipid syndrome (APS) are among most prevalent and still highly controversial syndromes. While clinical relevance of antiphospholipid antibodies (aPL) was recognized more than 30 years ago, definite classification criteria for antiphospholipid syndrome were given at the International Workshop in Sapporo, Japan 1998 [4] and revised 2006 in Sidney, Australia [5]. Very interesting proposal of APS criteria based on biological mechanisms is presented lately aiming at simplicity and greater accuracy and, at the same time, avoiding non-specific formulations [6] (**Table 1**). Recent investigations have also shown that, beside characteristic thrombotic or obstetric symptoms, there is growing number of systemic non-criteria manifestations (for example, thrombocytopenia, livedo reticularis, skin ulcerations, pseudovasculitis, migraine and epilepsy) correlating with certain type of aPL and with important predictive role [7, 8]. It is likely that a prominent place among these manifestations belongs to components of MetS, but it is still to be proved. The prevalence of APS in the general population is estimated to be around 2–4%.

Initial Reaven's postulate in 1988, which draw attention to the causal association between insulin resistance with ensuing hyperinsulinemia and cardiovascular diseases [9], was followed by numerous definitions of MetS. Three of them, i.e. definitions given by World Health Organization (WHO) [10], the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) [11] and International Diabetes Federation (IDF) [12], were most frequently used and still neither of them is obsolete. While all three definitions share central obesity, atherogenic dyslipidaemia and arterial hypertension as common criteria, WHO definition put the insulin resistance in focus of metabolic syndrome while an obligatory criterion requested by IDF definition is elevated waist circumference (WC) with population- and country-specific cut-offs (**Table 2**). All of these three definitions are very similar but different enough, especially when used for the assessment of prevalence of MetS in some other entities, in this case, among patients with APS. Even the latest joint attempt of several major

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professional organizations (the IDF Task Force on Epidemiology and Prevention, National Heart, Lung and Blood Institute, American Heart Association, World Heart Federation, International Atherosclerosis Society and International Association for the Study of Obesity) to unify interconnected cardio-metabolic risk factors into a universal definition of metabolic syndrome did not seem to be final [13].

<table>
<tr><th>Sapporo criteria[4]</th><th>Sidney criteria[5]</th><th colspan="2">Newly proposed minimalistic criteria[6]</th></tr>
<tr><td colspan="2">Clinical criteria (at least one)
1. Vascular thrombosis:

≥ 1 clinical episodes of arterial, venous or small vessel thrombosis, confirmed in any tissue or organ by appropriate imaging studies or histopathology, but without significant inflammation in the vessel wall.</td><td>Thrombotic APS

see Sapporo/Sidney criteria.</td><td>aβ2GP1-domain I IgG

β2GP1-dependent LA

LA

aβ2GP1-independent LA

aFII (aFII/PS) IgG

aA5 IgG</td></tr>
<tr><td colspan="2">2. Pregnancy morbidity
(a) ≥ 1 unexplained early pregnancy loss at or beyond the 10th week with normal fetal morphology documented by ultrasound or direct fetal examination;
(b) placenta-mediated late pregnancy complications (before 34 weeks) causing ≥ 1 fetal death;
(c) ≥ 3 unexplained early pregnancy loss without maternal anatomic and hormonal abnormalities as well as without maternal and paternal chromosomal abnormalities.</td><td>Obstetric APS

1. Unexplained early pregnancy loss (at or beyond 10th week)

Placenta-mediated late pregnancy complications (before 34 weeks) with fetal death</td><td></td></tr>
<tr><td colspan="2">3. Laboratory criteria (at least one)</td><td rowspan="2">aβ2GP1-domain I IgG

β2GP1-dependent LA

LA</td></tr>
<tr><td>Detected on ≥ 2 occasions, at least 6 weeks apart:

β2-glycoprotein I–dependent aCL IgG and/or IgM isotype antibodies, in medium or high titer;

LA detected according to the guidelines of the International Society on Thrombosis and Hemostasis.</td><td>Detected on ≥ 2 occasions, at least 12 weeks apart:

LA detected according to the guidelines of the International Society on Thrombosis and Hemostasis;

aCL antibody of IgG and/or IgM isotype;

anti- β2-glycoprotein I antibody of IgG and/or IgM isotype.</td></tr>
</table>

Table 1. Antiphospholipid syndrome definitions.

Similar ambiguity exists concerning the definition of adequate circulating VitD level, as well as of its deficiency and insufficiency. Earlier definition of VitD insufficiency by its blood level of <20 ng/mL (50 nmol/L), given by the World Health Organization (WHO) [14], has been recently accepted by most researchers as a definition of the deficiency of this vitamin [15, 16]. Its insufficiency is defined as a VitD concentration between 20 and 30 ng/mL (50 and 75 nmol/L), while its concentrations >30 ng/mL (75 nmol/L) are regarded as sufficient [17, 18].

The WHO definition[10]	**NCEP ATP III definition**[11]	**IDF definition**[12]
Insulin resistance *plus ≥ 2 of:*	*≥ 3 of:*	
	Central obesity	*plus 2 of:*
	Atherogenic dyslipidaemia	
	Hypertension	
Microalbuminuria	Impaired fasting glucose/Glucose intolerance/Diabetes	

Table 2. Metabolic syndrome definitions—similar but different enough.

3. Experience from Serbian National APS Registry

3.1. Patients and methods

Study included a total of 137 APS patients, attending outpatient clinic of the University Medical Center Bezanijska kosa, all Caucasians, who were previously enrolled in Serbian National APS Registry. These patients represented only the part of those so far included in this Registry, which is still growing and is still unable to appraise the prevalence of APS among general population in Serbia. Among studied patients, 68 were PAPS patients (59 females, nine males, mean age 43.51+10.58 years) and 69 sAPS patients (61 females, eight males; mean age 47.83+15.67 years). All studied APS patients have met 2006 updated Sydney criteria [5] which requested the presence of at least one clinical criteria (i.e. vascular thrombosis or multiple and recurrent foetal losses) and at least one of antiphospholipid antibodies (aPL), i.e. lupus anticoagulant (LA), anticardiolipin (aCL) and/or anti-β2-glycoprotein 1 (β2GP1) antibodies. Most of our sAPS patients had APS associated with systemic lupus erythematosus (SLE) (n=60; 87%), while the rest had Sjögren's syndrome (n=8; 11.5%) and ankylosing spondylitis (n=1; 1.5%). Mean duration of these rheumatic diseases in sAPS patients was 5.69+2.87 years, ranging from 1 to 13 years.

Characteristics of two subgroups of APS patients were compared with 50 MetS patients (35 females, 15 males; mean age 47.68+11.66 years). The presence of metabolic syndrome among studied patients was determined according to the International Diabetes Federation (IDF) clinical definition [12]. An obligatory criterion for MetS requested by this definition is abdominal obesity defined by elevated waist circumference (WC) with gender- and ethnic-specific cut-offs, meaning 94 cm for males, and 80 cm for females belonging to European population. Besides abdominal obesity, two or more of the four additional criteria (a) hyper-

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triglyceridemia >150 mg/L, confirmed or already treated; (b) high density lipoprotein (HDL) cholesterol <40 mg/dL in males or <50 mg/dL in females; (c) blood pressure >130/85 mmHg, newly diagnosed or already treated; (d) impaired fasting glycaemia, >100 mg/dL or previously diagnosed diabetes) are necessary for the diagnosis.

For every participant, clinical data concerning thrombotic events, their appearance, management and follow-up were obtained from medical charts review. As thrombotic events, the following were recorded: superficial and deep venous thrombosis, pulmonary embolism, peripheral arterial occlusion, cerebral vascular accident and myocardial infarction.

After an overnight fast, height (m), weight (kg) and waist circumference (cm) were measured in every participant with underwear and without shoes. Waist circumference (WC) was measured at the level of the umbilicus while the participant was standing and breathing normally. Body mass index (BMI) was calculated according to the widely accepted formula dividing body weight by the square of individual's height. Morning samples of venous blood (3–5 mL) were withdrawn from every participant for the analysis of serum glucose and lipids. Serum vitamin D levels were determined in every participant .

The study was approved by the Institutional Ethics Committee. All examinations were conducted according to the most recent amendment of Declaration of Helsinki (Edinburgh, 2000), only after obtaining an informed consent for participation in the study from every subject.

Statistical analysis was performed using the STATISTICA 10 software program. Descriptive statistics was used. Prevalence of MetS as well as of its individual components, within studied groups of patients was expressed as percentage. Testing significance of their differences, the Student's t-test and Fisher's exact test were used, considering p value <0.05 statistically significant.

3.2. Results

3.2.1. Prevalence of MetS among patients with APS

Metabolic syndrome was observed in high percentage of patients with APS. Its prevalence did not differ significantly between PAPS (36.76%) and sAPS (42.03%) patients (p=0.53).

Anthropometric and metabolic syndrome characteristics among studied groups are given in **Table 3**. Borderline statistical significance of the difference in WC value was observed when two subgroups of APS patients were compared with MetS patients (F=2.77, p=0.065), while BMI has differed highly significantly between these groups (F=9.765, p=0.0001). In spite of slightly lower BMI and slightly higher WC in PAPS patients, neither BMI (p=0.434) nor WC (p=0.275) did differ significantly between two subgroups of APS patients.

Atherogenic dyslipidaemia, represented by hypertriglyceridemia and low HDL cholesterol, was the most prevalent characteristic of metabolic syndrome among PAPS patients. In spite of this, prevalence of low HDL cholesterol among PAPS patients were significantly lower than in MetS patients (48.3% vs. 70%, p=0.02). Prevalence of hypertriglyceridemia (45.59% vs.

42.03%, p=0.67) and low HDL cholesterol (48.53% vs. 53.62%, p=0.55) did not differ significantly between PAPS and sAPS patients. Hypertension was significantly less prevalent among these patients compared with MetS (23.53% vs. 58%, p=0.0002) and even with sAPS (23.53% vs. 52.17%, p=0.0007) patients. The least prevalent characteristic of metabolic syndrome among patients with APS was hyperglycaemic disorder. Compared with MetS patients in whom impaired fasting glycaemia, glucose intolerance or diabetes were present in as much as 36%, these disorders were observed in only 5.88% of PAPS patients (p=0.0001) and 4.35% of sAPS patients (p<0.0001).

	MetS	**PAPS**	**sAPS**
BMI (kg/m^2)	32.09+6.14	27.81+5.98	28.54+4.22
WC (cm)	93.67+14.36	90.73+9.18	88.53+11.91
TG > 150 mg/dL (%)	58	45.59	42.03
HDL < 40/50 mg/dL (%)	70	48.53*[*]	53.62
Hypertension (%)	58	23.53**[**]	52.17§[§]
IFG, IGT, DM (%)	36#[#]	5.88**[**]	4.35

[*]p < 0.05, PAPS vs. MetS.
[**]p < 0.01, PAPS vs. MetS.
[#]p < 0.01, sAPS vs. MetS.
[§]p < 0.01, PAPS vs. sAPS.

Table 3. Anthropometric and metabolic syndrome characteristics among studied groups.

3.2.2. Prevalence of thrombotic events among APS patients with or without MetS

Compared with patients with metabolic syndrome, prevalence of thrombotic events was significantly higher among patients with PAPS (52.94% vs. 22%, p=0.0009) and sAPS (56.52% vs. 22%, p=0.0003). Thrombotic events were reported with similar prevalence in PAPS and sAPS patients (p=0.674).

When compared with APS patients without characteristics of MetS, thrombotic events were significantly more frequent among MetS positive patients with sAPS (75.86% vs. 42.5%, p=0.0075).

Although higher among MetS positive, compared with MetS negative patients with PAPS, difference of prevalence of thrombotic events among these two subgroups of PAPS patients did not reach statistical significance (68% vs. 44.19%, p=0.0622).

3.2.3. Vitamin D status among APS patients depending on MetS and/or thrombotic events

Low VitD status (insufficiency or deficiency) was highly prevalent among PAPS (insufficiency in 27.94% and deficiency in 36.76%) and sAPS patients (insufficiency in 30.43% and deficiency in 40.58%), as well as among patients with pure MetS (insufficiency in 20% and deficiency in 32%).

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In comparison with patients with pure MetS (28.58+14.32 ng/mL), VitD concentrations were lower in PAPS (25.76+12.18 ng/mL) and sAPS patients (23.81+11.22 ng/mL), but with statistically significant difference only between these concentrations in sAPS patients and patients only with MetS (p=0.04).

Significantly lower VitD level was observed in those with coexisting MetS (MetS +), compared with those without it (MetS -) both in PAPS (MetS +: 22.0+8.52 vs. MetS -: 27.0+13.49 ng/mL, p=0.05) and sAPS patients (MetS +: 18.83+9.16 vs. MetS -: 27.42+11.28 ng/mL, p=0.0012).

Also, significantly lower VitD level was observed in APS patients with thrombotic events (TE+), compared with those without these events (TE -), both in PAPS (TE +: 20.61+12.18 vs. TE -: 31.56+12.72 ng/mL, p=0.0001) and sAPS patients (TE +: 20.67+10.43 vs. TE -: 27.9+11.04 ng/mL, p=0.007).

In 11 (22%) of patients with MetS, but without APS, some thrombotic event was confirmed. In those patients, VitD levels were also significantly lower than in those without these events (TE +: 18.45+10.66 vs. TE -: 31.43+13.63 ng/mL, p=0.003).

However, both in PAPS and sAPS patients, with coexisting MetS, previous thrombotic events did not influence serum VitD levels (PAPS: p=0.12; SAPS: p=0.93).

4. Relationship between antiphospholipid syndrome and metabolic syndrome

Estimation of prevalence of MetS in general population seems to depend to a substantial degree on the used definition, at least in certain countries or in certain ethnic groups [19–22]. Its prevalence varies between <10% in China and as much as 60% among women of Samoa [23]. Different prevalences of MetS, ranging between 18 and 48%, were also recorded among populations of different European countries and regions [20–22, 24–26]. It is interesting to emphasize that even in populations in which comparable prevalence of MetS was found using each of three already mentioned definitions, level of agreement between them was not good. As could be expected, worse agreement was found between WHO-NCEP ATP III and WHO-IDF than between NCEP ATP III-IDF definitions because of the central obesity as common denominator of the last two definitions [20, 21, 23]. This observation raised the possibility that in fact different individuals were identified as having MetS by different definitions of this syndrome [23].

In a search for factors that contribute to the manifestations of APS, MetS came into a focus surprisingly late. Data on coexistence of these two syndromes are still relatively scarce, particularly considering that of MetS and primary APS (PAPS).

4.1. Metabolic syndrome in primary antiphospholipid syndrome patients

Recently, prevalence of MetS among PAPS patients has been assessed by Medina et al. [3] and Rodrigues et al. [27]. Both surveys were performed in Hispanics among whom MetS has the

highest prevalence [28]. Defined by the IDF criteria, the prevalence of MetS among 71 Brazilian PAPS patients was 33.8% [27]. Comparable prevalences of MetS were recorded among 58 Mexican PAPS patients, using NCEP ATP III (34.5%) or IDF definitions (37.9%), while it was only 17.2% when WHO definition was applied [3]. It has been hypothesized that these cases, identified by WHO definition, were insulin resistant and with more severe forms of MetS [3, 29]. However, in investigation conducted by Medina et al., prevalence of MetS among PAPS patients was higher than in corresponding general population (17.2% vs. 13.6%) when WHO definition was used [3]. Same as in general population without APS [20, 21, 23], among PAPS patients agreement between WHO and NCEP ATP III definitions of MetS was low (κ value 0.394), moderate between WHO and IDF definitions (κ value 0.427), while only between NCEP ATP III and IDF definitions agreement was good (κ value 0.851) [3].

Regarding individual components of MetS, atherogenic dyslipidaemia was most prevalent among Mexican PAPS patients, being present in approximately half of them [3]. Significantly higher mean triglyceride levels and significantly lower mean HDL levels were previously reported among PAPS patients in comparison with controls [30–33]. Some specific autoantibodies could influence lipoprotein levels and effects in these patients. These antibodies may interfere with paraoxonase (PON) activity of HDL and, indirectly, beta-2-glycoprotein I (beta-2-GPI) [32, 33], thus promoting LDL oxidation. Relationships between lipid profile, certain anti-lipoprotein antibodies, inflammatory markers and clinical manifestations of PAPS were meticulously investigated [31–33], but on relatively small number of patients and with inconsistent results. Delgado Aves et al. have not demonstrated any correlation between the observed decrease in PON activity and either aPL nor antibodies against HDL (anti-HDL) in PAPS patients [33]. However, pro-inflammatory and prothrombotic roles were proposed for anti-HDL, being present in higher titre among asymptomatic persistently aPL positive subjects, as well as in PAPS patients with thrombotic events, when compared with patients with inherited thrombophilia and healthy controls [32]. It has been also hypothesized that hypertriglyceridemia could be the result of decreased degradation as a consequence of an inhibition of lipoprotein lipase (LPL) by aPL [3]. Currently, there are only scarce data on prevalence of antibodies against LPL (anti-LPL) in PAPS patients, speaking against their existence and influence [31].

Different authors have observed similar prevalences of hypertension among PAPS patients (22.4 and 26.3%) [3, 31], not differing significantly from that in controls (20%). Nevertheless, among PAPS patients, hypertension was significantly more frequent in those with arterial thrombosis, with which it was independently associated [31]. It is interesting that in spite of highly prevalent insulin resistance (32.8%), hyperglycaemic disorders were present in only 5% of PAPS patients [3].

4.2. Metabolic syndrome in patients with antiphospholipid syndrome associated with autoimmune rheumatic diseases.

The literature data on coexistence of MetS and numerous rheumatic diseases (i.e. systemic lupus erythematosus, rheumatoid arthritis, Sjögren's syndrome, ankylosing spondylitis, osteoarthritis, gout) are fairly extensive [1, 34–42]. The prevalence of MetS among patients with

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these disorders ranges between 14 and 62.8% [1, 3]. Qualifier "antiphospholipid syndrome associated with certain autoimmune rheumatic disease" (sAPS), which replace currently obsolete term "secondary APS", refers mainly to the systemic lupus erythematosus (SLE) despite the still unscrambled puzzle of their relations [5].

It has been speculated that high prevalence of MetS among these patients might be the consequence of certain pharmacologic interventions, particularly of chronic corticosteroid therapy [43]. However, the presence of MetS in as much as 16% of 1494 young (35.2+13.4 years) SLE patients with rather short disease duration (24.1+18.0 weeks) seems to be enough to reject this relationship as causal [2]. Nevertheless, it should be kept in mind that duration and magnitude of corticosteroid exposure could aggravate well-known cardiovascular risk factors clustering as characteristics of MetS.

On the other hand, other pharmacological interventions, primarily methotrexate (MTX) use in patients with rheumatoid arthritis, have been depicted as independent factors for reduced prevalence of MetS in these patients, especially those older than 60 years [44, 45]. This beneficial effect of MTX was attributed to its anti-inflammatory, as well as to some still unclear drug-specific effects, i.e. affecting adenosine levels and, concomitantly, glucose and lipid metabolism, or decreasing homocysteine levels as an indirect effect of simultaneous use of folic acid [44]. However, other authors reported somewhat conflicting results not being able to confirm decreasing prevalence of MetS in subjects treated with MTX, among total of 353 patients with rheumatoid arthritis [46].

5. Vitamin D and thrombosis

Prothrombotic state is one of the well-known characteristics of both antiphospholipid and metabolic syndrome. It has rather complex pathogenesis in which VitD status has an important role affecting primarily immune-mediated thrombosis. Indirect proofs for this relationship are as follows: (a) existence of nuclear VitD receptors in vascular smooth muscle cells, endothelial cells, cardiomyocytes, platelets, as well as in most types of the immune cells [47–51], and (b) expression of cytochrome P450 enzyme, CYP27B1 activity by the same cell types, enabling local synthesis of biologically active form of VitD, 1,25(OH)2D [52].

There is substantial experimental data indicating that VitD plays significant role in maintenance of physiological balance between thrombosis and haemostasis [47]. It has been demonstrated that VitD exerts following actions:

- in monocytes, expression of tissue factor (TF) is downregulated, while the expression of thrombomodulin (TM) is upregulated [53];
- in vascular smooth muscle cells, the expression of TM is upregulated, but there is also downregulation of plasminogen activator inhibitor-1 (PAI-1) and thrombospondin-1 (THSP-1) [54];
- in endothelial cells, platelet activation is attenuated as well as the expression of vascular cell adhesion molecule-1 (VCAM-1) [55].

Net result of numerous effects of this vitamin on different haemostatic factors is its antithrombotic role. Prothrombotic state that exists in VitD receptor knockout animal models proves the importance of these extra-skeletal effects of VitD as well as the observation that all of them are VitD receptor-mediated [47, 56].

However, there are still relatively few indirect and even less direct clinical evidences for the association between VitD status and thrombotic events in humans. First of them came from the epidemiological studies in which have been observed that cardiovascular morbidity and mortality depended on season of the year and latitude [47, 57, 58]. Seasonal variations were also demonstrated for tissue plasminogen activator (tPA) antigen, fibrinogen, D-dimer and von Willebrand factor (vWF) concentrations in 6538 British subjects without significant cardiovascular disorders, aged 45 years [59]. In this population, negative correlation between VitD level and tPA, fibrinogen and D-dimer concentrations was observed indicating that at least some of the seasonal variations of these thrombotic markers could be attributed to the VitD status. More direct proof for the association between VitD status and thrombosis came from the research conducted in huge population of 18 791 subjects from general population of Copenhagen [60]. Authors have observed that every quartile of a decrease in VitD concentrations was accompanied by an increase in risk of venous thromboembolism (VTE), resulting in a 37% increased VTE risk between subjects with the VitD concentrations, in the lowest quartile and those in highest quartile.

Recent publication which retested the seasonality of different cardiovascular events in regard to VitD levels, in the Scottish Heart Health Extended Cohort (SHHEC), brings a dose of confusion in previously proposed relations. Namely, it failed to prove that seasonal appearance of cardiovascular events resembled seasonal variations in serum VitD concentrations nor that these events expressed more pronounced seasonality in those with lower VitD concentrations, compared with those with its higher concentrations [61]. But, during follow-up, significant correlations were observed between lower baseline concentrations of VitD and subsequent incident cardiovascular morbidity and incident cardiovascular and all-cause mortality [61].

Results of recent trials assessing the effects of VitD supplementation on the risk of thromboembolism were inconclusive [62–64]. In the Multiple Environmental and Genetic Assessment (MEGA) case-control study which included 2506 patients with venous thrombosis, thrombotic risk was 37% lower in those supplemented with various vitamins including VitD [62]. However, in a large cohort of postmenopausal women (n=36282) from the Women's Health Initiative, daily supplementation with calcium and VitD failed to reduce the overall risk of thromboembolism [63]. Even when high doses (300,000 IU) of VitD were given intramuscularly, in a small group of patients with proven deep vein thrombosis and pulmonary embolism, observed reduction in plasma concentrations of P-selectin and high-sensitive C-reactive protein (hs-CRP) did not reached statistical significance [64]. Additional information could be expected from the ongoing Vitamin D and OmegA-3 Trial (VITAL) and that is why the results of this investigation are eagerly awaited [65].

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6. Role of vitamin D in metabolic syndrome

Currently, increasing prevalence and co-existence of obesity, MetS and hypovitaminosis D represent serious public health concern [66, 67]. New data have considerably changed hierarchy of MetS components, with the shift of the focus from obesity and insulin resistance, firstly toward fatty liver and now toward VitD deficiency [68].

It is still questionable if relationship between VitD status and obesity is unidirectional or bidirectional, with the accumulating evidence favouring the influence of VitD on body composition and not vice versa. Namely, few years ago tempting hypothesis on essential role of VitD in evolvement of obesity has been postulated [68]. It started from a situation that is completely opposite to the "thrifty genotype hypothesis" proposed long ago [69] and gave the feasible explanation not only for obesity and MetS epidemic in adults but also for their growing prevalence among children [70]. According to this hypothesis, we are living in "obesogenic" environment, loaded with energy resources and unsuitable for efficient metabolism. It has been proposed that VitD as an ultraviolet (UV)-B radiation sensor (i.e. decline in its concentrations) could induce shift toward "winter metabolism", characteristic for MetS [68]. If this is true, then it could be expected that VitD supplementation may be efficient in prevention and treatment of obesity and MetS. Significant decrease in body fat mass after 12 week of VitD repletion (25 μg of cholecalciferol daily), compared to placebo (−2.7+2.0 kg vs. −0.4+2.0 kg, $p<0.001$), could be the proof for this presumption [71]. It was also speculated that VitD deficiency during pregnancy could lead to the epigenetic changes predisposing, in that manner, new-born children to obesity and MetS later in life [68, 70]. Experimental support for these assumptions is the expression of VitD receptors on adipocytes and its involvement in adipogenesis which is regulated by the intracellular concentrations of VitD [72], as well as inhibition of lipid accumulation in adipocytes and their atrophy achieved by the knock-down of VitD receptors [72, 73].

Nowadays, VitD deficiency is common even in general population (49%), but significantly more prevalent ($p=0.006$) and quite similar in overweight/obese patients with MetS (72%) and without MetS (69%) [74]. Premise that exaggerated adiposity could lead to VitD insufficiency or deficiency by its seclusion within adipose tissue could not be confirmed. It has been shown that VitD concentrations varied considerably (range 4–2470 ng/g) in the subcutaneous abdominal fat of 17 severely obese patients (BMI=48.7+8.1 kg/m2) undergoing bariatric surgery [75]. In spite of the average weight loss of 54.8 kg after one year and continuous VitD supplementation with more than 2500 IU/day, mean serum VitD concentrations did not change significantly during this period (23.1+12.6 vs. 26.2+5.36, $p=0.58$) [75].

Most of the studies have confirmed that serum VitD concentrations significantly inversely correlated with obesity parameters, BMI ($r=-0.159$, $p=0.007$) [74], or waist circumference ($p<0.001$) [76] as well as with serum triglycerides ($r=-0.149$, $p=0.012$) [76]. In the lowest quartiles of VitD concentrations corresponding to its severe deficiency, odds ratio (OR) for hypertriglyceridemia was 2.74 (95% CI: 1.64–4.57) [77]. This association between serum concentrations of VitD and triglycerides could be explained by the activation of lipoprotein lipase by VitD in adipocytes [76]. No significant relation could be demonstrated between VitD status and total-

(r=-0.044, p=0.461) [74], low density lipoprotein (LDL)- (r=-0.005, p=0.932) and high density lipoprotein (HDL)-cholesterol (r=0.065, p=0.276) [74]. Interestingly, hypothesis was proposed ten years ago stating the possibility that statins could be the analogues of VitD, acting via same receptors, particularly when we are talking about their mutual effect of enhancement of immune competence [78]. So, it seems that this absence of association between VitD status and parameters of cholesterol metabolism made this hypothesis shaky to some extent.

Another component of MetS for which association with VitD status has not been unequivocally confirmed is hypertension. Variability of blood pressure driven by the seasons or latitude speaks for the existence of this association, as well as the results of experimental studies pointing to VitD as an inhibitor of the renin-angiotensin-aldosterone axis [79, 80]. Negative correlation between VitD concentrations and blood pressure was demonstrated in most but not all of the surveys. This negative association was stronger in subjects younger than 50 years [81–83], while the absence of any relationship between VitD status and hypertension was also registered in some of the trials [74, 76, 84, 85], particularly those conducted in older subjects [84, 85]. However, in postmenopausal women with the VitD concentrations in the lowest quartiles corresponding to its severe deficiency, odds ratio (OR) for hypertension was 1.81 (95% CI: 1.15–2.85) [77].

7. Role of vitamin D in antiphospholipid syndrome

Although APS represents acquired, autoimmune condition, its pathophysiology and, especially pathophysiology of thrombosis in APS is highly heterogeneous, involving different genes and acquired factors [86], VitD insufficiency/deficiency being among them.

Same as for relationship between MetS and APS, much more is known about the impact of VitD status on antiphospholipid syndrome, associated with autoimmune rheumatic diseases, than on primary antiphospholipid syndrome. Patients with PAPS represent the population of particular interest for the investigation of interrelations with components of MetS and/or VitD status since these patients, unlike those with sAPS, were not treated with drugs (i.e. corticosteroids, immunosuppressants) which may affect expression of most of the MetS characteristics as well as VitD level.

One of the first announcements on the prevalence of VitD insufficiency or deficiency in PAPS and their impact on its manifestations dated from 2010 [87]. This letter to the editor presented the results of research conducted by Brazilian investigators in the group of forty-six PAPS patients, younger than 60 years, in whom the VitD levels were assessed in the autumn, when it was expected to be highest. VitD deficiency was found in 11% and insufficiency in 74% of these PAPS patients, resembling the findings of Italian authors [88] which have reported the prevalence of VitD deficiency in 17% and insufficiency in 60% of PAPS patients. It is interesting that Brazilian authors have noticed that VitD insufficient PAPS patients tended to be more overweighed than those with adequate VitD level [87]. Also, it seems that thrombotic APS is characterized with significantly lower concentrations of VitD than purely obstetric clinical syndrome (20.8 vs. 33.3 ng/ml, $p<0.01$) [88] highlighting once again the role of this vitamin in

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thrombosis. High prevalence of VitD deficiency among patients with APS (49.5%) and its significant correlation with thrombotic events were confirmed by Israeli authors [68]. In vitro, they have also demonstrated VitD ability to inhibit anti-β2-glycoprotein I autoantibody (anti-β2-GPI Ab)-mediated TF expression [89].

Seasonal variations in VitD concentrations were demonstrated in PAPS patients same as in healthy controls, with preserved differences in its level between these two groups through all seasons [88, 90]. These differences were most pronounced during summer, while they were not statistically significant only during the spring. This observation was somewhat surprising, given the lack of banning from sun exposure in these patients. That sun avoidance is not a cause of highly prevalent VitD deficiency and insufficiency in PAPS patients was indirectly demonstrated in previous Italian studies [88, 90] by observed absence of any difference in VitD levels between antinuclear antibodies (ANA)-positive and negative PAPS patients.

Until now, there is no valid explanation for the probable cause-and-effect relationship between insufficient VitD level, on one side, and PAPS or sAPS, on the other. There are only assumptions, and even they are much better clarified for sAPS [91–93], especially that accompanying SLE [91, 94, 95]. It is obvious that low levels of vitamin D in sAPS could not be attributed purely to banning of sun exposure or the use of certain medication in these patients. In an Israeli and European cohort of patients with SLE, significant negative correlation ($r=-0.12$, $p=0.018$) was demonstrated between the serum VitD concentrations and disease activity, assessed by SLE disease activity-2000 (SLEDAI-2K) and European Consensus Lupus Activity Measurement (ECLAM), which were converted into standardized z-value [94]. Severe VitD deficiency was found in 17.89% of 123 SLE patients with short disease duration, while the presence of renal disease (OR 13.3, 95% CI 2.3–76.7, $p<0.01$) and photosensitivity (OR 12.9, 95% CI 2.2–75.5, $p<0.01$) were its strongest predictors [95]. Investigation conducted in a small group of young women with newly diagnosed SLE, from one of the sunny places in Iran, gave very interesting results. VitD deficiency was highly prevalent among these patients, mild in 12.5%, moderate in 62.5% and severe in 17.5% of them [96]. It was much more pronounced in them than in general population of the similar age in that region, in whom mild VitD was present in 15.5%, moderate in 47.1% and severe in 7.1%. Very interesting was also an observation that serum VitD concentrations showed significant negative correlation with another disease activity score, the British Isles Lupus Assessment Group (BILAG) ($r=-0.486$, $p=0.001$), in spite of the short duration of disease [97]. Hypothetical explanation for the low serum concentrations of VitD in SLE patients by the existence of inhibiting anti-VitD antibodies in circulation could not be confirmed by the literature data [97, 98]. Their existence could be proven in 4–6% of patients with SLE and 3.5% of APS patients. Its association with anti-dsDNA ($p=0.0004$) could point to its potential role in this condition, but being only one of 116 different antibodies present in SLE patients characterized by the polyclonal B lymphocyte activation, it is still uncertain if it is pathogenic [97]. It seems that their presence did not affect VitD levels in these patients [97, 98], and it was speculated that they could be the consequence of high-dose VitD consumption rather than the cause of this vitamin deficiency [99].

Once again, it is important to emphasize that VitD deficiency is more pronounced in more severe APS phenotypes, i.e. thrombotic APS [88]. It could be speculated that supplementation

of this vitamin in these very patients may have certain beneficial effects [88, 99], but there is still no prospective studies proving them. Hypothesis of statins as VitD analogues has not still been tested in well-designed, randomized prospective trials [78]. However, since its proposal, there have been many experimental and small clinical studies confirming statins therapeutic value in APS patients, particularly in those with its thrombotic form [99–103]. So, future studies are badly needed to determine all the aspects of VitD repletion in APS prevention/therapy (choice between VitD precursors, its active form or VitD analogues, their dosage and treatment goals).

8. Key messages

- Prevalence of metabolic syndrome in APS, primary or associated with certain rheumatic diseases, is high.
- Atherogenic dyslipidaemia is the most prevalent characteristic of metabolic syndrome in APS patients.
- Prevalence of thrombotic events was significantly higher in APS patients with coexisting metabolic syndrome, compared with APS patients without metabolic syndrome characteristics.
- Among APS patients, prevalence of vitamin D deficiency was significantly higher in patients with coexisting metabolic syndrome, compared with those without it.
- Among APS patients, vitamin D level was also significantly lower in patients with previous thrombotic events than in those without them.
- In the contemporary literature, there are much more data in favour of pathogenic than therapeutic role of vitamin D in thrombotic events characterizing APS and/or metabolic syndrome. So, prospective studies designed to test all the aspects of VitD repletion in prevention and/or therapy of thrombotic events in APS and/or metabolic syndrome are badly needed.

9. Conclusions

Elucidating interrelationships between vitamin D deficiency, metabolic syndrome phenotype and thrombotic events in APS patients open up the possibility of distinguishing those subjects with the particularly high cardiovascular risk and ensuing need for the strict control of modifiable risk factors and vitamin D supplementation.

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Chapter 10

Therapeutic and Prophylactic Potential of Vitamin D for Multiple Sclerosis

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Additional information is available at the end of the chapter

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Abstract

A plethora of investigations demonstrated that vitamin D (VitD) has a broad immunomodulatory potential. It induces tolerogenic dendritic cells *in vitro* leading to the development of regulatory T cells that have a key role in immunomodulation of autoimmune diseases including multiple sclerosis (MS). Studies showed that many MS patients present lower serum levels of VitD than healthy subjects. In addition, VitD supplementation has been associated with a reduced relative risk of developing MS. Considering the alterations in VitD levels in patients and also the immunomodulatory properties of VitD, it would be interesting to evaluate VitD potential as a tolerogenic adjuvant in experimental models of MS. In this context, our research team has been investigating strategies employing VitD to establish an *in vivo* tolerance state toward central nervous system antigens in experimental autoimmune encephalomyelitis (EAE). We observed that the association between a myelin peptide and VitD determined both therapeutic and prophylactic effects on EAE development.

Keywords: vitamin D, multiple sclerosis, experimental autoimmune encephalomyelitis, immunomodulation, myelin peptides

1. Introduction

The immune system is well known by its ability to defend the host against infections. In this sense, it is academically subdivided into innate and adaptive immune responses. Innate immunity is the first defense line and includes the microbicidal activity of macrophages and

polymorphonuclear cells. Host defense against microorganisms is dependent upon recognition of pathogen-associated molecular patterns, mainly by toll-like receptors (TLRs) present in these cells. Otherwise, adaptive immunity requires specific antigen recognition by B and T lymphocytes. Differently from B cells that can directly recognize the antigens, T cells require previous antigen processing and interaction of epitopes with major histocompatibility complex proteins that are then expressed at the surface of antigen-presenting cells (APCs) as, for example, dendritic cells (DCs). Due to their strong potential for proliferation and activation, B and T cell activity needs to be regulated. A special T-cell subpopulation called regulatory T (Treg) cell plays a major role in controlling inflammatory immune responses. To maintain its homeostasis, the immune system has to manage a balance between inflammatory and anti-inflammatory responses. The imbalance of these immune responses leads to the development of many diseases such as autoimmune pathologies. In this context, other T-cell subpopulations such as T helper type 1 (Th1) and type 17 (Th17) cells, which are inflammatory, and type 2 cells (Th2), which are predominantly anti-inflammatory, are also involved. Besides its ability to eliminate pathogens and restore the host homeostasis, the immune system has also a mechanism to hamper the development of an immune response against the body's own tissues. This mechanism, called self-tolerance, can be disrupted by the combination of a variety of genetic, environmental, and immunological factors that lead to autoimmunity. The relevance of vitamin D (VitD) in multiple sclerosis (MS), which is an autoimmune disease involving the central nervous system (CNS), is discussed in this chapter.

2. VitD metabolism

The history of VitD is strongly linked to rickets and its treatment with cod liver oil. In 1922, McCollum [1] coined the term vitamin D to refer to the antirachitic factor found in cod liver oil [2]. For this reason and for a long time, the most widely accepted physiological role of VitD was related to calcium and phosphorus metabolism and bone mineralization [3]. However, since the 1980s, many researches implicated VitD on the cardiovascular, endocrine, and central nervous system (CNS), as well as on the immune system physiology. The active form of VitD (1α,25-dihydroxyvitamin D_3) determines pleiotropic effects in human body through binding to vitamin D receptor (VDR), which is a member of the steroid hormone receptor superfamily found in a variety of human cells. The biological effects of VitD can be elicited by non-genomic and genomic mechanisms depending on the cell location of VDR. The non-genomic (rapid) mechanisms consist in VitD direct effect on the cells through membrane VDR binding. These effects include, for example, the activation of protein kinase C in different organs [4]. The genomic mechanism is determined by intracellular VDR that heterodimerizes with retinoic X receptor after binding to active VitD. This heterodimer is then translocated to the nucleus leading to activation or inhibition of a vast diversity of genes [5].

Some of the most important aspects of VitD epidemiology have been established by the scientist Michael Holick and his collaborators. As many people do not have an adequate

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sunlight exposure due to skin cancer risk, sedentary lifestyle, darker skin, or during the winter in countries far from the equator, there is an increasing number of persons with VitD deficiency around the world [6]. In the past few years, VitD deficiency has been associated with the etiology of many chronic diseases, like Crohn's disease, infections of the upper respiratory tract, cancer, myocardial infarction, Alzheimer's disease, autoimmune diseases, and others [7]. According to current knowledge, VitD serum levels should be between 30 and 100 ng/mL in healthy humans. VitD insufficiency is related to levels between 21 and 29 ng/mL, whereas a pronounced VitD deficiency is considered in individuals whose VitD levels are below 20 ng/mL. On the other hand, serum levels over 150 ng/mL can determine intoxication VitD intoxication [8]. Excessive oral intake of VitD may cause a hypervitaminosis condition with toxic effects such as hypercalcemia and hypercalciuria. Theories concerning the mechanisms of VitD toxicity involve elevated plasma concentration of VitD itself or its metabolites that culminates in overexpression of a variety of genes [9]. Although solubility of vitamins (fat or water) has no direct effect on toxicity, the ability of fat-soluble vitamins such as VitD to accumulate in the adipose tissue determines their higher toxic potential than water-soluble vitamins. For example, subcutaneous fat necrosis releases tissue-accumulated VitD that leads to hypervitaminosis and its toxic effects [10].

The highest amounts of VitD are synthesized by the skin exposed to sunlight. Ultraviolet radiation converts 7-dehydrocholesterol in pre-vitamin D3. Then pre-vitamin D3 suffers a spontaneous thermal isomerization into vitamin D3, named cholecalciferol [11]. Due to this essential role of sunlight, this vitamin has been called "sunshine vitamin" [12]. Smaller amounts of VitD can be obtained from intake of certain foods such as mushrooms, fish, milk, and eggs [13]. To become a metabolically active hormone, cholecalciferol needs to be hydroxylated twice. The first hydroxylation takes place in the liver and converts cholecalciferol into 25-dyhidroxyvitamin D (calcidiol) via the enzyme 25-hydroxilase [14]. Plasma calcidiol levels are usually used as a parameter of VitD status because it increases in proportion to VitD intake [15]. After that, calcidiol binds to a carrier molecule, known as the vitamin D-binding protein, to be systemically transported to tissues that express 1α-hydroxylase (CYP27B1) [16]. The second hydroxylation, which generates the bioactive metabolite 1,25-dihydroxyvitamin D3 (calcitriol), occurs at the renal proximal tubular cells that are rich in CYP27B1 [17]. This reaction involves the sequential reduction of flavoprotein, renal ferredoxin, and cytochrome P-450 [18]. A critical physiological role in skeletal homeostasis is mediated by calcitriol. Concisely, hypocalcemia stimulates parathyroid glands to release parathyroid hormone, which activates renal CYP27B1 enzyme function, resulting in calcitriol production. Besides, parathyroid hormone stimulates osteoclast maturation to release calcium and phosphate from the bones. Calcitriol also reduces renal calcium excretion and increases calcium absorption from foods in the intestine. When normal calcium levels are obtained, calcitriol exerts a feedback regulation in the parathyroid gland, downregulating CYP27B1 activity to avoid VitD intoxication [14]. Besides the kidneys, 1α-hydroxylase has been reported in many tissues including bone, placenta, prostate, and parathyroid gland. In addition, several cancer cells and immune cells, such as macrophages, T lymphocytes, and DCs, are also able to produce this enzyme [19,20].

3. Immunomodulatory properties of VitD

First evidences of VitD role in the immune system regulation date from the 80s. Haq [21] demonstrated that active VitD, but not its non-active form, blocked the production of IL (interleukin)-2 and consequently inhibited T-cell proliferation. Based on this downmodulatory effect, the potential of VitD to increase organ survival in experimental allograft transplantation was also evaluated. First studies in this field were based on the *in vitro* immunosuppressive effects of VitD and its analogs. One of the most evident toxic effects of high VitD doses, which are usually required to avoid transplant rejection, is hypercalcemia. To avoid this and other toxic effects such as bone resorption, many efforts were done to develop synthetic structural analogs of active VitD that still preserved its immunomodulatory properties [22]. When tested *in vivo*, a 20-epi-vitamin D3 analog did not prolong renal allograft survival in Lewis rats and also led to the development of hypercalcemia [23]. These authors emphasized the importance of more experimental studies to evaluate the potential of VitD and its analogs to prevent graft rejection. Later, Hullett et al. [24] successfully demonstrated that Lewis rats orally receiving active VitD presented prolonged survival heart allografts without hypercalcemia. Over the years, a much broader role of VitD in the immune system was disclosed and the mechanisms underlying its immunomodulatory effects were progressively elucidated. Currently, calcitriol is largely known to modulate both innate and adaptive immunity through its binding to VDR, which is present in a multitude of immune cells. Although VitD can bind to both genomic and non-genomic targets, the most important immunomodulatory properties are elicited by genomic mechanisms [25].

It is well known that VitD stimulates the innate immune system by enhancing the antimicrobial ability of monocytes and macrophages. This effect is mainly associated with TLRs activation and increased release of cathelicidin and IL-1β by these cells [26]. Clinical evidences suggested a strong correlation between a poor VitD status and an increased susceptibility to infections. VitD has also been linked to more severe infectious diseases [27–29]. Moreover, Nouari et al. [30] recently demonstrated that active VitD can enhance the microbicidal activity of human monocyte-derived macrophages against *Pseudomonas aeruginosa*.

Conversely, VitD has an inhibitory effect on the adaptive immune system. It directly targets APCs, which are a very important link between the innate and adaptive immunity. In this sense, conventional APCs as DCs are profoundly affected by VitD. The mechanisms underlying the effects of VitD on DC function were recently reviewed by Barragan et al. [31]. *In vitro* treatment with active VitD or its analogs inhibits both differentiation and maturation of human and murine DCs leading to changes in its phenotype and function [32]. The immature or semi-mature state induced by VitD is generally characterized by a decreased expression of co-stimulatory molecules such as CD40, CD80, and CD86. This state determines a tolerogenic DC phenotype associated with reduced IL-12 and increased IL-10 production. The addition of VDR agonists or active VitD during differentiation of DCs *in vitro* determines a reduction in subsequent T-cell proliferation and also in interferon-gamma (IFN-γ) production [33]. Tolerogenic DCs are also able to induce the development of Treg cells that are mainly characterized by the expression of CD4 and CD25 molecules and production of anti-inflammatory

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cytokines such as IL-10 and transforming growth factor-β (TGF-β) [34]. As mentioned before, Treg cells play a major role in controlling inflammatory immune responses. The main mechanisms underlying their suppressive activity include the induction of inhibitory molecules such as cytotoxic T-lymphocyte antigen 4, the production of inhibitory cytokines that leads to impaired T-cell expansion and the release of granzymes and perforin that trigger T-cell death [35]. Chambers et al. [36] demonstrated that addition of active VitD on human CD4+ T lymphocytes significantly increased the expression of forkhead box protein P3 (Foxp3) that characterizes Treg cells.

The direct effect of VitD on T cells was the first evidence of the immunomodulatory activity of this hormone. Active VitD suppresses Th1 inflammatory immune response through inhibition of IL-2 and IFN-γ production, which are the main cytokines produced by this Th cell subset. This subject was revised by Lemire et al. [37]. These authors described that VitD preferentially inhibited Th1 functions having little effects over Th2 cells. At that time, they already suggested that this vitamin could have a potential therapeutic application in Th1-mediated diseases as is the case of some autoimmune pathologies.

Many inflammatory responses are also related to the development of Th17 cells and its signature cytokine named IL-17. It is largely known that this T-cell subpopulation is involved in the pathogenesis of a variety of inflammatory and autoimmune disorders [38]. In this context, Th17 cell pathogenicity is frequently related to a Th17-Th1 functional plasticity that is regulated by the cytokine milieu [39]. The immunomodulatory effects of VitD on Th17 cells are not clear and depend upon the disease. Most of what is known concerning VitD effect on these cells is based on experimental studies. For example, oral treatment with active VitD prevented and partly reversed experimental autoimmune uveitis in mice. This effect was related to both decreased IL-17 production and impaired development of Th17 cells [40]. Moreover, Chang et al. [41] demonstrated that active VitD treatment protected mice from experimental autoimmune encephalomyelitis (EAE) by inhibiting the differentiation and further migration of Th17 cells to the central nervous system (CNS). Even though the effect of VitD on animal models is evident, human data are controversial and there is not a consensus in the literature yet.

Data on the effects of VitD on the development of Th2 cells are also conflicting. This T-cell subset is able to suppress Th1 inflammatory immune response through the production of anti-inflammatory cytokines such as IL-4 and IL-5. A direct effect of active VitD on Th2 cells was demonstrated by Boonstra et al. [42]. Even in the absence of APCs, these authors observed an increased frequency of IL-4-, IL-5-, and IL-10-producing murine CD4+ T cells after *in vitro* stimulation with VitD. In addition, there was a decrease in the frequency of IFN-γ-producing cells. However, Staeva-Vieira and Freedman [43] demonstrated that active VitD inhibited the *in vitro* production of both, IFN-γ and IL-4 by murine CD4+ T cells.

Other T-cell subsets such as CD8+ T cells and natural-killer T cells (NKT) are also targets of VitD. Chen et al. [44] demonstrated that active VitD signaling through VDR is essential to control pathogenic CD8+ T cells in inflammatory bowel diseases. The importance of VDR was also highlighted by Yu et al. [45] who demonstrated a critical role of VDR expression in the development of induced NKT cells from mice fed with synthetic diets containing active VitD.

There are few studies concerning the impact of VitD on B cells. *In vitro* assays indicated that the active form of VitD inhibited the production of immunoglobulin E and increased IL-10 production by B cells [46,47]. Similarly to the effect over DCs, active VitD also downregulated the expression of co-stimulatory molecules at the surface of human B cells. Drozdenko et al. [48] demonstrated that the antigen-presenting function of B cells was compromised by *in vitro* addition of active VitD to B and T cell co-cultures. The authors detected a reduced expression of the co-stimulatory molecule CD86 in B cells along with diminished T-cell expansion and lower cytokine production by these cells. A general scheme indicating some of the most relevant effects of VitD on innate and adaptive immunity is displayed in **Figure 1**.

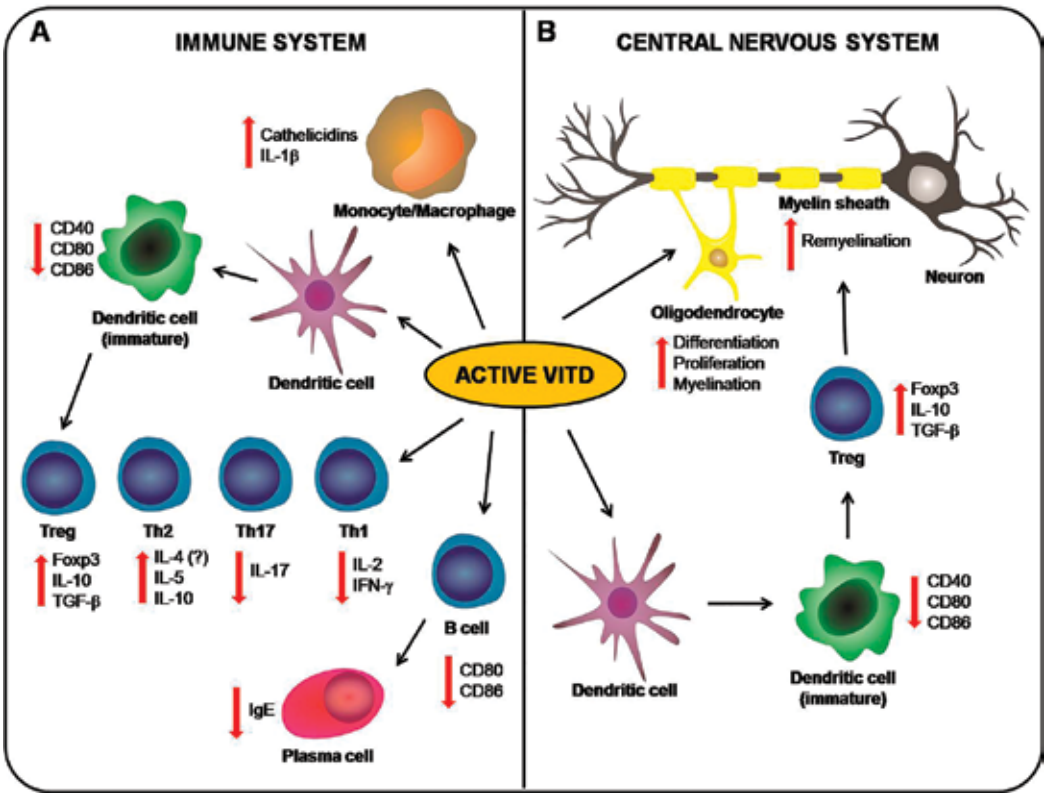

Figure 1. VitD action on the immune and the central nervous systems. (A) Effect of active VitD on the innate and the adaptive immunity cells and (B) direct and indirect effects of active VitD on the central nervous system.

The immunomodulatory potential of VitD has been widely explored in the field of autoimmune diseases. Epidemiological studies demonstrated that low VitD is correlated with a higher incidence of autoimmune diseases. Besides, genetic factors as VDR polymorphisms are also linked to autoimmune disorder susceptibility. The association between VitD and systemic and organ-specific autoimmune diseases, including multiple sclerosis (MS), was carefully reviewed by Agmon-Levin et al. [49].

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4. Epidemiological evidence that VitD is relevant in MS

MS is an autoimmune disease characterized by the activation of self-reactive T cells specific for CNS antigens. This immune response triggers an initial inflammation in brain and spinal cord that is then followed by demyelination, axonal damage, and scar formation [50]. The pathogenic immune response observed in MS is mainly mediated by Th1 and Th17 [51]. About 85% of MS patients present with a biphasic disease characterized by alternating episodes of neurological disability and recovery, which is entitled as relapsing remitting MS (RRMS). Within 20–25 years, 60–70% of these patients progress to a secondary-progressive disease that is characterized by progressive neurological deterioration. Approximately 10% of the patients display a disease course classified as primary progressive MS, which is characterized by a continuous decline in neurological performance without any recovery episode [52]. Magnetic resonance imaging (MRI) is playing a prominent role in the diagnosis and also in the analysis of MS therapy efficacy [53]. As mentioned before, autoimmune diseases result from the interactions of environmental and genetic risk factors. Environmental risk factors considered essential for MS development include infections and non-infectious factors that comprise differences in diet and other behaviors, such as cigarette smoking and sunlight exposure [54,55]. The development of MS has been strongly associated with viral and bacterial infections [54,56]. More recently, a possible relationship between MS and Candida species was proposed [57–59]. Our research team recently demonstrated that previous infection with *Candida albicans*, a commensal and opportunistic human pathogen, aggravates the clinical signs of EAE [60].

Epidemiological data on MS incidence and prevalence drew attention to a possible link between the geographical distribution of the disease and exposure to the sun, UV radiation/intensity, and VitD levels. This sunshine hypothesis also known as latitude-gradient effect was initially proposed by Limburg [61] that suggested a correlation between higher MS occurrence and increasing distance from the equator. According to the World Health Organization [62], the highest prevalence of MS occurs in Europe (80 per 100,000 people) and the lowest prevalence in Africa (0.3 per 100,000). More recently it was reported that, until 2013, the number of MS was higher in northern hemisphere and lower in southern hemisphere, with the exception of Australia and New Zealand [63]. A latitudinal variation was also identified in the continents. For example, geospatial analysis carried out in North American regions showed an inverse correlation between MS and UV radiation, that is, higher MS rates have been associated with lower UV radiation due to a south-north latitudinal gradient [64]. Interestingly, a series of lifestyle changes that include sun evasion associated with skin protection and extra time indoors, or increased charter tourism to warmer countries during the winter, seems to abolish latitude effects on UV radiation exposure [65]. According to these authors, this association between sun exposure and MS can be determined by distinct effects: by the VitD generated by sun exposure, by direct sun effects, or by a combination of both. These possibilities are reinforced by data from experimental animals and also from dietary studies in human populations. Dermal application of VitD ointments and UV radiation in VDR knockout mice were both able to induce Treg cells [66]. Further study indicated that these UV-induced Treg

cells were able to migrate to the CNS of mice with EAE where they downregulated the inflammatory activity [67].

A lower prevalence of MS in some northern countries, which in a general way are expected to have a higher number of patients with the disease, could be explained by VitD-related dietary factors. For example, VitD sufficiency could be achieved through a traditional diet that includes fatty fish and cod liver oil. This possibility has been suggested to explain the reduced risk of MS in Norway that is located at the north of the Arctic Circle [68]. The relevant role of dietary VitD intake in MS was examined in two large cohorts of women: the Nurses' Health Study (NHS; 92,253 women followed between 1980 and 2000) and the Nurses' Health Study II (NHS II; 95,310 women followed between 1991 and 2001). The authors concluded that intake of VitD from supplements had a protective effect on the risk of developing MS [69]. A recent study with 953 MS patients indicated an inverse association between MS risk and the dose of cod liver oil during adolescence, suggesting that this stage of life is an important susceptible period for adult-onset MS, reinforcing the importance of dietary VitD as a risk factor for MS [70]. Altogether these data supported the possibility that MS patients could have lower levels of VitD. Regarding this, the largest study to date compared VitD levels present in Iranian MS patients (n = 700) to the ones found in healthy individuals (n = 1000) and demonstrated that VitD levels were significantly lower in patients with MS [71]. Strong evidences also support the likelihood that low VitD levels can be related to disability and progression of this disease. In a study with 267 patients, lower serum VitD levels were also associated with higher rates of both relapse and disability [72]. Other authors showed an association between a low VitD status at the start of RRMS and the early conversion to secondary progressive MS [73]. The possible effect of VitD levels in the therapeutic efficacy of interferon beta 1b(IFN-β-1b), fingolimod (FTY), and glatiramer acetate (GA) was also investigated. Among patients treated with IFN-β-1b, higher VitD levels were associated with a reduced risk of relapse [74], whereas lower VitD levels early in the disease course correlated with a strong risk factor for long-term MS activity and progression [75]. In a similar way, in FTY-treated patients, higher VitD levels were associated with an approximately 50% reduction in new inflammatory events and in relapses [76]. By contrast, there was no significant benefit of higher VitD levels with respect to inflammatory events, relapses, or disability progression in GA-treated patients [76]. The strong correlation between low VitD levels and higher MS susceptibility reinforces the hypothesis that VitD deficiency leads to MS and/or disease progression and stimulates new researches focused on supplementation of these patients with VitD.

5. Supplementation of MS patients with VitD

The recent identification of VitD as a risk factor for MS susceptibility, and more recently as a potential modifier of disease course, inspired several clinical trials in relapsing MS [77]. It has been proposed that VitD supplementation is a low-cost and a low-risk intervention that may potentiate the efficacy of certain treatments against MS, without the risk of provoking serious adverse events as occurs with other combination therapies [76]. In effect, many patients are being already supplemented with VitD. However, it is not known whether supplementation has a significant impact on MS progression. A clinical trial (NCTO1339676) employing oral

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supplementation with active VitD (20,000 IU/week, cholecalciferol, Dekristol) administered once a week during 12 months together with IFN-β-1b resulted in reduction of MRI lesions in the brain of MS patients [78]. In another clinical trial (NCT 00785473), this same dose (20,000 IU/week, cholecalciferol, Dekristol) was administered during 24 months in RRMS patients under treatment with IFN-β-1b, GA, or natalizumab. Even though the patients presented a significant increase in serum VitD levels, the markers of systemic inflammation were not modified. The authors suggested that the anti-inflammatory effects of VitD supplementation are limited to RRMS patients with VitD insufficiency or to earlier stages of the disease [79]. A higher dose of VitD3 (50,000 IU/week) administered by oral route during a short period (2 months) reduced disability in RRMS patients and surprisingly upregulated IL-6 and IL-17 gene expression in the peripheral blood mononuclear cells of these patients [80]. Similarly, the same VitD dose (50,000 IU) administered by oral route every five days for 3 months in 94 RRMS patients under treatment with IFN-β-1b reduced disability of these patients but also increased IL-17 serum levels in comparison to a placebo group [81]. Investigations in this area suggested that changes in IL-17 levels could be related to the adopted VitD doses. For example, Golan et al. [82] demonstrated that IL-17 serum levels were significantly increased in a lower dose group (800 IU/per day), whereas patients that were taking higher doses (4370 IU/per day) presented heterogeneous IL-17 responses: 40% of them had decreased serum IL-17 levels, whereas 45% had increased IL-17 levels after three months of supplementation. These authors suggested that IL-17 data must be interpreted with caution as serum IL-17 is not an established biomarker of MS disease activity. Furthermore, IL-17 serum levels before treatment with IFN-β could not be correlated to disease activity parameters [83]; IL-17 also showed a trend toward higher levels in MS patients with inactive disease compared to those with active disease [84]. More recently, 40 patients with RRMS were randomized to receive 10,400 IU or 800 IU of cholecalciferol daily for 6 months. Mean increase of VitD levels from baseline to the ones detected at final visit was larger in the high-dose group than in the low-dose one and adverse events were minor and did not differ between the two groups. Interestingly, in the high-dose group, but not in the low-dose one, there was a reduction in the proportion of IL-17+CD4+ T cells. The authors concluded that daily cholecalciferol supplementation with 10,400 IU is safe and well tolerated in patients with MS and determines *in vivo* pleiotropic immunomodulatory effects [85]. Considering that IL-17 is an important cytokine involved in MS pathogenesis, further studies are needed to clarify the role of VitD on these unexpected elevated IL-17 levels. Therefore, until nowadays it is not possible to consider IL-17 as a biological marker for VitD levels in human body.

The researches done so far strongly suggest that VitD supplementation could be useful in MS treatment. However, the exact doses to be prescribed to patients presenting different clinical symptoms are still waiting to be determined [86]. Regarding the side effects of VitD that include hypercalcemia [87] and the imbalance in serum concentration of parathyroid hormone [88], monitoring serum VitD would also be extremely important. In spite of the findings that VitD directly regulates the nervous system development and function [89], there is no scientific evidence to support its use as a monotherapy for MS in clinical practice [90]. Recent human trials concerning VitD supplementation in MS patients suggest that higher VitD doses are more efficient to control the symptoms and disease inflammatory markers. Nonetheless, to fix the

ideal dose, it is essential to measure VitD serum levels before supplementation and to follow up the patients by constantly monitoring side effects. It is important, however, to highlight that the ideal dose could vary from one patient to another. The possible use of VitD analogs devoid of side effects must be also evaluated. World Health Organization (WHO) and Multiple Sclerosis International Federation (MSIF) published in 2008 the first Atlas of MS [62], correlating the epidemiology, diagnosis, and therapy. To the best of our knowledge, WHO did not define a specific VitD dose to treat MS.

6. Therapeutic effect of VitD in EAE

Experimental autoimmune encephalomyelitis (EAE) is an animal model universally employed to investigate mechanisms of inflammation in the CNS in the context of MS. EAE is mainly induced in rodents either by active immunization with CNS antigens associated with adjuvant or by passive transfer of CNS-specific T cells. Most of the therapeutic procedures adopted nowadays were initially tested in murine EAE [91]. In 1991, it was demonstrated that VitD administration every other day for 15 days, starting 3 days before EAE induction, significantly prevented disease development and prolonged the survival of SJL/J mice [92]. This was the first report concerning the therapeutic potential of VitD on EAE. To avoid undesirable hypercalcemia *in vivo*, the immunomodulatory activity of VitD analogs were confirmed and they were equally efficient to suppress EAE development [93,94]. Since then, EAE has been widely employed to understand the mechanisms involved in VitD efficacy against MS. In this regard, one of the first studies was done with the Lewis rat model. The authors observed that VitD administered after the beginning of clinical signs determined significant clinical improvement. This therapeutic effect was associated with a striking decrease in the number of CD4+ cells, macrophages, and activated microglia in the CNS [95]. VDR is also essential for the beneficial effects of VitD on EAE since VitD treatment was not able to prevent disease manifestations in VDR-knockout mice [96]. The efficacy of VitD over EAE has also been attributed to effects on cells from the innate immunity. It decreases macrophage accumulation [97], inhibits chemokine synthesis and inducible NOS, and also suppresses CD11b+ monocyte recruitment into the CNS [98]. NKT cells also contribute to the protective effect of VitD on murine EAE. All mice lacking NKT cells [CD1d(−/−)] presented EAE symptomatology upon VitD administration, whereas the same treatment completely avoided EAE development in wild-type mice [99]. More recent data revealed that VitD administration induces tolerogenic DCs in the lymph nodes, which leads to suppression of encephalitogenic T cells, resulting in less inflammatory response in the CNS [100].

Critical effects of VitD on CD4+ T cells have been reported, whereas it is not evident if this vitamin affects CD8+ T cells, which express the highest concentrations of VDR. The effect of VitD on CD8+ T cells in EAE was evaluated in one report. The authors demonstrated that VitD inhibits EAE development even in mice lacking functional CD8+ cells, suggesting that they were not essential for VitD-suppressive effect in murine EAE [101]. The conception that the CD4+ T-cell subset was the main VitD target during EAE therapy was then established. VitD treatment triggered a reduction in the total number of lymphocytes, while the amount of IL-4

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and TGF-β-1 transcripts increased in the CNS of EAE mice [102]. Still regarding anti-inflammatory cytokines, VitD therapy was reported to be much less effective in preventing EAE symptoms in IL-4-deficient mice [103] and also failed to inhibit EAE in mice with a disrupted IL-10 or IL-10R gene [104]. A more recently described profile of CD4+ T cells termed Th17 plays a critical role in numerous inflammatory conditions and autoimmune diseases. In this context, researchers showed that VitD can inhibit the differentiation and migration of Th17 cells to the CNS, ameliorating EAE symptoms [41,105].

After the first demonstration that VitD leads to induction of CD4+CD25+Foxp3+ cells with suppressive activity *in vitro* [106] and that these regulatory cells are directly involved in the natural resolution of EAE [107], many studies validated the correlation between VitD treatment and the increment of a Foxp3+ regulatory profile in EAE [99,103,104] (**Figure 1B**). The potential for reversing inflammatory and demyelinating processes in the CNS has been attributed to an augmented generation of Foxp3+ Treg cells in the periphery and their further migration to the CNS [100,108]. New therapeutic approaches have also been tested to improve VitD efficacy in EAE. A synergistic effect was found by association of VitD with estrogen, which determined more CD4+Helios+Foxp3+ Treg cells and fewer CD4+ T cells among CNS mononuclear cells, preventing EAE development [109]. In addition to the large contribution of VitD immunomodulatory activity in EAE, this treatment can also directly act on neural cells promoting CNS remyelination and other neuroprotective effects (**Figure 1B**). *In vitro* assays indicated that this vitamin significantly enhanced proliferation of neural stem cells and their differentiation into neurons and oligodendrocytes [110]. In addition, VitD treatment modulated autophagic activity and neuroapoptosis in EAE mice. As autophagy is an evolutionarily conserved cellular catabolic process that recycles damaged organelles and its inhibition causes neurodegeneration in mature neurons, this process plays an essential role in maintaining neuronal homeostasis [111]. In summary, VitD controls EAE symptoms through reduction of inflammatory immune response and elicitation of a regulatory profile. As EAE reproduces specific features of the histopathology and neurobiology of MS [112], highlighting these mechanisms in rodent models is essential to translate VitD supplementation to MS patients.

Emphasis has been given to specific therapies, that is, to procedures that target CNS antigen and that would be, therefore, more efficient and devoid of side effects. In this context, MOG administration by different routes as intravenous [113], oral [114] or nasal [115], was able to suppress EAE symptoms. Various formulations containing myelin antigens were tested to control EAE. MOG conjugated with nanoparticles [116], mannan, [117] or inserted into a plasmid DNA [118] reduced EAE symptoms through induction of Foxp3+ Treg cells and dowmodulation of Th17 and Th1 cells. Our research group has been working in this context. Considering that an antigen from the CNS can provide the required specificity and that VitD is endowed with a strong downmodulatory potential, we anticipated that VitD could work as a tolerogenic adjuvant. Differently from the conventional immunogenic adjuvants that reinforce the immune response, the denominated tolerogenic adjuvants have the ability to downmodulate or modify the specific immune response when associated with specific antigens. Confirming this hypothesis, we recently demonstrated that a combined therapy with MOG + VitD blocked EAE development. This elevated efficacy was correlated

with reduced production of IL-6 and IL-17 by spleen and CNS cell cultures stimulated with MOG, reduced splenic DC maturation, and also a striking decline in CNS inflammation [119] (**Figure 2**).

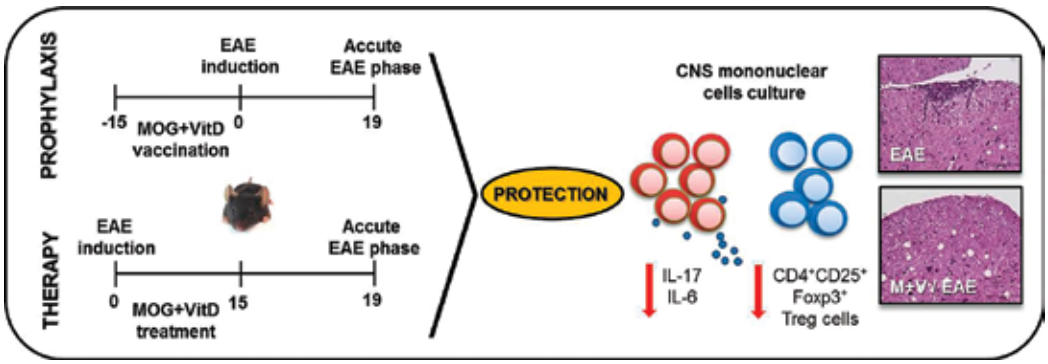

Figure 2. MOG + active vitamin D3 association strategy for EAE prophylaxis and treatment. C57BL/6 mice were vaccinated or treated with this association and the effect on EAE was evaluated in the acute EAE phase. Both strategies decreased production of inflammatory cytokines by CNS mononuclear cells, frequency of CD4+CD25+Foxp3+ Treg cells, and inflammation in the CNS.

7. Prophylactic effect of VitD on EAE

Prophylactic strategies in EAE, and also in other autoimmune pathologies, are based in the concept of "inverse vaccination." This procedure refers to the use of an immunization protocol that, differently from classical vaccination, aims to achieve an antigen-specific tolerogenic state [120]. Even though the term "inverse vaccination" could also be used as a therapeutical strategy, in this text we applied it only in the context of prophylactic vaccination. The majority of the prophylactic strategies in EAE have been done by administration of a diversity of MOG formulations delivered by distinct routes. A few examples of these procedures and the main histological and immunological findings are illustrated in **Table 1**.

The prophylactic potential of VitD (or analogs) alone or associated with other pharmaceuticals has been tested in EAE. The adopted experimental protocols are not standardized and therefore, different amounts of VitD are administered by distinct routes. Time periods chosen for VitD administration in relation to EAE induction are also variable and some procedures consist in prolonged administration periods, even reaching the disease clinical phase. However, a general consensus is that VitD is able to improve clinical disease manifestation and also to trigger evident effects on the CNS and the immune system. Some of the effects observed in mice with EAE that were previously injected with VitD are exemplified in **Table 2**.

Peptide formulation	Animal model	Effects	References
Plasmid DNA vaccines encoding MOG35–55	C57BL6/J mice	↓Microglia/macrophage activation, astrogliosis, and axonal damage ↑CD4+CD25+Foxp3+ Treg	Fissolo et al. [118]
MOG35–55 conjugated to mannan, intradermally	C57BL/6 and SJL/J mice	↓Demyelination ↓Inflammatory infiltrates	Tseveleki et al. [117]
Tolerogenic DC pulsed with MOG40–55	C57BL/6 mice	↑IL-10 production by MOG-stimulated splenocytes ↑CD3+CD4+CD25+FoxP3+ cells	Mansilla et al. [121]
MOG35–55-PLGA + IL-10-PLGA, subcutaneously	C57Bl/6 mice	↓IL-17 and IFN-α production by splenocytes↓Demyelination score	Cappellano et al. [122]

Table 1. MOG prophylactic procedures in EAE.

Route	Animal model	Effects	References
Diet	CD8+ $^{-/-}$ mice	Protection independent of TCD8+ cells	Meehan and DeLuca [101]
Intraperitoneally	C57BL/6 mice	↓MyD88, IRF-4, IRF-7 and NF-kB expression ↓Several TLRs	Li et al. [123]
Oral, gavage	C57BL/6 mice	Intact blood–CNS barrier ↓Inflammatory infiltrates in the CNS	Grishkan et al. [124]
Intraperitoneally	C57BL/6 mice	↓Demyelination ↑Beclin-1 expression in neurons	Zhen et al. [111]

Table 2. Vitamin D3 prophylactic procedures in EAE.

The combination of VitD with other substances as calcitonin [125], IFN-β [126], bisphosphonate [127], rapamycin [128], and cyclosporine [129] has determined cooperative effects over EAE control. We recently tested the association of VitD with MOG as a prophylactic approach to control EAE development. Again, in this procedure, we explored the concept of VitD as a tolerogenic adjuvant. This concept and its potential application to trigger self-tolerance in autoimmune diseases were conceived by Kang et al. [130]. These authors validated this hypothesis by demonstrating that FK506 (tacrolimus) associated with MOG was prophylactic in encephalomyelitis [131]. In this context, we hypothesized that active VitD could also behave as a tolerogenic adjuvant if associated with a CNS-specific antigen. Vaccination with MOG associated with VitD, before EAE induction in C57BL/6 female mice, determined a significant clinical improvement characterized by absence of clinical score and no body weight loss. An impressive reduction in CNS inflammation, DC maturation and also cytokine production by CNS and spleen cell cultures was detected in these vaccinated animals [132]. As described in Section 6 of this chapter, this combination of MOG with VitD was also very efficient as a therapeutic procedure in the EAE model. This prophylactic and therapeutic potential of the MOG/VitD association in EAE is illustrated in **Figure 2**. The possible use of VitD as a tolero-

genic adjuvant in association with other self-antigens, as a strategy to control autoimmune pathologies, warrants future investigation. In our opinion, the fact that VitD is already accepted for human supplementation will facilitate its adoption for MS treatments based on its association with neuronal self-antigens.

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Chapter 11

Nanoparticles for Delivery of Vitamin D: Challenges and Opportunities

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Additional information is available at the end of the chapter

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Abstract

In addition to the traditional role of calcium homeostasis and bone mineralization, calcitriol, the active metabolite of vitamin D, also displays other metabolic activities as antiproliferative, pro-differentiating, anti-inflammatory, immunomodulatory, and antineoplastic effects. Thus, the awareness that vitamin D insufficiency/deficiency may be associated with various diseases has grown. Also nowadays, vitamin D is recognized as a potential therapeutic agent in anticancer therapy. However, its administration presents some drawbacks such as high toxicity and low bioavailability. Thus, the use of nanotechnology may overcome these problems associated with vitamin D administration, allowing to decrease its toxicity in healthy tissues and increasing its bioavailability. In this chapter, an overview on vitamin D and its metabolic activity is presented, as well as a review of nanosystems for the encapsulation of vitamin D for different applications, such as food and pharmaceutical industries.

Keywords: vitamin D, calcitriol, nanotechnology, drug-delivery systems, nanoparticles

1. Introduction

Vitamin D (VD) was firstly identified as a vitamin and now is recognized as a prohormone. VD is a precursor to its active and biologically functional metabolite, a lipophilic seco-steroid hormone known as calcitriol [1].

In the epidermis, VD is produced in the form of cholecalciferol due to the action of sunlight [2]. Once produced, VD is translocated into the bloodstream. However, VD does not remain in

circulation for a long time as it is almost instantly stored on the adipose tissue or metabolized in the liver. As cholecalciferol is inert, it must be metabolized in the liver and the kidney through two hydroxylation processes to be converted to its active form, calcitriol [3]. Due to their structural similarities, calcitriol acts like classical steroid hormones binding to the vitamin D receptor (VDR) regulating target gene expression via both genomic and nongenomic pathways [4].

Despite its well-known regulation of calcium homeostasis and bone mineralization functions [5, 6], in the late 1970s VD was found in tissues not previously considered targets of VD action, which came to disclose that this hormone may carry out several other functions [3]. Calcitriol is nowadays associated with many additional actions including antiproliferative, pro-differentiating, anti-inflammatory, and immunomodulatory effects. For example, this hormone has the ability to suppress prostaglandin actions and enhance pro-inflammatory cytokines production, displaying a role in ceasing inflammatory process [7, 8]. Also, several studies support that VD may play a major role in tumor's pathogenesis, progression, and therapy [8–11].

Still, vitamin D deficiency is a worldwide well-recognized problem with health consequences. Due to the very limited dietary sources of VD and insufficient exposure to sunlight in northern regions, between 30 and 60% of the European and North American population suffer from VD deficiency [12]. Hence, formerly in western diets VD was added to food and beverage products such as milk, soft drinks, and bread, to increase its nutritional value. However, the acknowledgment of VD's high toxicity associated with the hypercalcemia phenomena [9, 13] and low bioavailability, since more than 75% of VD intake is catabolized and excreted before being converted to its active form or before its storage [2, 14], raised several issues to its administration resulting in the forbiddance of food fortification with VD.

However, the recognition that vitamin D deficiency as a health risk leads to the development of new functional foods and therapies using nanotechnologies for VD incorporation into foods and pharmaceutical formulations without reducing its bioavailability or activity. Thus, this chapter is dedicated to provide a systematic overview of VD and its activity, as nanocarriers for the delivery of VD.

2. Vitamin D and nanotechnology

2.1. Vitamin D: an overview

Vitamin D cannot technically be considered a vitamin in its true meaning. More than a micronutrient, vitamin D is a precursor to its active form, calcitriol. The latter is a lipophilic seco-steroid hormone [10]. Vitamin D is derived from a steroid precursor, a cholesterol-like molecule. A seco-steroid molecule is very similar to a steroid, but with a few differences in its skeleton. Whereas a steroid molecule core is constituted by 20 carbon atoms assembled in four fused rings (A–D), three cyclohexane rings (A, B and C) and one cyclopentane ring (D), the seco-steroid has its B ring broken [15, 16]. **Figure 1** shows the difference between these two

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types of molecules. In more recent years, it was revealed that vitamin D is not responsible for all biological activities linked with it, but actually it only represents a precursor to its active and biologically functional metabolite, known as calcitriol [5, 11]. Calcitriol is indeed the one, which displays several biologic activities formerly thought as vitamin D responsibility. Due to their similarities, calcitriol acts like classical steroid hormones. It binds to the vitamin D receptor regulating target gene expression via both genomic and nongenomic pathways [10].

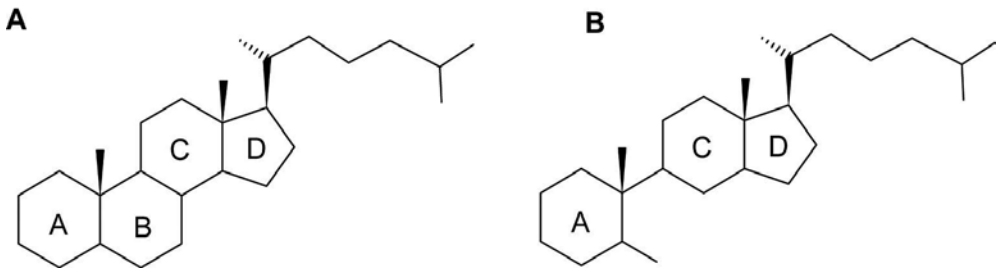

Figure 1. General chemical structures of (A) steroid and (B) seco-steroid (drawn in ACD/ChemSketch®).

In humans' bloodstream, vitamin D displays two main chemical forms: D2 or ergocalciferol, and D3 or cholecalciferol. The first one comes from the dietary source and can be found in some kinds of food such as salmon and cereals. On the other hand, the latter is produced in the epidermis from the action of sunlight and represents 95% of total blood's vitamin D [2]. These two forms exhibit chemical differences in their side chains (**Figure 2**). D2 has an extra methyl group at C24 and an extra double bond between C22 and C23.

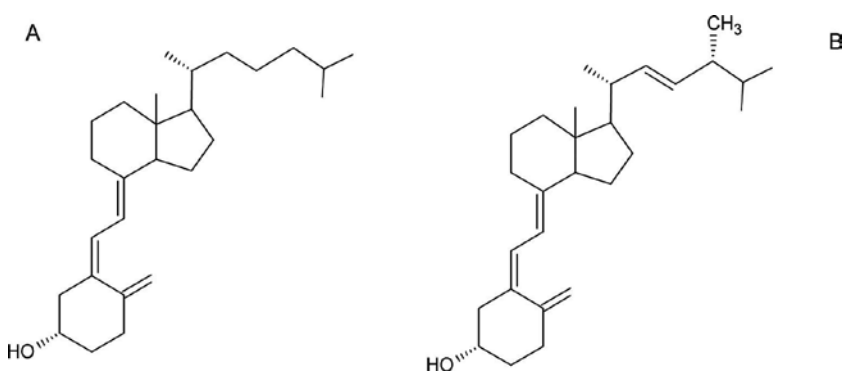

Figure 2. Chemical structures of (A) cholecalciferol and (B) ergocalciferol (drawn in ACD/ChemSketch®).

These structural changes, between D2 and D3, are reflected in their affinity for the carrier known as vitamin D-binding protein (DBP). Despite their metabolites' biologic activity being comparable, the fact that vitamin D3 has a higher affinity for DBP leads to the observation that in humans vitamin D3 potency is three times higher than vitamin D2's [15–17].

Due to this, the amount of work and research involving vitamin D3 is far superior, and all evidences reported to date on the efficacy of vitamin D for food fortification and the prevention of cancer and other diseases have been based on vitamin D3 [18]. For that reason only vitamin D3 will be covered for the next sections of this chapter.

2.2. Vitamin D metabolism

To better explain the process mentioned above, it is important to lay out the reaction steps involved in vitamin D synthesis. 7-Dehydrocholesterol is the precursor of pre-vitamin D. During exposure to the sunlight, UV radiation breaks the B ring of the precursor to form pre-vitamin D. Pre-D is rapidly isomerized by the body temperature into vitamin D (cholecalciferol), as it is schematized in **Figure 3** [11, 15, 19]. Once formed, cholecalciferol is translocated from the plasma membrane into the bloodstream where it bounds to DBP [19]. However, vitamin D does not remain in circulation for a long time as it is almost instantly stored on the adipose tissue or metabolized in the liver [3].

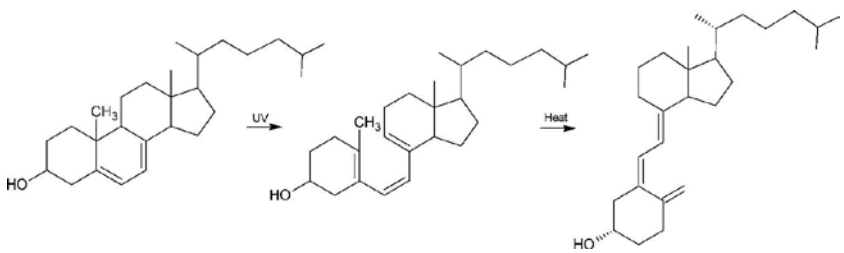

Figure 3. Image illustrating the production of cholecalciferol in epidermis (drawn in ACD/ChemSketch®).

Depending on its degree of hydroxylation, cholecalciferol can be found with three different chemical structures: calciol, calcidiol, and calcitriol. The first in order of appearance in the sequence of metabolic pathways, calciol, is the unhydroxylated and inactive form. Calcidiol is the monohydroxylated [16] and is the major blood circulating form [19] at concentrations in the range of 10–40 ng/ml [3]. Calcitriol is the dihydroxylated and active form responsible for all the vitamin D known biological actions [16]. These three different molecules can be compared in **Figure 4**.

Summarizing, cholecalciferol is inert and must be metabolized in the liver and the kidney through two hydroxylation processes to be converted to its active form. Thus, the first step of this activation, the hepatic 25-hydroxylation, inserts a hydroxyl group in C25 of cholecalciferol, thereby creating 25-hydroxyvitamin D3 (25-OH-D3). This step of 25-hydroxylation is mediated by a 25-hydroxylase enzyme (CYP2R1). Later in the kidney, 25-hydroxyvitamin D3 1α-hydroxylase (CYP27B1) enzyme is responsible for the insertion of one more OH group into the C1 of the A ring, converting it into calcitriol [3, 15, 20, 21]. In **Figure 4** also, the overall process of vitamin D activation is schematized.

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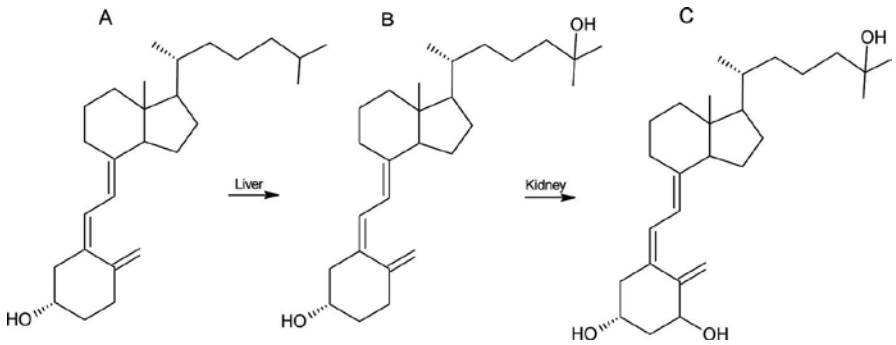

Figure 4. Representation of cholecalciferol three different chemical structures: (A) calcio, (B) calcidiol, and (C) calcitriol. This scheme also represents the overall chemical reactions involved in vitamin D activation (drawn in ACD/ChemSketch®).

Uncontrolled levels of calcitriol in the bloodstream may subsequently result in hypercalcemia phenomena [3, 11], related to a high risk of calcification of soft tissues especially intestine, kidney, and heart leading to organ failure and even death. As a result, the human body has a control mechanism that allows the inactivation of calcitriol. As this process of inactivation intends, the prevention of hypercalcemia therefore is upregulated by the administration of vitamin D, high levels of calcitriol itself, and high levels of serum calcium [3]. Hence, 24-hydroxylase enzyme (CYP24) inactivates calcitriol by hydroxylation (**Figure 5**). This reaction may occur in the liver or in any other target tissue, such as bone or intestine. The obtained inactive form, calcitroic acid, is metabolized and excreted. As the product is 10 times less biologically active than calcitriol, it has low affinity for VDR. For this reason, it is the main biliary excretory of vitamin D, since it is easily eliminated [3, 11].

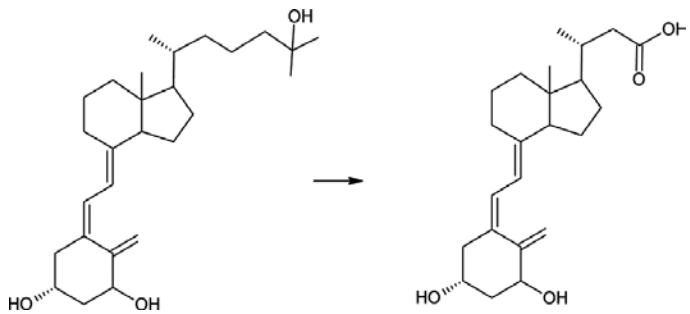

Figure 5. Image illustrating the inactivation of calcitriol, through conversion in calcitroic acid (drawn in ACD/ChemSketch®).

2.3. Molecular actions of vitamin D's metabolites

Calcitriol exerts its effects through a nuclear hormone receptor known as VD receptor. This receptor is a transcription factor that regulates gene expression that mediates the hormone

biologic activity, and in more recent years was found in tissues that are not involved in maintaining calcium homeostasis and bone health. In fact, VDRs have a broad tissue distribution, being present in organs such as heart, stomach, pancreas, brain, skin, gonads, and immune system cells [22].

Thus, despite its well-known regulation of calcium homeostasis and bone mineralization functions, calcitriol is nowadays associated with many extraskeletal effects including antiproliferative, pro-differentiating, anti-inflammatory, and immunomodulatory effects [7]. In fact, recent studies proved that VD receptors are present in activated macrophages and lymphocytes. Binding of these receptors is directly responsible for the activation of antimicrobial genes [23]. Also, this hormone has the ability to suppress prostaglandin actions and enhance pro-inflammatory cytokines production, displaying a role in ceasing inflammatory process [8].

VD is also associated with the regulation of the proliferation of several cells, as cardiac muscle cells [22].

2.3.1. Vitamin D in calcium homeostasis and bone mineralization

It is well established that vitamin D stimulates intestinal absorption of calcium by activating the signaling pathways for calcium transport across the plasma membrane. VD also stimulates calcium mobilization from bone playing an important role in initiating bone remodeling and repairing.

Recent studies proved that all skeleton cells (chondrocytes, osteoblasts, and osteoclasts) contain the receptors for both VD receptor and the enzyme CYP27B1 required for calcitriol synthesis. Therefore, it was proved that VD plays a major role in the activation of osteoblasts to the osteoclast cells to resorb bone. Also, activated osteoclasts induce the reverse transport of calcium from the bone to plasma.

VD active metabolite is also responsible for altering the expression of several skeletally derived factors as growth hormone that can exert effects on bone homeostasis [3, 24].

2.3.2. Vitamin D and its antineoplastic activity

Several studies support that vitamin D may play a major role in tumor's pathogenesis, progression, and therapy [8–11]. In fact, as Garland and coworkers state, more than 3000 research studies have been published investigating vitamin D and its metabolites antineoplastic activity [18]. The types of cancer where most of the anticancer actions of vitamin D have been studied are the breast, prostate, and colon cancers [10].

As mentioned above, calcitriol exerts its effects through the VDR. This receptor is widely distributed among tumor cells, regulating calcitriol antineoplastic activity [8, 9, 15]. Therefore, several pathways by which vitamin D metabolites may prevent, treat, or stop tumor growth have been described. The most discussed mechanisms are (1) inhibition of tumor cell growth, (2) inhibition of angiogenesis and tumor metastasis, (3) triggering apoptosis, (4) enhancing of "traditional" anticancer agents therapeutic action, and (5) anti-inflammatory effects [8–11].

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1. VDR activation by calcitriol can inhibit tumor cell proliferation by inducing cell cycle arrest in the G_1/G_0 phase [8, 10, 11]. The cell cycle is divided in five different phases. The first stage is called the G_0 phase, a resting phase where the cell has left the cycle and has stopped dividing. Then, the cell enters in the G_1 phase, a checkpoint to ensure that all cellular mechanisms are ready for DNA synthesis that occurs in the next stage, the S phase. Another checkpoint follows the S phase, the G_2 gap. Finally, cell division—mitosis—occurs in the M phase [25]. VDR activation by calcitriol can also inhibit tumor cell proliferation through inducing malignant cells differentiation in a variety of cell lines [8, 9, 11].

2. Calcitriol also inhibits angiogenesis by reducing the proliferation of vascular endothelial cells [8, 11] and regulating the expression of key molecules, such as serine proteinases, metalloproteinases, extracellular matrix proteins, and integrins [8, 9]. Another calcitriol antineoplastic activity is related to reducing the invasive and metastatic potential of tumor cells [8–10]. Inhibition of tumor metastasis is due to increased expression of E-cadherin, a tumor suppressor associated with the metastatic potential of cells, and inhibition of angiogenesis itself [9].

3. Apoptosis triggering of tumor cells occurs through activation of the intrinsic pathway of apoptosis by increasing the expression of proapoptotic proteins and decreasing the expression of antiapoptotic proteins, or by directly activating effectors caspases [10, 11]. Apoptosis may also be induced by the inhibition of telomerase enzyme [11]. This programmed cell death is characterized by causing the disruption of mitochondrial function, cytochrome release, and production of reactive oxygen species [10, 11].

4. Vitamin D can also potentiate the antitumor actions of a number of more "traditional" anticancer agents [8, 11].

5. Inflammatory mediators such as cytokines, chemokines, prostaglandins, and reactive oxygen and nitrogen species enhance tumorigenesis through the activation of multiple signaling pathways in tumor tissue. Hence, anti-inflammatory effect of calcitriol mentioned at the section above can also be considered as an antineoplastic activity [8, 10].

However, calcitriol exhibits antitumoral activity only at supraphysiological doses (10^{-9} to 10^{-6} M *in vitro* and $>10^{-9}$ M *in vivo*) associated with a high risk of hypercalcemia [9, 13].

Not only calcitriol plays a major role in tumor's pathogenesis, progression, and therapy but also the enzymes involved in its metabolism have urged a serious interest in several research projects. Tumor cells, likewise other vitamin D target tissues, exhibit enzyme 24-hydroxylase responsible for calcitriol inactivation. Therefore, vitamin D activity will be reduced. Some studies address this problem through a combination therapy with 24-hydroxylase inhibitors alongside with calcitriol administration. However, this co-addition may also results in an increase in the risk of hypercalcemia effects. For these reasons, several authors argue that structural analogs of calcitriol that resist 24-hydroxylation may be a more useful cancer therapy [8].

It is documented that the enzyme 1-α-hydroxylase, which converts calcidiol into calcitriol, is also present in cancer tissues such as breast and prostate cancer. Therefore, calcidiol also could

be administrated as a therapeutic agent, increasing local drug concentration without systemic side effects associated to calcitriol high levels [8, 13].

2.4. Vitamin D deficiency/insufficiency and health risks

Vitamin D deficiency/insufficiency is a worldwide well-recognized problem associated with an increased risk for several acute and chronic diseases [2]. VD deficiency is a resulting consequence of the modern lifestyle with the prevalence of obesity, increased information, and consciousness on harmful effects of UV radiation leading to an increased sun protection and sun avoidance [23]. Due to the very limited dietary sources of VD and insufficient exposure to sunlight in northern regions, between 30 and 60% of the European and North American population suffer from VD deficiency [12].

Thus, several adverse outcomes are nowadays firmly associated with the VD insufficiency problems. Concerning the musculoskeletal system, low bloodstream levels of VD are related to low bone mineral density leading to rachitic with increased fracture risk. This bone illness is associated more directly with the circulating VD levels than to the dietary calcium intake [23].

VD insufficiency is also a risk factor for the development of various cardiovascular diseases. As VD regulates the proliferation of cardiac muscle cells, its deficiency is associated with the increased risk for coronary artery disease, heart failure, and peripheral artery disease. Studies also showed that VD deficiency leads to the development of hypertension, ventricular hypertrophy, and coronary artery calcification [22].

VD deficiency can also be related to the appearance of microbial diseases since VD plays a major role in promoting antiviral activity [23].

As mentioned before, VD plays a major role in tumor's pathogenesis; therefore, its deficiency increases the risk of developing several types of cancer [26]. In fact, studies show the relationship between VD deficiency and at least 15 types of cancer, including breast, colon, rectal, gastric, and ovarian cancer. VD insufficiency leads to an impairment of antimitogenic, proapoptotic and prodifferentiating signaling pathways that have been implicated in the pathogenesis of these types of cancer [21].

2.5. Nanoparticles for the encapsulation of vitamin D

The recognition that vitamin D deficiency as a health risk leads to the development of new functional foods and therapies using nanotechnologies for VD incorporation into foods and pharmaceutical formulations without reducing its bioavailability or activity.

Nanomedicine has dictated trends in the last decades, and its influence is notorious in several fields, since nanomaterials exhibit unique physicochemical properties due to their small size and larger surface area. Nanoparticles (NPs) are colloidal carriers with dimensions on the nanoscale (10^{-9} m) with unique physicochemical properties as small size, larger surface area, stability, varied composition, biocompatibility, and biodegradability [27, 28]. Encapsulating molecules in a nanocarrier allows to increase their bioavailability and bioaccumulation in the target site, and decreases their toxicity. The fulfillment of these main goals allows maximizing

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therapeutic effects and minimizing side effects [27, 28]. Nanoencapsulation of several compounds can be achieved using a wide variety of different nanocarriers. At the moment, the most studied NPs are liposomes, polymeric NPs, dendrimers, lipidic NPs, micelles, carbon, and silica nanotubes.

Among polymeric NPs, poly(D,L-lactide-co-glycolide) (PLGA) is probably the most popular ones. PLGA has become one of the most attractive candidates for a range of applications due to being biocompatible, biodegradable, and Food and Drug Administration (FDA)-approved, and having adjustable biodegradation rate and tunable mechanical properties [29]. PLGA allows the controlled and sustained release of VD for several days, increasing VD's bioavailability and enhancing its therapeutic effect [30]. However, its use faces a few limitations due to their poor loading capacity, allowing the delivery of only about 10% (w/w) of VD, as already described [30]. The characteristic initial burst release can be another major pitfall since a large amount of VD is lost before reaching the target tissue [30]. Also, the many required steps for NP production such as centrifugation and dialysis are expensive and difficult to scale up. PLGA NPs also exhibit a size-dependent cytotoxicity. Small PLGA NPs may trigger the generation of reactive oxygen species, mitochondrial depolarization, and inflammatory cytokines release. Another drawback of these polymeric NPs is the challenge of hydrophilic molecules entrapment, since those rapidly partition into the aqueous phase during NPs preparation. For that is necessary to use appropriate preparation methods as the double emulsion technique [29].

On the other hand, liposomes offer an advantage in the encapsulation of hydrophilic molecules, since they have the ability to carry hydrophilic and hydrophobic drugs within the aqueous vesicles and lipid bilayer membranes, respectively. Liposomes are probably the most popular among the nanosystems studied for nanomedicine applications. Liposomes are small spherical vesicles composed of concentric bilayers of self-assembled phospholipids in aqueous medium and can be classified into different categories by their number of bilayers and size.

Multilamellar vesicles (MLVs) are composed of a structure of concentric phospholipid bilayers separated by water compartments. In unilamellar vesicles (UVs), the liposomes exhibit only a single phospholipid bilayer enclosing the aqueous compartment. These unilamellar vesicles can also be classified into two different types: the small unilamellar vesicles (SUVs) and the large unilamellar vesicles (LUVs). Liposomes can be produced in a broad range of sizes, from 15 to 2000 nm. The most used technique for the preparation of liposomes is Bangham's method. This method consists in the preparation of lipid moisture and evaporating it to form a lipid film. Then, the film is hydrated to form liposomes. The product of hydration is large MLV. Liposomes can be downsized by a variety of techniques, including sonication or extrusion. While usually sonication yields SUV (in the range of 15–50 nm), extruded liposomes are usually LUV [31].

Liposomes are nontoxic and biocompatible causing no harmful effects to the human body, as they are very similar in structure and composition to the cell membrane phospholipids. Such nanocarriers are considered excellent systems for drug-controlled release due to their structural flexibility, size, composition, and fluidity/permeability of the lipid bilayer versatility. Also, their surface is easily functionalized to their polar head groups [32]. However, liposomes

present some disadvantages as well as low solubility, short half-life, and high production costs. Also, the phospholipids can undergo oxidation, and in several cases leakage of encapsulated molecules is verified, especially in low-molecular-weight molecules [33].

Since all systems have advantages and disadvantages, the choice of the most suitable nanosystems must take into account the molecule to be encapsulated and further application.

Several studies using nanocarriers for VD delivery both for therapeutic and for food fortification usage have been reported. Some of these studies are summarized in **Table 1** and **Table 2** and discussed below. These nanosystems will allow maintaining the physical and chemical stability of VD, protecting the molecule from extreme temperatures, light, and oxygen that food and pharmaceutical products may be exposed to.

Nanocarrier	Indication	Development phase			Ref.
		Physicochemical studies	Release studies	Cellular studies	
Liposomes	Cheese fortification	EE, TEM, VD recovery rate	n/a	n/a	[33]
Alginate NPs	Oral administration	FTIR, NMR, DLS, TEM, EE	SGF	n/a	[34]
Chitosan-zein NPs	Food fortification	SEM, FTIR, DSC, DLS, ELS, EE, stability	SGF	n/a	[35]
O/W emulsion	Cheese fortification	Stability, VD recovery rate	n/a	n/a	[36]
Micelles	Food and beverage fortification	Stability, DLS	n/a	n/a	[37]
Chitosan/soy protein NPs	Food fortification	DLS, ELS, SEM, EE, FTIR	SGF	n/a	[38]
Protein NPs	Food and beverage fortification	DLS, stability	n/a	n/a	[39]
Chitosan micelles	Food fortification	FTIR, NMR, XRD, DSC, TEM, AFM, ELS, DLS, stability, EE	SGF and PBS	Fibroblast mouse cell line (L929); MTT assay	[40]
Nanoemulsion	Food and beverage fortification	DLS, stability	n/a	n/a	[41]

Note that n/a stands for not applicable, AFM for atomic force microscopy, DLS for dynamic light scattering (used for size determination), DSC for differential scanning calorimetry, EE for NPs encapsulation efficiency, ELS for electrophoretic light scattering (used for zeta potential determination), FTIR for Fourier transform infrared spectroscopy, NMR for nuclear magnetic resonance, PBS for phosphate buffered saline, SEM for scanning electron microscope, SGF for simulated gastrointestinal fluid, TEM for transmission electron microscope and XRD for X-ray diffraction. SRB is a cellular proliferation assay (colorimetric) and MTT is cellular viability assay (colorimetric).

Table 1. Currently developed nanosystems for the entrapment of vitamin D for food fortification.

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Nanocarrier	Indication	Development phase			Animal studies	Ref.
		Physicochemical studies	Release studies	Cellular studies		
PLA NPs	Cancer treatment	DLS, EE, stability	PBS	Human breast cancer cells (MCF-7); MTT assay; cellular uptake (Fluorescence microscopy)	n/a	[13]
PLGA NPs	Cancer treatment	DLS, ELS, TEM, stability	PBS	Human pancreatic cell lines (S2-013 and hTERT-HPNE); lung cancer cell line (A549); SRB assay, cellular uptake and morphology (confocal microscopy); flow cytometry (cell cycle analysis)	n/a	[45]
HAp-PLGA NPs	Osteogenesis and bone tissue differentiation	XRD, FTIR, DLS, ELS	n/a	Mouse calvarial preosteoblastic cell line (MC3T3-E1), confocal microscopy	Rats with osteoporosis and induced bone defects, pathohistological analysis of bone tissue after sample injection	[44]
Quantum dots	Cancer diagnosis and treatment	FTIR, AFM	n/a	Mouse myoblast cell line (C2C12), confocal microscopy, luciferase activity assay (gene expression)	n/a	[43]

Table 2. Currently developed nanosystems for the entrapment of vitamin D for therapy.

As VD is a lipophilic component, it is necessary to be incorporated into aqueous media to become suitable for food and beverage products; also, fat removal during food products processing results in the removal of fat-soluble micronutrients, as VD [2]. Thus, the fortification of food and beverages with VD can be envisaged by its encapsulation in nanoparticles. It is important that the developed delivery nanosystem does not alter the physical, chemical, or sensory properties of the food or beverage product that it is incorporated.

Banville and coworkers used liposomes for the supplementation of Cheddar cheese with vitamin D. The attained nanosystems allowed to maintain the stability of VD for up to 5 months. The group used multilamellar liposomes to achieve high encapsulation efficiency values (approximately 80%). VD-loaded liposomes were added to milk before cheese produc-

tion, and the authors verified that VD was recovered in cheese with high recovery rates (60%) when compared to control conditions. The entrapment of VD in the liposomes allowed to obtain Cheddar cheese enriched with high levels of VD not altering the chemical composition of the fortified cheese [34].

Li and colleagues developed alginate derivate NPs, as a carrier for oral administration of VD, to enhance its water solubility improving its bioavailability. Alginate was modified with oleoyl chloride yielding oleoyl alginate ester conjugate for the NP preparation. The group verified that by increasing the concentration of used oleoyl chloride, the size of the obtained NPs decreased from 500 to 300 nm, approximately. The developed system exhibited high encapsulation efficiency values (approximately 70%) and maintained their structural and chemical properties in simulated gastrointestinal fluids. The NPs exhibited a controlled and sustained release of VD in the simulated human body fluids. The attained results proved that the developed nanosystem is a suitable oral carrier for the delivery of VD [35].

Luo and coworkers encapsulated VD into chitosan-zein NPs for food fortification to increase its stability and health-promoting properties during processing and storage. The group prepared zein nanoparticles with a chitosan surface's coating. Zein has been extensively studied for its ability to form biodegradable, biocompatible, and nontoxic NPs. Coating with chitosan significantly enhanced the NPs encapsulation efficiency from 50 to 90% approximately. Calcium was used as a cross-linker, and its influence in the NPs mean size was assessed. The group verified that the mean size varied from 80 to 200 nm, increasing the size with the increase of calcium concentration. The prepared NPs showed a controlled release of VD in both PBS medium and simulated gastrointestinal fluid proving to be a suitable system for the oral delivery of VD [36].

Tippetts and colleagues incorporated VD in oil-in-water emulsion, using milk protein emulsifiers to fortify milk for cheese production. The authors verified that the retention of vitamin D in cheese was enhanced when using the nanoemulsion comparatively with free vitamin. The obtained results proved that this nanosystem is suitable for milk fortification with VD for cheese production [37].

Ziani and colleagues developed surfactant-based colloidal delivery systems for VD, and other lipophilic active agents, encapsulation for food and beverage products fortification strategies. The group prepared oil-in-water emulsions and studied the influence of the surfactant type on the incorporation of VD into the surfactant micelles. The surfactants were Tween 20, 60, and 80, respectively. The study provided valuable knowledge for the rational design of delivery systems for food fortification [38].

Teng and colleagues successfully incorporated VD in carboxymethyl chitosan and soy protein complex nanoparticles to improve water solubility, absorption, and protection for food products fortification. The effect of pH and chitosan/soy protein mass ratio on the formation of nanoparticles was studied. The attained nanovehicles exhibited sizes around 200 nm and encapsulation efficiency values around 90%. The prepared nanoparticles showed a successful release of VD in simulated gastric fluid and under simulated intestinal condition proved to be a suitable VD nanocarrier for food industry application [39].

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Abbasi and coworkers developed protein isolate nanoparticles for the encapsulation of VD. The group concluded that the incorporation of VD in this nanocarrier allows delaying its degradation during storage time, and therefore they can be considered as enriching agent in beverages, fruit drinks, or low-fat food [40].

Li and coworkers encapsulated VD in chitosan-derived micelles to improve the solubility, efficacy, and stability of VD for functional food products. The attained nanosystem showed mean diameters around 200 mm and encapsulation efficiency values of approximately 50%. The prepared micelles also exhibited a biphasic release profile, with an initial rapid release, followed by a sustained release. The cytotoxicity of the nanocarrier was assessed using fibroblast mouse cells, and the results showed that the chitosan micelles had low cytotoxicity against the studied cell lines, proving to be biocompatible [41].

Guttoff and colleagues developed stable delivery systems for VD with high oral bioavailability based on nanoemulsions. The main goal of this work was to incorporate VD into aqueous-based food products, such as waters or juices. The authors assessed the influence of several experimental conditions on the nanoemulsion-obtained characteristics, such as the surfactant-to-oil ratio and surfactant type. The group prepared nanoemulsions with droplet diameters under 200 nm, stable for at least 1 month at storage conditions (room temperature). The results suggested that the developed nanosystem will be suitable for food and beverage fortification with VD [42].

Despite calcitriol's multiple medicinal benefits, its low bioavailability and high toxicity continue to be highlighted as major challenges in developing formulations for clinical use. In fact, two pharmaceutical formulations for Rocaltrol® (registered trademark of Roche Pharmaceuticals) are available with different administration pathways, oral and intravenous, for the treatment of refractory malignancies. However, they are inappropriate for cancer treatment due to several technical issues, as the difficulty to maintain active systemic levels [13, 43]. Also, several studies indicate that more than 75% of vitamin D intake is catabolized and excreted before being converted to its active form or before its storage. After being absorbed by the intestinal mucosa, vitamin D suffers first-pass effect being conducted by the portal vein to the liver where it is metabolized by hepatic 24-hydroxylase enzyme. This enzyme inactivates calcitriol by hydroxylation, yielding calcitroic acid as an inactive metabolic product. Therefore, vitamin D concentration is greatly reduced before it reaches the systemic circulation, and consequently before it reaches target tissues [14].

Also, some studies, intending to use VD as a therapeutic agent, aiming to increase its bioavailability avoiding first-pass effect, and decreasing its toxicity by ensuring specific action on target cells, have been reported. As calcitriol exhibits antitumoral activity only in supraphysiological concentrations as mentioned above [9, 13], its encapsulation on NPs could address the toxicity issue. One of the main advantages in using NPs to cancer therapy is the enhanced permeability and retention (EPR) effect verified in tumor tissues. The NPs take advantage of the increased permeability of blood vessels in tumor tissues, whereas lymphatic drainage is decreased which increases the concentration of loaded nanoparticles in the tumor tissue. As the EPR effect does not occur in healthy tissues, it is thus possible to target tumor cells, reducing VD's toxicity in healthy tissue [44].

Almouazen and partners encapsulated calcidiol in poly-lactic acid (PLA) nanoparticles to ensure specific action on malignant cells avoiding side effects as hypercalcemia. The authors developed nanocapsules with about 200 nm of mean diameter. Cellular studies showed a significant growth inhibition when calcidiol was entrapped in the PLA nanocapsules, when compared to free calcidiol, proving that the nanocarrier enhanced the intracellular delivery of vitamin D on breast cancer cells. The attained results showed that PLA nanocapsules are a suitable choice for the controlled delivery of calcidiol [13].

Bonor and coworkers developed calcitriol-conjugated quantum dots to study the distribution of calcitriol in mouse cancer cells. The designed tool is suitable for imaging drug-tumor interactions and to deliver drugs to tumors and metastasized sites [45].

Ignjatović and colleagues prepared hydroxyapatite (Hap) and PLGA-based nanoparticles for the local delivery of VD to enhance osteogenesis and bone tissue differentiation. The attained NPs exhibited mean diameters of 100 nm and a biphasic release profile. *In vitro* biocompatibility studies were conducted using osteoblastic cells. In animal studies, the authors verified that osteogenesis and bone structure differentiation were enhanced when VD was delivered by the developed system [46].

Ramalho and colleagues developed PLGA nanoparticles for the delivery of calcitriol for an antitumor therapy application. Initially, the authors used cholecalciferol as drug model for calcitriol to assess the influence of several experimental conditions, such as sonication time and VD/polymer ratio, on the NPs physicochemical properties. After achieving the optimized experimental conditions, the group synthesized calcitriol-loaded PLGA NPs with spherical form and mean diameters smaller than 200 nm as shown in **Figure 6**, and stable for several weeks at storage conditions (4°C). The attained nanosystems exhibited encapsulation efficiency values of approximately 60%. The prepared PLGA NPs exhibited a biphasic release profile, with an initial burst release in the first 24 h, followed by a slower and controlled release for 7 days. Human cancer cell lines were used to evaluate the toxicity of VD-loaded PLGA NPs. The obtained nanoparticles formulation was successfully internalized by the target cells and enhanced the vitamin's antitumor effect, showing a clear efficacy in the therapeutic effects as cell cycle arrest and major changes in cell's morphology [30].

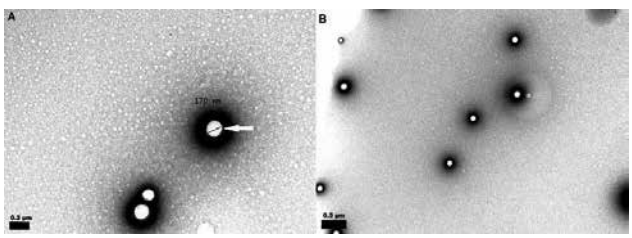

Figure 6. TEM images: (a) unloaded PLGA nanospheres; scale bar: 200 nm; (b) VD-loaded PLGA nanospheres; scale bar: 500 nm [30].

These developed systems reported in the literature allowed maintaining active doses of VD for long periods of time, due to their controlled and sustained release. These nanosystems also

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showed the ability to reduce and destroy tumor cells, taking advantage of the EPR effect verified in tumor tissues. As the EPR effect does not occur in healthy tissues, it is thus possible to target tumor cells, reducing VD's toxicity in healthy tissue [44]. None of the works here discussed reported the use of functionalized NPs. Modification of the NPs' surface with antibodies or other specific molecules would allow to address a more efficient therapy, enabling a targeted distribution into specific tissues.

3. Conclusion

With the growing awareness of vitamin D health benefits, as well of the harmful risks associated to vitamin D insufficiency, finding new solutions has become urgent within the scientific community. In more recent years, nanotechnology has emerged as a suitable answer to these issues, allowing to take advantage of the beneficial effects of this micronutrient, while overcoming some of the disadvantages associated with its administration. Nanoparticles provide protection from external conditions, and increase the stability and solubility of the molecule. Also, nanoparticles allow decreasing its toxicity associated with the hypercalcemia phenomena, and allowing circumventing the multidrug resistance problem hindering the molecule efflux out of the cells. Only a few nanosystems have been described for different applications, such as food and beverage fortification and as therapeutic agents, as shown in this chapter. It would be essential to conduct more substantial and insightful studies to support the great potential of nanotechnology for the delivery of vitamin D. Also, it would be valuable to optimize the already-described systems to make them more efficient and specific to a specific target tissue.

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Vitamin D, a fat-soluble vitamin, also called as “sunshine vitamin” is derived mostly from sun exposure and food, and for normal activation, it has to undergo two hydroxylation reactions. Vitamin D affects more than 2000 genes in the body. Serum level of 25(OH) D is an ideal indicator of vitamin D status in our body. Vitamin D deficiency leads to various diseases. On a therapeutic point of view, vitamin D helps to treat many diseases. The book “A Critical Evaluation of Vitamin D - Clinical Overview” targets the principles, mechanisms, and clinical significance of vitamin D. This book covers four sections: “Vitamin D in Cardiovascular and Renal Diseases”, “Vitamin D in Age and Neurological Diseases”, “Vitamin D and Cancer” and “Therapeutic Measurements of Vitamin D”. Each of these sections is interwoven with the theoretical aspects and experimental techniques of basic and clinical sciences. This book will be a significant source to students, scientists, physicians, healthcare professionals and also other members of this society who are interested in exploring the role of vitamin D in human life.

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